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Agglutination of Jelly Coat and Cortical Granule Components and the Block to Polyspermy in the Amphibian *Xenopus laevis*

(sulfated glycoproteins/lectins)

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ABSTRACT **A block to polyspermy in amphibians is established at fertilization by the conversion of the vitelline envelope to the fertilization envelope. In *Xenopus laevis* a major ultrastructural change in the envelope at fertilization is the appearance of an electron-dense layer, termed the F layer, between the envelope and the innermost jelly coat layer, J_1. The F layer is derived, at least in part, from materials released from the cortical granules. Further definition of the origin and chemical nature of the F layer was sought by using isolated cortical granule (CG) exudate and jelly coat layer J_1. In double diffusion experiments, the isolated components interacted in an agglutination reaction producing a band of precipitation. The agglutination involved α-galactoside residues and metal ions (Ca^{++}). Employing chemically modified jelly, we demonstrated that sulfhydryl-disulfide interchanges were not involved in the agglutination and, with ^{35}S-labeled jelly, that the agglutinating J_1 component possessed sulfate esters. Both the CG exudate and the J_1 components contained carbohydrate, as evidenced by their lectin reactivity. A number of ionic polymers, both natural and synthetic, were tested as chemical analogs of CG exudate and J_1; none gave an agglutination band. Dissolved jelly coat material from eggs of two different species of frogs agglutinated with CG exudate, while jelly from sea urchin eggs and hyaluronic acid from mammalian eggs did not. Thus, the agglutination reaction was chemically and phylogenetically specific. An electron-dense layer, similar to the F layer, formed on the outer surface of the vitelline envelope when jellied unfertilized eggs were immersed in CG exudate; such eggs were not fertilizable. We suggest that in *Xenopus laevis*, and perhaps other organisms as well, an agglutination type of reaction between cortical granule components and egg integuments is a participant in the structural and molecular events establishing a block to polyspermy.**

The eggs of most animals are surrounded by one or more coats or integuments through which a sperm must pass in fertilizing an egg (2, 17, 18, 21, 22). Egg integuments can be cellular, e.g., cumulus oophorus of mammalian eggs, or noncellular, e.g., jelly coats and vitelline envelopes of sea urchins and amphibians, and the zona pellucida of mammals. The function or biological action of these integuments is multiple and includes effects on the sperm and the egg such as agglutination, capacitation, activation, the provision of sperm binding sites for adherence of the sperm to the egg, and as a block to polyspermy (2, 12, 17, 18, 20, 27).

Abbreviations: FE, fertilization envelope; VE, vitelline envelope; CG exudate, cortical granule exudate; F layer, fertilization layer; J_n, nth jelly coat layer; EDTA, ethylenediaminetetraacetate.

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In the case of the South African clawed toad, *Xenopus laevis*, the egg is surrounded by a vitelline envelope adjacent to the plasma membrane and by an oviductally secreted jelly coat composed of three layers designated J_1, J_2, and J_3 in order of their deposition around the egg (8). When a *Xenopus* egg is penetrated by a sperm, dramatic surface changes take place (10). The cortical granules immediately beneath the plasma membrane fuse with the membrane and release their contents into the perivitelline space between the plasma membrane and the vitelline envelope. Under the influence of the cortical granule material, the vitelline envelope (VE) is converted into the fertilization envelope (FE) with the pronounced appearance of a new electron-dense structure termed the fertilization (F) layer between the VE and the innermost jelly coat layer J_1 (Fig. 1*a* and *b*). In amphibians, conversion of VE to FE has been ascribed a functional role in preventing penetration of more than one sperm into the egg, a block to polyspermy (2, 4, 9, 18, 19, 22).

Electron and light microscopy studies (10) indicate that the F layer is derived at least in part from material released from the cortical granules. In consideration of the structural location of the F layer (in between the VE and J_1) and of the structural alteration in the F layer when the jelly coat was removed, the possibility that egg integuments might be involved in F layer formation was suggested (10). This paper further defines the components responsible for the formation of the F layer. The reactivity of isolated jelly layer J_1 and isolated CG exudate suggests that F layer formation involves a specific agglutination or aggregation reaction.

