IMMUNOLOGIC STUDIES ON THE EFFECT OF OVIDUCAL JELLY COMPONENTS IN THE FERTILIZABILITY OF RANA PIPIENS EGGS

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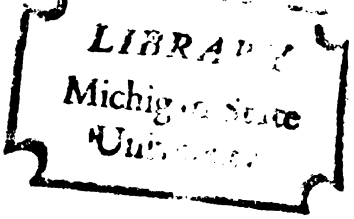
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ABSTRACT

IMMUNOLOGIC STUDIES ON THE EFFECT OF OVIDUCAL JELLY COMPONENTS IN THE FERTILIZABILITY OF *RANA PIPIENS* EGGS

By Carmen Celia Umpierre

In an attempt at elucidating further the possible roles and relative importance of gamete surface components in Anuran sperm-egg jelly interaction during fertilization, two sets of experiments were conducted.

In the first part, *Rana pipiens* eggs were pretreated with antisera specific for jellies obtained from different regions (whole, upper, middle, and lower) of *R. pipiens* oviduct before fertilization. Statistical analysis of the data indicated that treatment of eggs with such antisera produced a significant decrease in percentage cleavage values as shown by comparison with the controls. Anti-middle oviducal jelly serum had the most inhibitory effect on cleavage of eggs, when compared with the effect of antisera specific for the other oviducal jellies. Such significant inhibitory effects upon cleavage were manifested even at a 1:45 serum dilution.

These results were interpreted as indicating that, due to an overlapping of antigenic components, middle oviducal jelly seems to play an important role in the initial egg-jelly-sperm interactions.

In the second part, R. pipiens anti-whole, anti-upper, anti-middle, and anti-lower oviducal jelly sera were absorbed with whole oviducal jellies of different species of Amphibians. Such absorbed sera were tested for their effects on cleavage of R. pipiens eggs. The results of such absorptions indicated that both common generic, as well as species-specific antigenic components are present in oviducal jellies. Both seem to be involved in the initial interactions between sperm and egg-jelly.

Common generic components are present in R. catesbiana whole oviducal jelly and R. pipiens lower oviducal jelly, and in R. clamitans whole oviducal jelly and R. pipiens upper oviducal jelly. In other words, the whole oviducal jellies of these two species of Rana seem to share common generic components with the lower and upper oviducal jellies of R. pipiens respectively, and, consequently, with the outer and inner egg-jelly layers.

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By

Carmen Celia Umpierre

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INTRODUCTION

Immunologic techniques are an effective means to detect the early events of fertilization. Various investigators have used them to study gamete surface components and their possible role in fertilization, cleavage, and early development. These macromolecular surface components readily elicit the production of antibodies when injected into an appropriate foreign species and such antibodies can be employed to identify, to determine the location, and isolate specific antigens and determine whether they perform an essential role in fertilization. It has been postulated that if an antigen or its active site plays a role in fertilization or subsequent development, the antibodies specific for that antigen will inhibit its normal function (Tyler, 1957; Edds, 1956; and Nace, 1955).

Antigen-antibody reactions suggest that these surface components may be involved in such processes as the

initial adherence of sperm to egg, the acrosomal reaction of the sperm, the penetration of the egg surface by the sperm, the initiation of the cortical reaction of the egg, and the establishment of the block to polyspermy (Monroy, 1965; Metz, 1967; Tyler, 1959, 1960, 1962; Perlman, 1957, 1959).

Lillie (1913, 1919) was the first to propose that interactions of an antigen-antibody type between surface components of sperm and egg seemed to form the basis for the initial adherence of the gametes in fertilization. This was suggested by observations of the phenomenon of agglutination of sperm by materials derived from eggs of the homologous species in sea urchins and in some other species of marine animals. To the principal interacting substances of egg and sperm, Lillie assigned the terms fertilizin and antifertilizin, respectively. He postulated a theory of fertilization in which the various events of fertilization, i.e. adherence of gametes, activation of the egg, establishment of the block to polyspermy, etc. were explained on the basis of interaction of groups present in these two types of molecules.

