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Cholinergic Sites in Skeletal Muscle. II. Interaction of an Agonist and Two Antagonists with the Acetylcholine Site†

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ABSTRACT: The equilibrium interactions of α-bungarotoxin, *d*-tubocurarine, and carbamylcholine with junctional and extrajunctional skeletal muscle acetylcholine receptors were examined. *d*-Tubocurarine is a competitive inhibitor of the bindings of α-bungarotoxin to the acetylcholine receptor. No substantive difference was observed in the association of *d*-tubocurarine with the junctional and extrajunctional receptors. In contrast, the carbamylcholine inhibition of toxin binding is not competitive. The data indicate that either the single set of α-bungarotoxin and *d*-tubocurarine binding sites contains two subsets of carbamylcholine sites or that the carbamylcholine binds in a cooperative manner to a single set of sites. In addition, the affinity of carbamylcholine for extrajunctional receptors may be higher than the affinity for junctional receptors.

In the preceding paper of this issue, a set of high-affinity binding sites in normal and denervated skeletal mixed muscle preparations was described. It was concluded based on those data and the results of other investigations that this set represented the interaction of α-bungarotoxin with the cholinergic receptor site that mediates muscle depolarization. In normal muscle preparations, greater than 95% of the sites in the set represent junctional receptors, and in 10-day denervated muscle preparations, approximately 95% of the sites are extrajunctional receptors (Almon and Appel, 1975). A difference was also observed between junctional and extrajunctional receptors with respect to their interaction with an IgG derived from the serum of some patients with myasthenia gravis.

Fundamental to both the definition of this set of high-affinity binding sites as the AcCh receptor and understanding its role in the cholinergic effector mechanism is the analysis of its interaction with cholinergic agonists. The pharmacological behavior of cholinergic agonists and antagonists provides a framework within which the molecular interactions can be interpreted. Since the binding of a cholinergic agonist to the acetylcholine receptor results in membrane depolarization and the binding of a cholinergic antagonist does not, a fundamental difference in the binding of the two types of agents is indicated. In addition, the prolonged exposure of the muscle to cholinergic agonists results in the phenomenon known as desensitization (Katz and Thesleff, 1957). The characteristics of cholinergic desensitization suggest that it represents an agonist-mediated receptor inactivation (Rang and Ritter, 1969a,b). It is possible that the inactivation reflects a metastable conformational change in the receptor induced by the agonist but not requiring its presence for interim maintenance (Rang and Ritter, 1969a).

Although the analysis of thermodynamic characteristics does not allow the examination of dynamic biological events, it does allow the assessment of equilibrium variations which may help explain the functional properties. In the present re-

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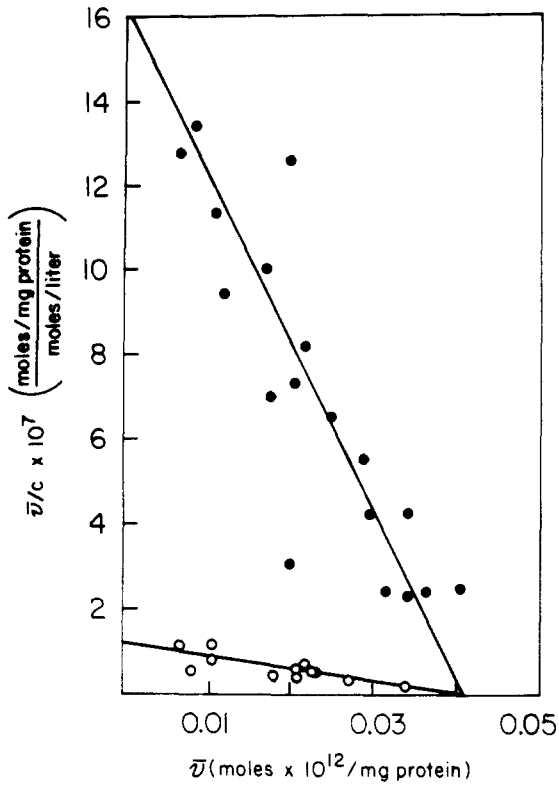

FIGURE 1: Scatchard analysis of the binding of [^{125}I]iodo-α-bungarotoxin to the high-affinity set of sites in a Triton X-100 preparation from normal innervated muscle. The control data are indicated by the closed circles and the experimental data in the presence of 8.7×10^{-6} M dTc are indicated by open circles. The intercept on the ($\bar{\nu}$) axes is the number of available sites (N). The slope of the line is the affinity constant (K). The data demonstrate a lower apparent affinity in the presence of dTc.