MATERIALS AND METHODS

Eggs from *Xenopus laevis* were obtained by injection of human chorionic gonadotropin as previously described (28). CG exudate was obtained by exposing dejellied eggs to a cycle of pH and temperature changes. Eggs (4000) were dejellied with 0.045 M mercaptoethanol, Ca^{++}-free DeBoers solution, suspended in 2–4 ml of ice-cold DeBoers solution, and 1% butyric acid was added to pH 4.5. After 3 min of gentle shaking, the solution was warmed to room temperature and adjusted to pH 9 with 0.05 M NaOH. The eggs were placed on ice and cold butyric acid added again to pH 4.5. Finally, the pH was adjusted to 7.8 at room temperature and the suspension gently shaken for 25 min. The solution was decanted from the eggs and centrifuged at 40,000 × *g* at 4°. The clear centrifugate was the CG exudate. This procedure usually resulted in less than 2% lysis of eggs.

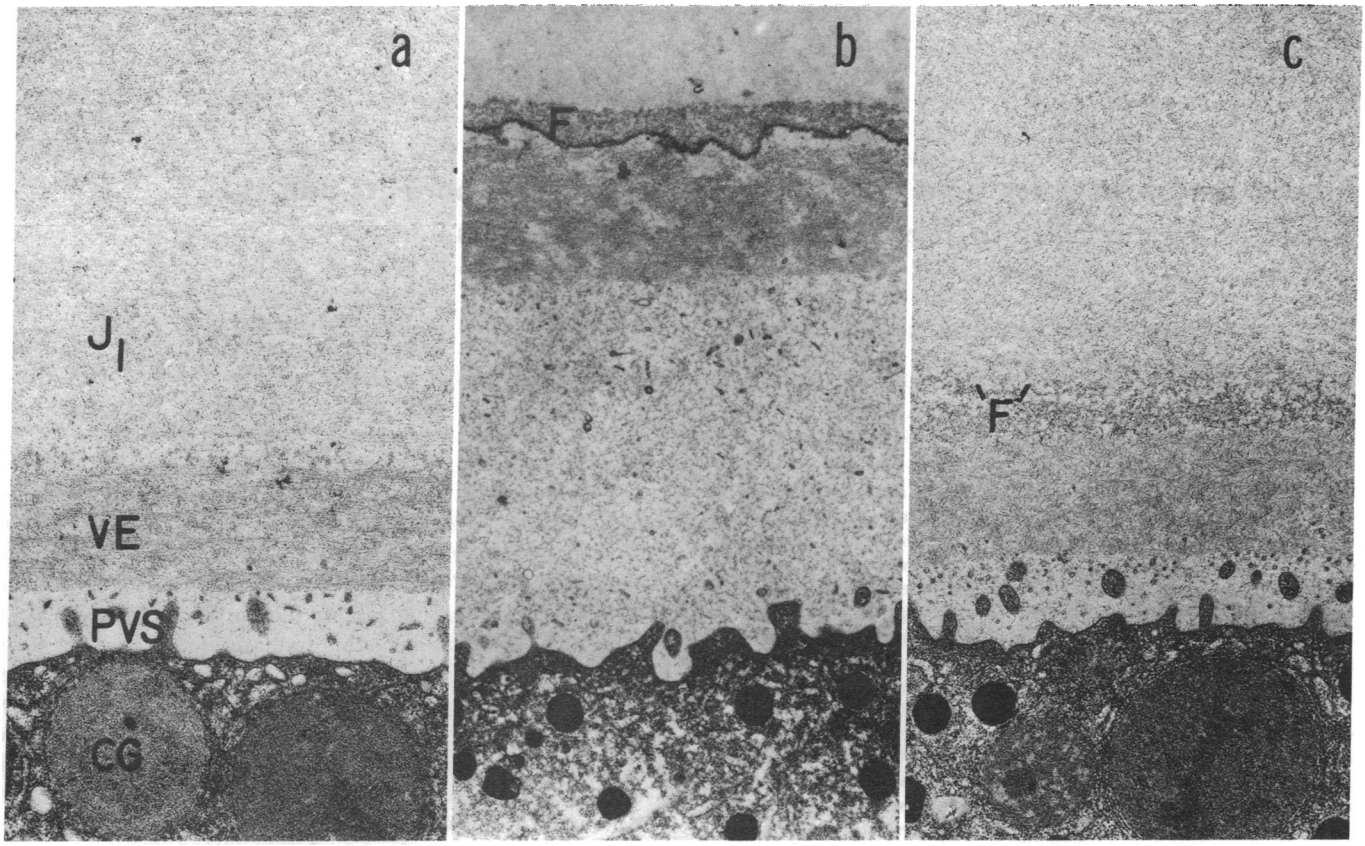

FIG. 1. Electron micrographs of the surface of *Xenopus laevis* eggs. Abbreviations used to denote the structures are: J_1, jelly layer J_1; VE, vitelline envelope; PVS, perivitelline space; CG, cortical granule; F, F layer of the fertilization envelope; 'F', F-like layer. Magnification is ×9,500. (*a*) Unfertilized egg, (*b*) fertilized egg, and (*c*) CG-exudate-treated unfertilized egg.