These observations have led to an extensive number of investigations on invertebrates, mainly with sea urchins,

concerning the role of these surface components in fertilization. Other studies demonstrate essential roles of fertilizin and antifertilizin, the effect of removal and addition of these materials on fertilization, and the effect of certain agents which block or inhibit fertilizin and antifertilizin (e.g. antisera) on the fertilizability of eggs or the fertilizing capacity of sperm (For reviews, see Tyler, 1959; Perlmann, 1959; Metz, 1961a, 1961b, 1967).

Although Lillie considered fertilizin to be continuously secreted by the egg while it remained in a fertilizable condition, later evidence has shown that fertilizin of sea urchins, and other marine animals, comprises the macromolecular material of the jelly coats of eggs (Tyler, 1940). It is also a constituent of the plasma membrane of the egg (Motomura, 1953; Hagstrom, 1956; Tyler, Seaton and Signoret, 1961). Antifertilizin has been found in the plasma membrane of the sperm (Tyler and O'Melveny, 1941), as well as in the interior of eggs (Tyler, 1940).

Tyler (1949) found fertilizin to be tissue and species-specific. A mixture of "anti-agglutinin" (sperm extract) and "agglutinin" (egg water) caused a visible reaction and neutralized sperm motility.

The fertilizability of eggs after mechanical or chemical removal of fertilizin from the egg (Tyler, 1941; Tyler and Metz, 1955), or after treatment of sperm with fertilizin (Tyler, 1941) has been studied.

Antisera specific for fertilizin, antifertilizin, and extracts of fertilized eggs, and of embryos at various developmental stages, were tested for effects on fertilization and early development (Tyler, 1959, 1963; Tyler and Brookbank, 1956a, 1956b). Cytotoxic and inhibitory effects were observed on unfertilized and fertilized eggs, among these was the inhibition of cleavage of eggs by antisera specific for fertilizin, or whole egg homogenates. The blocking action involved inhibition of nuclear as well as cytoplasmic division. Inhibition of cleavage by the antisera was interpreted as due to blocking of specific antigenic sites in the egg jelly by complementary antibodies in the antiserum.

Perlmann (1957, 1959) and co-workers, studying on the effect of antisera specific for egg extract and jelly coat preparations on sea urchin eggs, found that antisera specific for egg material rendered sea urchin eggs unfertilizable. However, in the eggs from a considerable proportion

of sea urchins, anti-egg serum induced parthenogenetic activation. Thus, in contrast with the commonly found inhibitory or cytotoxic effect of antibodies on cells, these investigators found that anti-egg serum had a stimulatory effect on sea urchin eggs. Four different types of antigens were detected in the surface layers of eggs, three of which seemed to have some function in sperm and egg attachment and egg activation.

In summary, fertilizin and antifertilizin are the specific receptor substances on the surfaces of the egg and sperm, respectively. It is the interaction of these complementary substances on their plasma membranes in a species-specific manner that accounts for the specific adherence of the sperm to egg and incorporation of sperm by a sort of pinocytotic process (Tyler, 1959, 1963, 1965).

Studies similar to those made on sea urchins have been done in Amphibian eggs (mostly Anurans) and they suggest that analogous substances (gamete surface components) and phenomena (antigen-antibody type of reaction) interact in the fertilization and development of this group. (For recent reviews, see Shaver, 1966, and Metz, 1967). Analysis of these surface components of the jelly coats of eggs with

respect to their possible role in fertilization has been the main concern of these studies. Such investigations, however, have not been so extensive as those done with sea urchins.

Various studies have revealed that the gelatinous envelopes around the eggs appear to be necessary for the normal interaction of gametes after insemination. It has been observed that jelly-less eggs of Anuran and Urodele species, either in the coelom of the female (before passage down the oviducts), or after physical (mechanical) or chemical removal of the jelly, are not generally fertilizable. (Bataillon, 1919; Good and Daniel, 1943; Kambara, 1953; Tchou-Su and Wang, 1956; Subtelny and Bradt, 1961; Katagiri, 1965, 1966; Barch and Shaver, 1960).