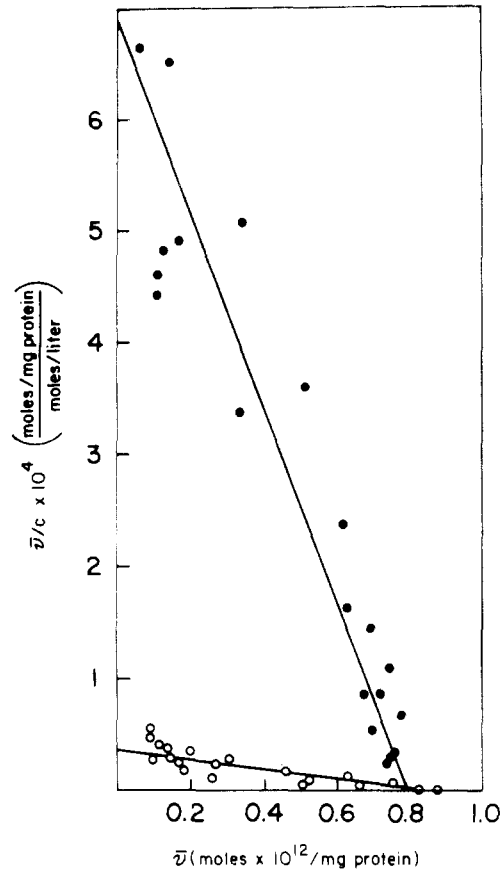

FIGURE 2: Scatchard analysis of the binding of [^{125}I]iodo-α-bungarotoxin to the high-affinity set in a Triton X-100 preparation from 10-day denervated muscle. The control data are indicated by closed circles and the experimental data in the presence of 8.7×10^{-6} M dTc are indicated by the open circles. The intercept on the ($\bar{\nu}$) axes is the number of available sites (N). The slope of the lines (least squares) is the affinity constant (K). The data demonstrate a lower apparent affinity in the presence of dTc.

port, we have analyzed the equilibrium interactions of a cholinergic agonist (carbamylcholine) and a cholinergic antagonist (*d*-tubocurarine) with the putative AcCh receptor defined in the preceding report. The experimental analysis is based on the effects of these agents on the binding of [^{125}I]iodo-α-bungarotoxin to the high-affinity set of sites described in the preceding report.

Materials and Methods

Materials. Diiodo-α-bungarotoxin was obtained and prepared as described in a preceding paper (Almon et al., 1974). *d*-Tubocurarine chloride (dTc) and carbamylcholine (Carb)[1] were obtained from Sigma. All other chemicals were reagent grade. Female white rats were obtained from West Jersey Biologicals and denervated as described in a preceding paper.

Binding Assay. Aliquots (0.1–0.5 ml) of a frozen Triton X-100 extracted muscle receptor fraction ($0.5–1 \times 10^{-12}$ mol of receptor) from normal innervated and 10-day denervated muscle were employed. Normal muscle preparations were studied after the affinity shift as discussed previously (Almon et al., 1974). Toxin concentration between 10^{-10} and 5×10^{-7} M was employed in these investigations of the high affinity cholinergic set of sites. The total reaction volume was 0.5–1.0 ml.

Inhibition Experiments. In experiments to characterize the effects of the cholinergic agonist (Carb), and the antagonist (dTc) on the binding of [^{125}I]iodo-α-bungarotoxin to the high-affinity cholinergic site, aliquots were first incubated for 30 min at 22 °C with the indicated concentration of one of the inhibitory ligands. The concentrations of the cholinergic ligands employed were chosen such that only partial inhibition is observed at the K_D of the set of binding sites. These data were derived from results detailed in an earlier report (Almon et al., 1974). After the initial incubation for 30 min at 22 °C, [^{125}I]iodo-α-bungarotoxin (100 μl) was added to the aliquots to the desired final concentration. Incubation was continued for an additional 16 h at 4 °C prior to assaying the amount of toxin bound at each free toxin concentration.

Analysis. The initial step in the analysis was to subtract the unsaturable nonspecific component (cf. preceding paper in this issue) from the uninhibited control isotherm and the isotherm reflecting the effect of the added low concentration of cholinergic agonist (Carb) or antagonist (dTc). The resulting control and agonist or antagonist altered isotherms were analyzed both in the log plot ($\bar{\nu}$ vs. log C) and by the method of Scatchard as applied previously for these type of data (Scatchard, 1949).