The innermost jelly layer, J_1, was obtained by first manually removing jelly layers J_3 and J_2 from the eggs with watchmaker's forceps under a dissecting microscope. The exposed jelly layer J_1 was then dissolved in 0.01 M dithiothreitol, 0.01 M Tris·HCl, 0.15 M NaCl, pH 8.0, and the jelly solution was removed from the eggs by decantation, centrifuged, and dialyzed against Tris·HCl, NaCl, pH 8.0, to remove the dithiothreitol.

Preparation of FE and VE utilized dejellied fertilized or partially dejellied unfertilized eggs. Eggs were lysed by passage through a 10-ml syringe with a 19-gauge needle. The ruptured envelopes were filtered from the suspension with a 33 mesh nylon screen, and thoroughly washed with distilled water, room temperature in the case of FE and 0° for VE. A second washing with 0.2 M mercaptoethanol, pH 9.5, was routinely used to remove adhering jelly from VE and from FE if the jelly was not completely removed by the initial dejellying step. The mercaptoethanol-treated envelopes were then again washed with distilled water. Envelopes were dissolved by heating suspensions in 0.05 M Tris, pH 9.7, for 10 min at 70°.

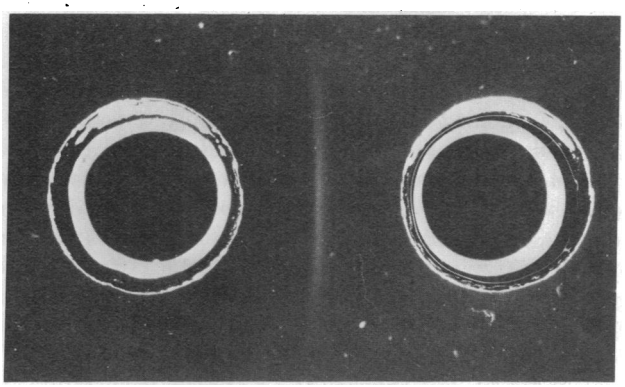

FIG. 2. Precipitin line formed between CG exudate and J_1. Concentrations of substances used were: J_1, 0.48 mg of hexose per ml; CG exudate, that obtained from 4000 eggs in 2 ml. The well on the right contains CG exudate, the well on the left, J_1.

The plant lectins were prepared from seed extracts carried through the ammonium sulfate stage of purification (3, 7, 16). Concanavalin A was a commercial preparation.