Coelomic, jelly-less frog eggs can be activated artificially, either by innoculation of cellular material (Bataillon, 1919), or by transfer of a blastula nucleus (Subtelny and Bradt, 1961). However, the latter investigators found that normal gastrulative processes and later differentiation were markedly enhanced by enrobing jelly-less embryos in gelatinous coats taken from uterine eggs.

Good and Daniel (1943) made similar observations with Triturus torosus, i.e. enrobement of coelomic eggs with egg jelly rendered the eggs fertilizable.

Kambara (1953) and Tchou-Su and Wang (1956) showed that oviducal eggs of Bufo bufo asiaticus could not be fertilized after removal of the jelly layers, but that re-enrobement with jelly restored their fertilizability. Kambara (1953) showed that dejellied eggs were rendered equally fertilizable when recoated either with jelly, or with a layer of gelatin or agar. He concluded that the role of the jelly coat in fertilization in the toad egg was to serve as physical substrate which elicited a thigmotactic response on the part of the spermatozoon, i.e., jelly envelopes supply a mechanical foothold which enables sperm to penetrate into the egg.

Contrary to Kambara's results, Katagiri (1965, 1966), working with Bufo bufo formosus, was unable to obtain an increase in the fertilizability of coelomic or dejellied eggs by artificial covering with gelatin, agar, or egg albumin. However, he found that a high percentage of chemically dejellied, uterine eggs were fertilized when they were inseminated in the presence of either dialyzed jelly material, pronase-digested jelly material, or polivinylpyrrolidone (PVP).

Additional evidence for a role of egg jelly in fertilization has come from experiments with antisera prepared

by immunizing rabbits with jelly obtained from uterine eggs (Shaver and Barch, 1960). Pretreatment of R. pipiens eggs with such antisera, before fertilization, markedly reduced their fertilizability, as expressed by the production of a significant decrease in cleavage (Shaver and Barch, 1960). Such inhibitory action was interpreted as resulting from blocking of jelly receptor sites and not by mechanical interference with sperm penetration, e.g. by precipitating the egg jelly, because an antiserum rendered non-precipitating (univalent) by papain digestion also inhibited fertilization (Shivers and Metz, 1962). It was also observed that when spermatozoa are treated with anti-jelly serum, there is a significant decrease (reduction) in the cleavage of normal eggs inseminated with them (Shaver and Barch, 1960).

Furthermore, Shaver and Barch (1966) have observed many times that the degree of inhibition of cleavage is positively correlated with the failure of treated eggs to undergo the prior events of early development, e.g. rotation of orientation, emission of polar bodies, etc. Thus, failure of treated eggs to cleave is most probably due to failure of sperm contact or penetration as a result or blocking of jelly-receptor sites.

Shaver, Barch and Shivers (1962), using the double diffusion technique, found that the antigenic components of R. pipiens egg jelly are tissue specific, i.e., they are specific to the egg jelly, and to the oviducal cells which secrete them, since absorption of the anti-jelly serum with sperm, or extracts of a variety of tissues, including ovary, failed to affect the inhibition of fertilization induced by anti-jelly serum.

Recently Shivers (as cited by Shaver, 1966) found that body-cavity (jellyless) eggs of R. pipiens can be successfully fertilized by spermatozoa (as evidenced by raising of fertilization membranes) which have had prior contact with uterine (jellied) eggs.

In conclusion, these experiments seem to indicate that an interaction between spermatozoa and jelly-coat materials is necessary before the former can fertilize frog eggs.