Results

d-Tubocurarine: Analysis of the effect of dTc on the binding of [^{125}I]iodo-α-bungarotoxin to the high-affinity set of cholinergic sites in skeletal muscle ($K_A \simeq 10^9$ M^{-1}) indicates that this cholinergic antagonist is a competitive inhibitor. Figure 1 presents the data for normal muscle; and Figure 2 presents

[1] Abbreviations used are: Carb, carbamylcholine; dTc, *d*-tubocurarine; AcCh, acetylcholine.

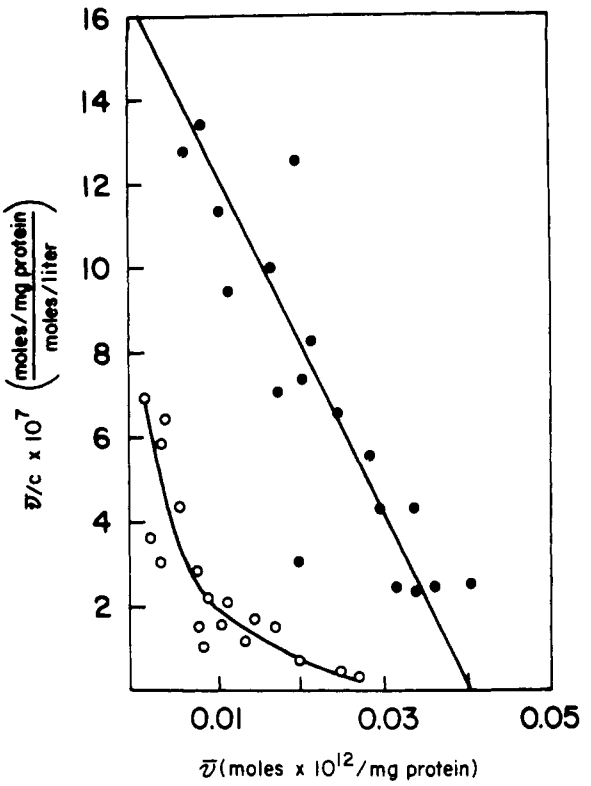

FIGURE 3: Scatchard analysis of the binding of [^{125}I]iodo-α-bungarotoxin to the high-affinity set in a Triton X-100 preparation from normal innervated muscle. The control data are indicated by the closed circles and the experimental data in the presence of 1.2×10^{-4} M Carb are indicated by the open circles. For the control data, the intercept on the ($\bar{\nu}$) axes indicated the number of available sites (N) and the slope is the affinity constant (K). The experimental data (O) do not approximate a straight line, indicating either that the sites are not independent (cooperativity) or that the sites are not identical (more than one set of sites).