Double diffusion experiments in two dimensions used 3-mm holes 3 mm apart with 1% agarose in 0.01 M Tris·HCl, 0.15 M NaCl, pH 8.0, at room temperature. Electron microscopy procedures were as previously described (10). Neutral hexose was determined by the phenol–sulfuric acid procedure using galactose as a standard (1).

All other biological preparations and chemicals were obtained from commercial sources unless otherwise noted.

RESULTS

Agglutination of CG Exudate and J_1. In double diffusion experiments, isolated jelly layer J_1 and CG exudate react with one another in an agglutination type of reaction producing a precipitin line (Fig. 2). A single precipitin arc appeared between 24 and 48 hr of diffusion using jelly layer J_1 (0.48 mg of neutral hexose per ml) and undiluted CG exudate. Dilu-

tion of CG exudate up to 1:8 gave the same result as undiluted solution.

Sugar Specificity. In analogy with plant lectin agglutination (for review see ref. 23) a number of sugars were incorporated into the agarose of the double diffusion plates and tested for their ability to inhibit the agglutination reaction of CG exudate and J_1. D-Glucose, D-mannose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, and L-fucose at 0.1 M did not affect agglutination; only D-galactose inhibited the reaction. Methyl galactosides were used to distinguish the potential reactivity of galactose anomers; only α-methyl galactoside inhibited the reaction, suggesting that J_1–CG exudate agglutination involves α-galactoside residues. The J_1-CG agglutination reaction was fully reversible; the precipitin line could be dissolved by overlaying a double diffusion plate with a D-galactose solution.

Metal Ion Involvement. The participation of calcium ion in the agglutination reaction was tested in a similar fashion to the sugars by adding calcium or sequestering agents to the agarose. Addition of 10 mM $CaCl_2$ greatly potentiated the reaction, producing a much sharper line of reaction between the CG exudate and J_1 in comparison to the situation where the Ca^{++} concentration was determined by the carry over from the CG exudate preparation (Fig. 2). Addition of 1 mM ethylenediaminetetraacetate (EDTA) or ethyleneglycol bis(β-aminoethyl ether)-*N*-*N*′-tetraacetate (EGTA) totally inhibited the agglutination reaction. The precipitation line could be dissolved by overlaying a 1 mM EDTA solution. Thus, it would appear that a metal ion(s) is involved in the agglutination reaction. Because of the difficulty in adequately removing calcium from the reactants and the agarose, metal specificity was not investigated further.

Plant Lectin Reactivity of CG Exudate and J_1. The double diffusion assay system was modified to incorporate a third well to test the reactivity of plant lectins with CG exudate and J_1. The CG exudate and J_1 showed variable reactivity with different lectins (Table 1). Thus, both components contain various sugars.

Inhibition of the lectin–CG exudate or lectin–J_1 agglutination reaction was obtained by adding various sugars to the agarose (Table 2). Based on the sugar specificity and metal requirements for agglutination with plant lectins (for review see ref. 23), expected results were obtained with three exceptions: (*1*) wheat germ agglutination of CG exudate and with J_1 was inhibited by D-GalNAc, whereas its specificity is for $(D\text{-}GlcNAc)_2$, (*2*) *Dolichos biflorus* agglutination with CG exudate was inhibited by EDTA, whereas its reactivity has not been reported to require a metal, (*3*) concanavalin A agglutination is metal-dependent and its reaction with J_1 was inhibited by EDTA but not the reaction with CG exudate. These results may be meaningful in terms of the specificity of the lectins used or they may be due to the impure substances used (lectins and metal-free reagents) or the lectins themselves may be ligands for CG exudate or J_1 agglutinins. Further purification of the lectins and improvements in the system used to detect their agglutination will be necessary to answer these questions. The lectin reactivity of the CG exudate and the J_1 layer can be used in purification and assay of the macromolecules composing these substances.