Further evidence as to an apparently essential role of egg jelly in fertilization is associated with the fact that uterine eggs fertilize readily. Uterine eggs, in contrast to coelomic, jelly-less eggs, are surrounded by various jelly layers, e.g. three visible ones in R. pipiens

(Nace et al., 1961), four in Hyla arborea japonica (Katagiri, 1963), and four in Rana temporaria (Katagiri, 1961). At least five antigenic components have been found in each of seven other amphibian egg jellies by agar-gel precipitin tests (Shivers, 1965). In R. pipiens, some of these components are not uniformly distributed throughout the jelly capsule, but are confined to certain jelly layers (Shivers, 1962). Localization of antigens in the jelly layers was done by treating eggs with fluorescein-conjugated anti-R. pipiens jelly serum.

The different jelly layers (and thus the antigenic components present in them) are deposited around the egg during their passage down the oviduct (Rugh, 1951). Oviducal gland cells are the source of these jellies (Lee, 1967; Humphries, 1964).

Other experiments have indicated the acquisition of fertilizability by eggs after passage down the ofiduct (Arnold and Shaver, 1962; Tchou-Su and Wang, 1956; and Kambara, 1953). Kambara (1953) observed that eggs of the toad (Bufo bufo asiaticus), having only the innermost layer of jelly, which is secreted in the upper region of the oviduct, were not fertilizable; however, fertilizability increased as the next three

layers were added. Tchou-Sou and Wang (1956), working with the same species, tested also for the relative importance for fertilizability of the outer and inner layers of jelly and found similar results.

Glick and Shaver (1963) have shown that R. pipiens eggs become progressively more fertilizable as they descend down the oviduct, being quite refractory in the upper segment, and attaining a degree of fertilizability of about half of uterine eggs in the middle and lower portions. These results were interpreted as suggesting that the jelly secreted around the eggs in the upper portion of the oviduct lacked some essential factor or factors necessary for completely successful sperm-jelly interactions, and that such factor(s) was(were) acquired only when the middle and outer layers of jelly were placed around the egg, in the corresponding regions of the oviduct.

Further investigations were done to see if different antigenic components could be localized in the jellies obtained from different levels of the oviduct. Barch and Shaver (1963) treated mature, uterine eggs of R. pipiens with antisera specific for upper and lower thirds of the oviduct, prior to insemination. The results showed that

the percentage of fertilizability and cleavage of eggs treated with antiserum specific for the lower segment is significantly lower than that of eggs treated with antibodies against the upper segment. Antisera specific for the middle oviduct were least inhibitory of fertilization as compared with the effect of antisera against either whole, upper, or lower oviduct. However, when eggs were treated with antibodies specific for antigens of the whole oviduct, the value for the fertilization and cleavage of eggs was several times lower than that for either of the isolated segments (upper and lower). This was interpreted as indicating a possible contribution of middle segment antigens toward the inhibitory effect of anti-whole serum.

Finally, it must be said that the antigens present in these oviducal extracts evidently represent antigens (receptor sites) present in the egg jelly, because anti-egg jelly serum produced the same immunoprecipitation-band pattern in agar-gel diffusion tests (Barch and Shaver, 1963).

In conclusion, it has been established that fertilizability of uterine eggs, at least in part, is due to the presence of various jelly components which are secreted around the eggs in their passage down the oviduct.

Furthermore, these different antigenic components produced at different oviducal levels seem to differ with respect to their effect upon the fertilizability of eggs.

The next step in the study of the possible role of egg jelly in fertilization was that of determining what jelly component or components were essential and what roles they perform. Shivers (1961), in a systematic analysis of agar diffusion plates, showed the patterns of egg jelly antigenic components in four species of Rana. Some of these jelly components were shared among all four species, some were common to 2 or 3 of the species, i.e. were non-species-specific antigens; and several were species-specific. Of the minimum of 5 different egg-jelly antigens found, 2 or 3 types of species-specific antigens were present in each species of Rana (Shivers, 1965).

Shivers (1965) also identified species-specific and shared (non-species-specific) jelly antigens in two species of Bufo (B. americanus and B. marinus) and in one species of Urodele (Ambystoma mexicanum).