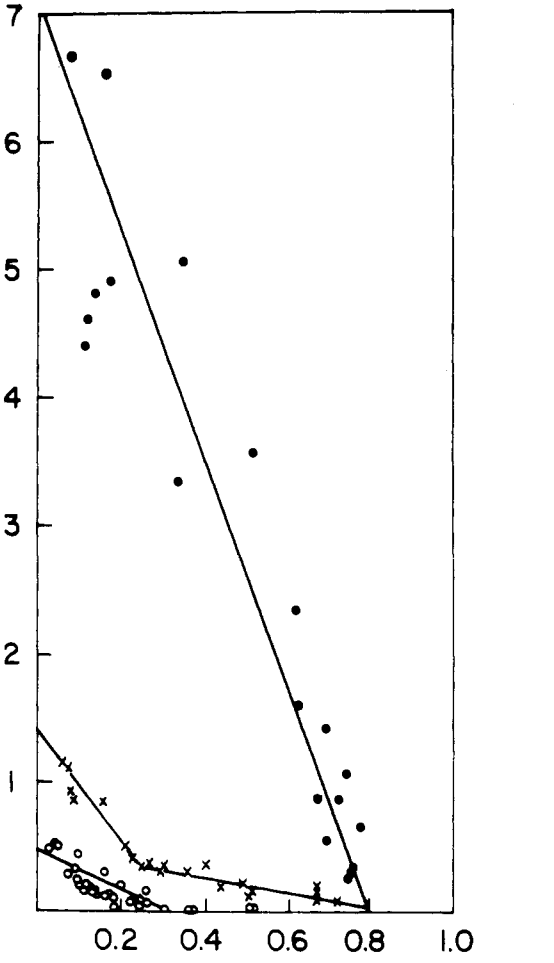

FIGURE 4: Scatchard analysis of the binding of [^{125}I]iodo-α-bungarotoxin to the high-affinity set of sites in a Triton X-100 preparation from 10-day denervated muscle. The control data are indicated by the closed circles. The experimental data in the presence of 1.3×10^{-5} M Carb are indicated by the X's and the experimental data in the presence of 1.2×10^{-4} M Carb are indicated by the open circles (O). The control data and the experimental data in the presence of 1.2×10^{-4} M Carb approximate a straight line, indicating a single set of identical sites. The number of sites in the set are: control, 8 pmol/mg of protein, plus 1.2×10^{-4} M Carb, 0.3 pmol/mg of protein. The experimental isotherm in the presence of the lower Carb concentration (3.5×10^{-5} M) has been analyzed as containing two sets of independent sites. The number of sites in the set with the highest apparent α-bungarotoxin binding affinity is 0.3 pmol/mg of protein. The number of sites in the set with the lowest apparent toxin binding is 0.5 pmol/mg of protein. It should be noted that a higher apparent toxin binding affinity indicates a lower Carb binding affinity.

the data for 10-day denervated muscle. In these figures the closed circles represent the control data in the absence of dTc, and the open circles represent the experimental data in the presence of 8.7×10^{-6} M dTc. The analysis of the toxin binding to both the normal and denervated preparations shows that dTc does not change the intercept of the $\bar{\nu}$ axes and thus does not change the number of available sites. Furthermore, in the absence and the presence of dTc, the data can be approximated by a straight line, indicating a bimolecular reaction of a homogeneous ligand population interacting with an identical, independent set of sites. On the other hand, the slope of the line in the presence of dTc for both the normal and denervated data plots is altered substantially. Two conclusions can be drawn from these data: (1) both antagonists (α-bungarotoxin and dTc) associate competitively and similarily with this set of cholinergic sites; (2) the similar effects of dTc on normal and denervated muscle indicate that the dTc binding affinity for this set of sites is on the same order in both normal and denervated preparations.

Carbamylcholine. In the next series of experiments, the interaction between Carb and the set of high-affinity cholinergic sites was investigated by analyzing the effect of this agonist on the α-bungarotoxin binding to the set. Figure 3 shows the Scatchard analysis of the data for normal muscle preparations. The closed circles representing the control isotherm approximate a straight line, indicating a single set of independent, identical sites. The intersect on the ($\bar{\nu}$) axes (N) is in the order of 0.04 pmol/mg of protein. In contrast, the open circles representing the experimental data in the presence of 1.2×10^{-4} M Carb do not approximate a straight line. This curvature in the experimental Scatchard analysis can have two interpretations. First, although the sites in the set are homogeneous and identical with respect to α-bungarotoxin, they may not be identical with respect to Carb. This interpretation would mean that the high-affinity set of toxin sites contains two subsets of Carb sites with different affinities. Second, the single set of toxin sites may not be independent for Carb. The curved experimental Scatchard would then indicate negative cooperativity in the Carb binding. More specifically, the occupation of each site with Carb reduces the probability of occupation of each successive site by Carb. The effect of Carb on the toxin binding to denervated muscle preparations appears to clarify the situation. Figure 4 shows those data. In this figure, the closed circles represent control data; the X's represent the addition of 3.2×10^{-5} M Carb; and the open circles represent the addition of 1.2×10^{-4} M Carb. The data show that the

higher concentration of Carb reduces the number of available sites (N) and increases the apparent K_D (the toxin concentration at which $\frac{1}{2}$ N is occupied). There is no substantial increase in the range of toxin concentrations over which the residual set of sites saturate (~2 log units). However, in the presence of the lower concentration of Carb saturation is not apparent, K_D cannot be similarily analyzed, and the magnitude of the range in toxin concentrations over which the isotherm extends is increased. The Scatchard analysis seems to indicate that the appropriate interpretation is that two subsets of sites with different affinities for Carb exist. Specifically, the data for both the control isotherm and experimental isotherm in the presence of the higher concentration of Carb approximate a straight line. The control intersects the ($\bar{\nu}$) axes at 0.8 pmol/mg of protein, and the experimental intersects at 0.3 pmol/mg of protein. The experimental (high concentration) would therefore appear to reflect the complete block of one subset of sites with an (N) of 0.5 pmol/mg of protein and a competitive reduction in the affinity of a second subset with an (N) of 0.3 pmol/mg of protein. This interpretation is supported by a two-site analysis of the isotherm in the presence of the lower Carb concentration. Extrapolation of the two major portions of this curved line to the $\bar{\nu}$ axes would indicate two subsets that competitively bind Carb and toxins. The first has an (N) of 0.3 pmol/mg of protein and a lower affinity for Carb (reflected by the higher apparent toxin-binding affinity). The second has an (N) of 0.5 pmol/mg of protein and a higher affinity for Carb (reflected by lower apparent toxin-binding affinity). However, it should be pointed out that the data do not permit a clear choice between the two possible interpretations.