TABLE 1. *Lectin agglutination with CG exudate and J_1*

Lectin	Sugar specificity	Agglutination with: CG exudate	Agglutination with: J_1
Wheat germ	$(D\text{-}GlcNAc)_2$	+	++
Dolichos biflorus	α-D-GalNAc	+++	−
Ulex europeus	L-Fuc, $(D\text{-}GlcNAc)_2$	−	−
Concanavalin A	α-D-Man	+	++

Presence of J_1 in Isolated FE. Additional evidence that J_1 was a participant in forming the F layer was obtained from immunological studies. Antibodies against total jelly (all three jelly layers) were prepared and tested for their ability to precipitate purified and dissolved VE and FE. Conversely, the reaction of J_1 with antibodies prepared against VE and FE was also examined. Double diffusion experiments using anti-total jelly serum gave a strong precipitin line against FE and J_1 with no spur formation; against VE no precipitin line was present. Using anti-FE serum, four precipitin lines were obtained against dissolved FE, one of which was continuous with the single J_1 precipitin line; VE did not exhibit this common line of interaction. Anti-VE serum showed lines of interactions against FE and VE but none against J_1. Thus, on the basis of the immunogenicity of the FE and its reactivity with antijelly serum, J_1 determinants are present in the FE.

Sulfur-Containing Components of J_1 and Their Reaction with CG Exudate. Jelly layer J_1 contains sulfated glycoproteins (J. L. Hedrick, A. J. Smith, E. C. Yurewicz, G. Oliphant, and D. P. Wolf, submitted for publication). It was, therefore, suspected that sulfate might be involved in the agglutination reaction of J_1 and CG exudate. However, addition of 0.06 M Na_2SO_4 to a double diffusion plate did not inhibit the agglutionation reaction between J_1 and CG exudate. Thus, it would appear the sulfate groups are not directly involved in the CG exudate–J_1 interaction.

TABLE 2. *Inhibition of lectin agglutination*

Substance added[a]	Lectin	Effect of lectin agglutination with[b]: CG exudate	Effect of lectin agglutination with[b]: J_1
D-Gal	Wheat germ	+	+
	Dolichos biflorus	+	0
	Concanavalin A	+	+
D-GalNAc	Wheat germ	−	−
	Dolichos biflorus	−	0
D-GlcNAc	Wheat germ	−	−
	Dolichos biflorus	+	0
D-Glc or D-Man	Concanavalin A	−	−
EDTA	Wheat germ	+	+
	Dolichos biflorus	−	0
	Concanavalin A	+	−

[a] Sugars were tested at 0.1 M and EDTA at 1 mM.

[b] +, agglutination present; 0, no reaction initially; −, inhibition of initially positive reaction.

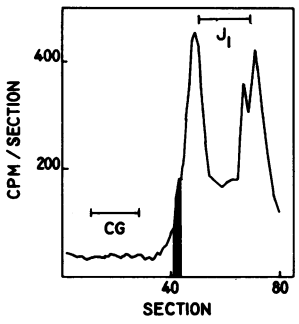

FIG. 3. Distribution of [^{35}S]J_1 on a diffusion slide and correlation with the band of agglutination. Double diffusion conditions were as described in the *text* except that the agarose slab (13 × 15 × 2 mm) was prepared on a microscope slide. The [^{35}S]J_1 was diffused for 74 hr and the CG exudate for 48.5 hr. The gel was sliced into 200-μm sections and counted for ^{35}S. The locations of the CG exudate and J_1 wells are indicated by the *brackets*. The position of the agglutination band is indicated by the *heavy black band* between the wells.