In an attempt to localize the species-specific and non-species-specific (shared) antigenic components of Rana pipiens egg jelly, Shivers (1962) made use of fluorescein-conjugated antibodies. When R. pipiens eggs were treated

with fluorescein-conjugated heterologous anti-jelly sera (Rana sylvatica and R. clamitans) only the innermost layer fluoresced. Following absorption with the heterologous jellies of the latter two species, anti R. pipiens jelly sera produced fluorescence in the outer and middle layers, but not in the innermost. Thus, it seemed that the innermost of the three readily visible layers contains only common components (non-species-specific antigens), whereas the middle and outer layers contained the species-specific antigens.

To evaluate the role of these species-specific and non-species-specific jelly antigens in fertilization, Shivers (1961, 1962) tested Rana pipiens eggs with heterologous (R. sylvatica, R. catesbiana, and R. clamitans) anti-jelly sera, and anti-Rana pipiens jelly sera absorbed with jellies from these 3 species to see if they inhibited fertilization. All of the heterologous anti-jelly sera (anti-R. clamitans, anti-R. sylvatica, and anti-R. catesbiana) inhibited fertilization of Rana pipiens eggs. Likewise, anti-Rana pipiens jelly serum, even following absorption with heterologous jelly, strongly inhibited fertilization of Rana pipiens eggs.

From these experiments Shivers concluded that at least two antigenic types are involved in fertilization. One type consists of non-species-specific (common) antigens confined to the innermost layer. The other type is comprised of species-specific antigens in one or both of the outer layers of jelly. Combination of either of these two antigenic types with homologous antibodies results in inhibition of fertilization.

Finally, it must be said that egg antigenic components of Anuran eggs are not limited only to the jelly layers. Lavin (1963) and Nace and Lavin (1963), upon treatment of coelomic (jellyless) eggs with antisera specific for mature ovaries and embryonic stages of Rana pipiens, found an antigen on the surface of mature oocytes and fertilized eggs. Antisera against this antigen (F) inhibited activation and cleavage.

On the basis of all of these experiments Shaver (1966) has formulated an hypothesis for the possible operation of jelly antigens in the fertilization of Anuran eggs. He suggests that egg jelly antigens are responsible for a series of reactions which involve the complexing of both species-specific and common (non-species-specific) combining sites

on the sperm surface and in the jelly layers, followed by reactions with complementary sites on the egg surface proper.

The present investigation was done with the purpose of elucidating further the possible roles of gamete surface components on Anuran sperm-egg jelly interactions during fertilization. It had two main objectives: the first part involved testing for the effects of treating eggs of Rana pipiens, prior to fertilization, with antisera specific for jelly material obtained from different oviducal levels of Rana pipiens. Such studies could reveal if there was any regional variation in the antigenic components of oviducal jellies corroborating previous investigations (Barch and Shaver, 1963).

The second part involved testing for the effects of treating Rana pipiens eggs with antisera of the same types used in the first part, but which had been previously absorbed with whole oviducal jellies of heterologous and homologous Amphibian species. Such experiments would determine the presence of common, as well as species-specific, antigenic components in oviducal jellies and their relative importance in initial interactions of sperm and egg-jelly.

MATERIALS AND METHODS

GENERAL PROCEDURE FOR OBTAINING JELLIES

Whole oviducts (having no eggs) were dissected from females of four species of Rana (Rana pipiens, R. clamitans, R. sylvatica, and R. catesbiana), two species of Bufo (Bufo marinus and B. americanus), and an Urodele species, Ambystoma mexicanum. The oviducts were cut into small pieces and placed in a beaker containing distilled water. Such a mixture was mechanically agitated for several hours to insure that the oviducal jelly, i.e. whole jelly, diffused into the medium, with its subsequent hydration. The mixture was filtered through glass wool to separate the jelly from any remaining oviducal tissue. The jellies were lyophilized in a Virtis freeze-mobile and stored in a dessicator for future use. All jellies used in these experiments were reconstituted by dissolving five mg. of the powdered jelly per ml. of one-tenth percent full strength Holtfreter's solution (standard antigen solution), and blended the mixture in an homogenizer for at least 30 minutes in a cold room (4-5°C).