Discussion

The antagonist dTc appears to act as a competitive inhibitor of [^{125}I]iodo-α-bungarotoxin binding. This conclusion is based on the observation that the only effect of dTc on the [^{125}I]-iodo-α-bungarotoxin binding isotherm is a parallel shift to a higher apparent dissociation constant. Beranek and Vyskocil (1967) presented data suggesting quantitative differences in the physiological sensitivity to dTc between normal junctional receptors and denervated extrajunctional receptors (Beranek and Vyskocil, 1967). Those data indicated that normal endplate potentials are more sensitive to dTc than are the extrajunctional AcCh potentials. In the present experiments, no appreciable difference was observed between normal and denervated muscle preparations with respect to dTc binding affinity. Previous analyses of the interaction of dTc binding with junctional and extrajunctional receptors have yielded conflicting results. Brockes and Hall (1975) reported a differential effect on the initial rate of binding of [^{125}I]monoiodo-α-bungarotoxin to junctional and extrajunctional receptors at 10^{-8} to 10^{-9} M dTc (Brockes and Hall, 1975). A kinetic analysis of those data suggested that the dissociation constant for the interaction of dTc with extrajunctional receptors was 5.5×10^{-7} M, and for junctional receptors was 4.5×10^{-8} M. However, using similar procedures, Alper et al. (1974) were unable to distinguish any difference in the effect of dTc on the rate of α-bungarotoxin binding to the junctional and extrajunctional receptors (Alper et al., 1974). Although our equilibrium data would tend to support the results of Alper et al., they do not provide a clear resolution to the apparent conflict between these two sets of kinetic data.

Carbamylcholine does not behave as a simple competitive inhibitor of α-bungarotoxin binding. Although the sites in the set are identical and independent with respect to the antagonists (α-bungarotoxin and dTc), they are not both identical and independent with respect to the agonist (Carb). Either two subsets of agonist binding sites with different affinities for the Carb or cooperativity are suggested by the data. The former interpretation suggests that one subset of agonist sites has a higher affinity for Carb than its comparable subset in normal muscle. The explanation of these differences between the agonist and the antagonist in their interactions with the AcCh receptor is not clear. It is probable, however, that these differences reflect the depolarization and/or desensitizing phenomena associated with the agonist binding.

Katz and Thesleff (1957) observed that the prolonged exposure of the motor end-plate to cholinergic agonists produced an appreciable loss of sensitivity to AcCh (Katz and Thesleff, 1957). Increased amounts of AcCh could not compensate for the loss in AcCh responsiveness. The rate and degree of desensitization were found to be related to the concentration of agonist. On the other hand, the course of recovery was independent of concentration used to produce desensitization. Further investigations showed that extrajunctional areas were more readily desensitized than junctional areas (Feltz and Mallart, 1971). More recently, it was shown that desensitized receptors are not vulnerable to cobra α-toxin (a close homologue of α-bungarotoxin) (Lester, 1972). Miledi and Potter (1971) reported data suggesting that Carb can desensitize detergent-extracted receptors with respect to α-bungarotoxin binding (Miledi and Potter, 1971). Desensitization, therefore, represents a condition under which the apparent number of available receptor sites is reduced. The present analysis of the effect of Carb on α-bungarotoxin binding suggests that the desensitization may be reflected by the removal of one subset of Carb binding sites. Lester (1972) reported that the combined treatment with agonist and toxin led to a decline over a period of time to an irreversible plateau. The level of this plateau was an inverse function Carb concentration. Although it is difficult to compare the dynamic state observed with intact cell preparations with the present equilibrium data, several analogies can be drawn. As noted in our previous report (Almon et al., 1974), the binding of α-bungarotoxin to the receptor in the detergent-extracted preparations is slowly reversible. In the dynamic intact cell state this would represent an irreversible binding over the period of hours studied. In the present experiments, the level of Carb was kept constant to maintain equilibrium. However, in previous experiments by Miledi and Potter (1971) the removal of AcCh following a desensitizing block did not restore the number of sites available to [^{125}I]-iodo-α-bungarotoxin to its original level within a period of several hours (Miledi and Potter, 1971). Future experiments analyzing the kinetics of the reversal of the Carb block of toxin binding to both subsets of Carb sites should indicate whether the equilibrium characteristics observed in the present experiments are a reflection of the desensitization process.