Evidence that sulfated macromolecules are involved in the agglutination reaction was obtained using ^{35}S-labeled J_1 in double diffusion experiments. [^{35}S]sulfate is incorporated into the jelly as sulfate esters when administered at the same time as the chorionic gonadotropin used to induce ovulation. Using such radioactive jelly and performing a double diffusion test, the distribution of radioactivity versus the distance from the J_1 well was determined and compared with the location of the precipitin line. A typical example is shown in Fig. 3. Double diffusion tests were performed at several concentrations of CG exudate and [^{35}S]J_1. In all cases, the observed single precipitin line could not be dissociated from the [^{35}S]J_1 diffusion gradient and, as indicated in Fig. 3, the precipitin line was associated with a discontinuity in the distribution curve of the [^{35}S]J_1. By allowing the radioactive J_1 to "prediffuse" into the agarose for various times before placing the CG exudate into its well, it was possible to compare the diffusion rates of the [^{35}S]J_1 macromolecules and the J_1 component interacting with the CG exudate component. The diffusion rate of the J_1-agglutinating component approximated the diffusion rate of the [^{35}S]J_1 macromolecules. In addition to these experiments, it has been observed that purified preparations of sulfated J_1 macromolecules (in contrast to the dissected J_1 layer used here) precipitate with the CG exudate (E. C. Yurewicz, A. J. Smith, and J. L. Hedrick, unpublished observations). Finally, sulfated jelly coat macromolecules with CG-exudate-agglutinating activity are selectively precipitated with cetyl pyridinium chloride, a quaternary amine used for the isolation of sulfated glycoproteins and mucopolysaccharides (for review see ref. 30). We conclude that the CG exudate component(s) is reacting with a sulfated J_1 component(s).

It has been shown that disulfide bonds are present in the jelly macromolecules (11). These bonds are reduced to free sulfhydryl groups when the jelly is dissolved in mercaptans; the sulfhydryl groups of the dissolved jelly were modified by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (6) and the CG exudate reactivity of the SH-substituted J_1 compared with that of nonmodified J_1. No difference was detectable, indicating that the oxidation state of the sulfhydryl groups of J_1 was not functionally important in the agglutination reaction.

J_1 and CG Exudate Analogs. Some of the J_1 macromolecular components are strongly acidic as shown by their electrophoretic migration, alcian blue staining characteristics, and the presence of sulfate (J. L. Hedrick, A. J. Smith, E. C. Yurewicz, G. Oliphant, and D. P. Wolf, submitted for publication). By chemical analogy and in view of the fact that eggs from many different organisms are surrounded by acidic substances [e.g., poly(fucose) sulfate jelly in the case of a sea urchin and hyaluronic acid in mammals, refs. 17 and 20], a number of acidic substances were tested as J_1 replacements in the agglutination reaction with CG exudate. The following were tested: hyaluronic acid, chondroitin sulfate, dextran sulfate, fucoidan sulfate, heparin sulfate, and poly(glutamic acid). At the concentrations tested (0.06 to 10 mg/ml, saturated solution in the case of fucoidan sulfate) none of them precipitated either CG exudate or J_1. Various basic substances were also tested as CG exudate substitutes; protamine, histone, and polylysine at concentrations between 0.015 and 1.0 mg/ml also, showed no precipitation.

To further define the specificity of the agglutination reaction, jelly coats from the sea urchin *Strongylocentrotus purpuratus* (15), the frog *Rana pipiens*, and the toad *Bufo boreas* were tested. The sea urchin jelly coat gave no agglutination with CG exudate. However, both the *Rana pipiens* and *Bufo boreas* jellies gave a single agglutinin line with CG exudate that was continuous (no spurs) with the precipitin line against J_1. Thus, the amphibian jellies contain reactive sites that are analogous to those of the *Xenopus laevis* jelly.