Whole oviducts were dissected from female R. pipiens, stretched out on a piece of glass, and cut into 3 equal pieces, i.e., an upper, a middle, and a lower region. Jellies from the segments of each oviducal region were obtained as described above.

PRODUCTION OF ANTI-JELLY SERA

The jelly antigen preparations consisted of 5 ml. of the standard antigen solution emulsified in an equal volume of Freund's complete adjuvant. One and a half milliliters of these emulsions were injected, half on each side, into the subscapular region of rabbits. An equal amount in incomplete Freund's adjuvant, was injected one week later. In 3-4 weeks, if antibodies could be detected on agar-diffusion plates, bleedings from the ear vein were begun and continued every other week. Antigen injections were repeated every six weeks to two months. Blood was collected from the rabbits prior to the first antigen injections and the sera were pooled and used as control sera.

Antisera specific for the jellies obtained from the upper, middle, and lower thirds, as well as from the whole

oviducts of all the species employed. Unfractionated antisera specific for the same antigen obtained from different rabbits were pooled before testing on the eggs. The presence of the antigenic components of the various jellies was detected by means of a modification of the Ouchterlony double-diffusion technique (Shaver, 1961). The plates were prepared employing 1% agar (Ionogar, obtained from Consolidated Laboratories, Chicago Heights, Illinois), and developed at room temperature (20°-22°C) for 5 days. When fully reacted, the plates were photographed for a permanent record.

All dilutions of sera used in treating eggs were made with one-tenth percent full strength Holtfreter's solution. Dilutions out to and including 1:5 were made for the first set of experiments, while for the second set dilutions out to and including 1:405 were used.

ABSORPTION PROCEDURES

Full strength antisera against jellies from the different oviducal regions, as well as from whole oviducts, of R. pipiens were absorbed with whole oviducal jellies obtained

from several Amphibian species (R. pipiens, R. sylvatica, R. clamitans, R. catesbiana, R. marinus, B. americanus, and A. mexicanum). Equal volumes of antiserum and of the jellies (5 mg/ml) were used in each absorption. The antigen-antibody mixtures were shaken well to insure even mixing and were let stand in the refrigerator (4°C) for a minimum of 24 hours. This was followed by centrifugation to remove any unabsorbed jelly. The supernatant was then used as a test antiserum. Unabsorbed anti-sera (used as controls in the absorption experiments) were mixed with an equal volume of one-tenth percent full strength Holtfreter's solution to maintain equal dilutions in the absorbed and unabsorbed sera.

EXPERIMENTAL PROCEDURE FOR THE EXTERNAL TREATMENT OF RANA PIPIENS EGGS

Mature eggs were obtained by inducing ovulation in Rana pipiens females at room temperature. Ovulation was induced in one of two ways: by injection of a total of 5 whole pituitaries for two successive days intraperitoneally (Rugh, 1934), or by injecting 0.25 cc of progesterone (Wright and

Flathers (1961) dissolved in oil (Mazola) into the dorsal lymph sacs plus an intraperitoneal injection of one pituitary, 24 hours before the experiment. All pituitary injections were made using one-tenth percent full strength Holtfreter's solution.

A small number (30-45) of eggs were squeezed directly onto glass slides. Then they were treated for two minutes with the sera to be tested. After two minutes, the eggs were washed thrice with one-tenth percent full strength Holtfreter's solution, and then fertilized with a sperm suspension (2 testes per 20 ml one-tenth percent full strength Holtfreter's solution). Ten minutes were allowed for insemination. Then the excess sperm solution was decanted from the slide and the fertilized eggs were immersed in aerated tap water in finger bowls. Egg cultures were kept at room temperature (18-20°C). After three hours, the eggs were examined to see if cleavage had occurred and the percentage of cleavage for each treatment was determined.