Acknowledgment

We thank Mr. Joe Vogel for technical assistance.

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Isolation, Physicochemical Properties, and the Macromolecular Composition of the Vitelline and Fertilization Envelopes from *Xenopus laevis* Eggs†

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ABSTRACT: As a step toward defining in molecular terms the sperm-triggered block to polyspermy reaction established by the egg at fertilization, vitelline (VE) and fertilization (FE) envelopes were isolated from eggs of the South African clawed toad *Xenopus laevis* and some of their physicochemical properties determined. Envelopes were isolated after lysis of the fertilized or unfertilized eggs by sieving techniques; isolated envelopes retained their in situ morphology as determined by electron microscopy. The isolated envelopes had different solubility properties and, in general, VE was more readily dissolved by aqueous solvents than FE, although both could be completely dissolved by detergents or chaotropic agents. Changes in envelope solubility correlated with the progression of the cortical reaction implicating a role for cortical granule material in modifying the solubility properties of the envelope. The VE and FE were composed of protein and carbohydrate with no lipid components detected. As determined by immunodiffusion experiments, the FE contained the same antigens as the VE plus components derived from the cortical granules and the innermost jelly layer, J_1. The macromolecular composition of the envelopes was determined by sodium dodecyl sulfate gel electrophoresis. The VE contained at least 11 glycoproteins with molecular weights ranging from 125 000 to <16 000 with two components (40 000 and 33 000) accounting for almost two-thirds of the total stainable material. The FE contained ten glycoproteins that had the same molecular weights as those in the VE. One glycoprotein component underwent a reduction in molecular weight from 77 000 to 67 500 when the VE was converted to the FE. This molecular weight change was interpreted as the probable result of limited proteolysis. In addition, the FE gel electrophoresis patterns contained macromolecular components derived from the cortical granules and jelly layer, J_1, consistent with the immunodiffusion experiments. These components were absent when the FE was prepared in the absence of Ca^{2+}, suggesting a role for Ca^{2+} in binding the VE, cortical granules, and J_1 components together. We concluded that the conversion of the glycoproteinaceous VE to FE at fertilization is caused by interaction of the VE with components from the cortical granules and jelly layer J_1. These interactions are of both a chemical and physical nature.

Establishment of a block to polyspermy at fertilization in some animal ova is effected by conversion of an extracellular vitelline envelope (VE) which is penetrable by sperm to a fertilization envelope (FE)[1] which is impenetrable by sperm (for reviews see Piko, 1969; Austin, 1968; Monroy, 1965). The transformation is produced by the so-called cortical reaction that is triggered by the first spermatozoon to contact the egg. The cortical reaction involves exocytosis of granules lying immediately beneath the plasma membrane of the egg. It is accompanied by an elevation or lift-off of the vitelline envelope from the egg plasma membrane (Nishihara and Hedrick, 1976).

In the case of the South African clawed toad *Xenopus laevis,* the conversion of the VE to the FE produces a new structure located between the innermost jelly coated layer J_1 and the former VE (Grey et al., 1974). The new structure has been termed the fertilization layer or F layer. It was postulated that F layer formation was via a precipitin reaction between a macromolecular component of the cortical granules and the innermost jelly coat layer, J_1 (Wyrick et al., 1974a,b; Wolf, 1974a). The FE has been shown to be impenetrable to sperm and to have a greatly reduced binding affinity for sperm in comparison with the VE (Grey et al., 1976), thus establishing its role in a block to polyspermy at fertilization.

Although the morphological alterations in the surface of the *Xenopus* egg have been well defined and their involvement in preventing supernumerary sperm penetration established, our

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[1] Abbreviations used are: FE, fertilization envelope; VE, vitelline envelope; J_1, jelly coat layer J_1; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; PAS, periodic acid-Schiff base.