Formation of the F Layer in Unfertilized Eggs. Three readily identifiable surface characteristics distinguish fertilized from unfertilized eggs; (*1*) the pressure of the F layer in fertilized eggs and its absence in unfertilized eggs, (*2*) the absence of cortical granules in fertilized eggs and their presence in unfertilized eggs, and (*3*) a fertilization-induced elevation of the envelope away from the plasma membrane (Fig. 1*a* and *b*). If the F layer were formed from interaction of CG exudate and a structurally distinct component of J_1, immersion of a jellied unfertilized egg in CG exudate should result in the appearance of the electron-dense F layer even though neither the cortical reaction nor elevation of the envelope has occurred. Fig. 1*c* illustrates that such a result was obtained. The electron-dense layer produced in this manner was not, however, identical to that of a fertilized or activated egg (Fig. 1*b*) in that the very dense component on the surface of the FE was absent.

Fertilizability of CG-Exudate-Treated Eggs. To test the potential biological function of the agglutination of CG exudate and J_1 components, jellied eggs were immersed in CG exudate solution for 20–40 min, washed, and a sperm suspension was added. In three different experiments with a total of 153 eggs, none of the treated eggs were fertilized. Untreated control eggs (325) were fertilized to the extent of 93%. We conclude that CG exudate agglutination with jelly coat prevents fertilization.

DISCUSSION

From previous model studies on the interaction of egg surfaces with antibodies and plant lectins (5, 20, 21, 25) it was postulated that an antibody–antigen or agglutination type of reaction might prevent sperm penetration of an egg. The experiments described here demonstrate that agglutination

of substances present and released upon fertilization can explain the appearance of a new structure that functions as a barrier to supernumerary sperm penetration. The chemical nature of the interaction of sulfated macromolecules composing jelly coat layer J_1 and CG exudate components involves α-galactoside residues, a metal ion (Ca^{++}), and apparently does not involve the sulfate ester groups on J_1 or disulfide–sulfhydryl interactions.

From a previous study (10) and from the fact that isolated VE and FE retain their *in vivo* structure (R. E. Wyrick and J. L. Hedrick, unpublished observations), it was concluded that the FE was derived from interaction of VE with the contents of cortical granules. FE is structurally composed of a VE component and an F layer, i.e., VE + CG exudate → VE component + F layer = FE. From the results reported here, the F layer is apparently produced from the interaction of J_1 components, CG exudate and metals. Hence, a more complete formulation is: FE = VE component + J_1–CG exudate–metal complex.

Based on the above, two postulates on how the FE might prevent sperm penetration are *(1)* by preventing the action of sperm lysins (associated with the acrosome) with their substrate (the envelope), or *(2)* by blocking or otherwise modifying the sperm binding sites on the VE as has been suggested in the case of the sea urchin and hamster (12, 20, 27). We have not as yet, however, detected any specific binding between isolated VE and sperm in the case of *Xenopus laevis*. Since we have isolated the components involved in the formation of FE, it should be possible to investigate at the molecular level the block to sperm penetration.

The agglutination of J_1 and CG exudate appears to be chemically and phylogenetically specific. Of the wide variety of substances tried as chemical analogs of CG exudate and J_1, none reacted. The question of phylogenetic relatedness was minimally investigated but the results are sufficient to be worthy of comment. The jelly coats of various amphibians are immunologically related (14, 24, 26) and it has been suggested that jelly coat components might function in determining species specificity of fertilization. Jelly from *Bufo boreas*, *Rana pipiens*, and *Xenopus laevis* are immunologically related (unpublished observations, U. A. Urch and J. L. Hedrick). The observations reported here that *Bufo boreas* and *Rana pipiens* jelly contain components that agglutinate *Xenopus laevis* CG exudate provides a means of identifying and potentially isolating the jelly coat macromolecular components so that their chemical and functional relatedness can be assessed.

Considering the general similarity of chemical substances found on the outside of eggs from different species (sulfated/acidic glycoproteins or mucopolysaccharides, refs. 13 and 20) and the structural and functional similarities of egg integuments, the interaction of CG exudate and egg integuments may be a fundamental process of fertilization. We suggest that in *Xenopus laevis*, and perhaps other organisms as well, an agglutination type of reaction between cortical granule components and egg integuments is a participant in the structural and molecular events leading to a block to polyspermy.