Percentage values were transformed into arcsin equivalents in accordance with the statistical test employed (i.e. an analysis of variance). In each of the 3 sets of experiments done, eggs from 20 or 21 different females were tested

against the different antisera, as well as against control sera (obtained by bleeding the rabbits before immunization) and one-tenth percent full strength Holtfreter's solution (normal treatment). The eggs from a female were not used more than once in testing each series of treatments. Furthermore, only those R. pipiens females, whose eggs showed from 75 to 100 percent normal fertilizability upon preliminary testing, were used here.

The data collected was analyzed statistically according to two methods:

(1) The method of analysis of variance (Snedecor, 1956; Bliss, 1967). This was used to determine whether the variations due to the factors being tested in the experiments, i.e., possible differences in the inhibitory effects of anti-jelly sera (unabsorbed, absorbed, and diluted sera) from different oviducal regions on egg cleavage; as well as variation due to interactions between factors, were both significantly different from chance at the 1% level.

(2) The sequential Q method (Snedecor, 1956) or Duncan Multiple Range test (Bliss, 1967). This was used to compare the order of variation in the percentages of cleavage of the eggs, after various treatments with the anti-jelly sera, e.g. with unabsorbed sera, absorbed sera and diluted sera.

RESULTS

EFFECTS OF ANTISERA SPECIFIC FOR DIFFERENT OVIDUCAL JELLIES ON THE FERTILIZABILITY OF *RANA PIPIENS* EGGS

Preliminary studies in this laboratory (Barch and Shaver, 1963) had revealed that if antisera prepared against whole oviduct homogenates of *R. pipiens* are tested against jelly antigens obtained from the upper, middle, and lower oviduct, regional antigenic differences were observed. Analysis of the double-diffusion plates showed the presence of common antigenic components between middle and lower jellies, which were not present in the upper jelly. Other antigenic components were common to the three regions. Also components unique to certain regions were observed.

In view of these results, the antisera specific for the jellies obtained from the different oviducal regions (whole, upper, middle, and lower) in this study were analyzed. Plate 1, Figure 1, shows the immunoprecipitation bands obtained when reacting such antisera with *R. pipiens*

PLATE I

Figure 1.--Ouchterlony plate showing immunoprecipitation bands obtained when testing Rana pipiens whole oviducal jelly (center well) versus various antisera specific for jellies obtained from different oviducal levels of R. pipiens (outer wells).

ovj----Antiserum specific for R. pipiens whole oviducal jelly.

upper--Antiserum specific for R. pipiens upper oviducal jelly.

mid----Antiserum specific for R. pipiens middle oviducal jelly.

low----Antiserum specific for R. pipiens lower oviducal jelly.

C------Non-immune rabbit serum (Control).

u------Immunoprecipitation band representing components unique to upper oviducal jelly.

ml-----immunoprecipitation band representing component(s) common to middle and lower oviducal jellies.