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1. Ashwell, G. (1966) *Methods in Enzymology*, eds. Neufield, E. F. & Ginsburg, G. (Academic Press, New York), Vol. 8, pp. 85–95.
2. Austin, C. R. (1968) *Ultrastructure of Fertilization* (Holt, Rinehart, and Winston, New York).
3. Burger, M. M. & Goldberg, A. R. (1967) *Proc. Nat. Acad. Sci. USA* **57**, 359–366.
4. Deuchar, E. M. (1966) *Biochemical Aspects of Amphibian Development* (Methuen, London), p. 11.
5. Dudkiewicz, A. B., Shivers, C. A., Franklin, L. E. & Fussell, E. N. (1972) *Amer. Zool.* **12**, 706.
6. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77.
7. Font, J., Leseney, A. M. & Bourillon, R. (1971) *Biochim. Biophys. Acta* **243**, 434–446.
8. Freeman, S. B. (1968) *Biol. Bull.* **135**, 501–513.
9. Graham, C. F. (1966) *J. Cell Sci.* **1**, 363–374.
10. Grey, R. D., Wolf, D. P. & Hedrick, J. L. (1974) *Develop. Biol.*, **36**, 44–61.
11. Gusseck, D. J. & Hedrick, J. L. (1971) *Develop. Biol.* **25**, 337–347.
12. Gwatkin, R. B. L., Williams, D. T., Hartmann, J. F. & Kniazuk, M. (1973) *J. Reprod. Fert.* **32**, 259–265.
13. Jeanloz, R. W. (1971) in *The Biology of the Blastocyst*, ed., Blandau, R. J. (University of Chicago, Chicago), pp. 131–138.
14. Katagiri, C. (1967) *SABCO J.* **4**, 33–43.
15. Lorenzi, M. & Hedrick, J. L. (1973) *Exp. Cell Res.* **79**, 417–422.
16. Matsumoto, I. & Osawa, T. (1969) *Biochim. Biophys. Acta* **194**, 180–189.
17. Metz, C. B. (1967) in *Fertilization*, eds. Metz, C. B. & Monroy, A. (Academic Press, New York), Vol. 1, pp. 163–236.
18. Monroy, A. (1965) *Chemistry and Physiology of Fertilization* (Holt, Rinehart, and Winston, New York).
19. Noble, G. K. (1954) *The Biology of Amphibia* (Dover, New York).
20. Oikawa, T., Yanagimachi, R. & Nicolson, G. L. (1973) *Nature* **241**, 256–259; Oikawa, T., Nicolson, G. & Yanagimachi, R. (1974) *Exp. Cell Res.*, in press.
21. Piko, L. (1969) in *Fertilization*, eds. Metz, C. B. & Monroy, A. (Academic Press, New York), Vol. 2, pp. 325–403.
22. Rugh, R. (1961) *The Frog: Its Reproduction and Development* (McGraw-Hill, New York).
23. Sharon, N. & Lis, H. (1972) *Science* **177**, 949–959; Lis, H. & Sharon, N. (1973) *Annu. Rev. Biochem.* **42**, 541–574.
24. Shaver, J. R. (1966) *Amer. Zool.* **6**, 180–189.
25. Shaver, J. R., Barch, S. H. & Attinase, L. C. (1973) *Biol. Reprod.* **8**, 112–118.
26. Shivers, C. A. (1965) *Biol. Bull.* **128**, 328–336.
27. Vacquier, V. D., Tegner, W. J. & Epel, D. (1972) *Nature* **240**, 352–353.
28. Wolf, D. P. & Hedrick, J. L. (1971) *Develop. Biol.* **25**, 348–359.
29. Wolf, D. P. & Hedrick, J. L. (1971) *Develop. Biol.* **25**, 360–376.
30. Yosizawa, Z. (1972) in *Glycoproteins*, ed. Gottschalk, A. (Elsevier, New York), part B, pp. 1000–1018.