PLATE I

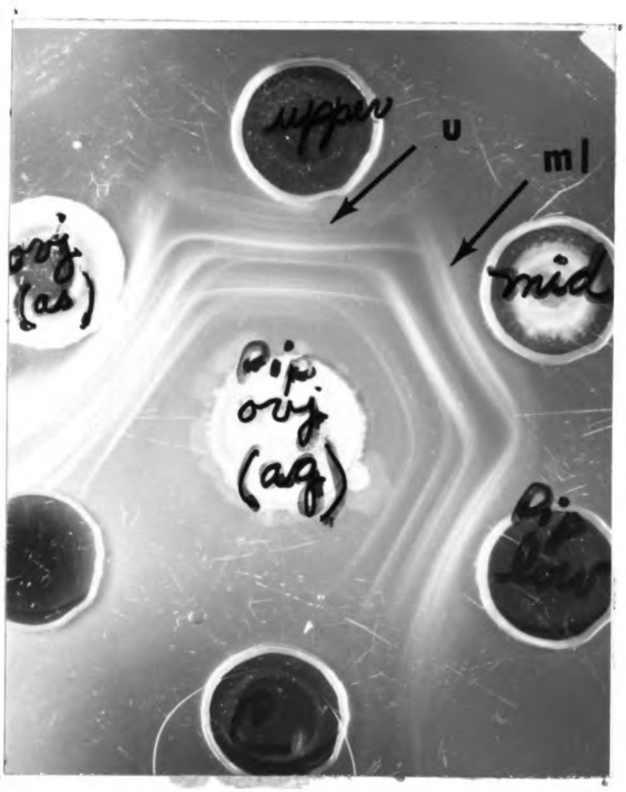


FIGURE 1

whole oviducal jelly (center well). The plate shows a component(s) common to jellies from middle and lower oviducal regions (See arrow- ml), and components unique to the upper region (See arrow- u).

Plate II, Figures 2, 3, 4, and 5, show the immunoprecipitation bands obtained when reacting dilutions (1:2, 1:3, 1:5) of the above antisera (anti-whole, anti-upper, anti-middle, and anti-lower oviducal jelly sera, respectively) with R. pipiens whole oviducal jelly (center well). Upon dilution of the four antisera tested, the immunoprecipitation bands get weaker (lighter). However, essentially the same number of bands remains upon dilution.

It must be pointed out here, as will be seen later (Text figure 1), that serum dilutions of 1:5 still have a significant inhibitory effect on egg cleavage, as compared with the controls, which show no inhibitory effect.

The results obtained after inseminating eggs that had been previously treated with antisera specific for the different oviducal jellies are presented in Table 1 and in text figure 1. For individual cleavage percentages see Appendix (Table I). Full-strength antisera specific for the jellies obtained from whole, upper, middle, and lower

PLATE II

Ouchterlony plates showing immunoprecipitation bands obtained when testing Rana pipiens whole oviducal jelly (center well) versus various dilutions of antisera specific for jellies obtained from different oviducal levels of R. pipiens (outer wells).

Figure 2.--Anti-whole jelly serum dilutions: 1:2, 1:3, and 1:5 (from left to right).

Figure 3.--Anti-upper jelly serum dilutions: 1:2, 1:3, and 1:5 (from left to right).

Figure 4.--Anti-middle jelly serum dilutions: 1:2, 1:3, and 1:5 (from left to right).

Figure 5.--Anti-lower jelly serum dilutions: 1:2, 1:3, and 1:5 (from left to right).

PLATE II

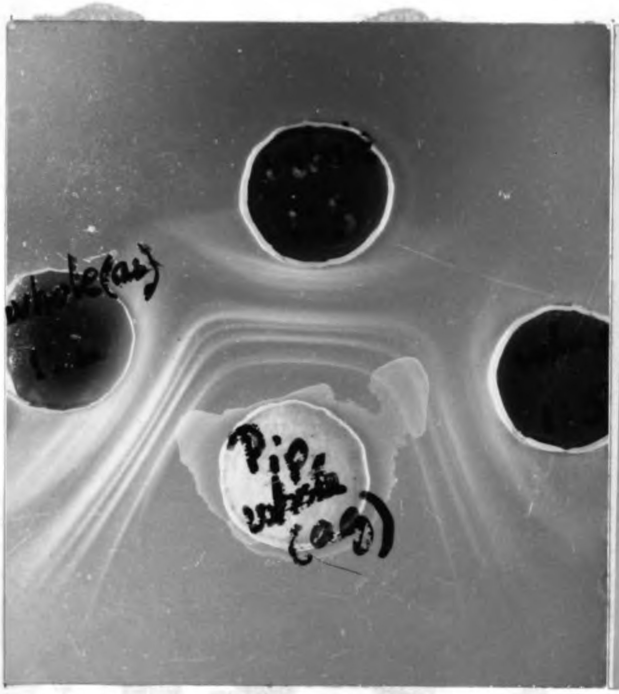


FIGURE 2

FIGURE 3

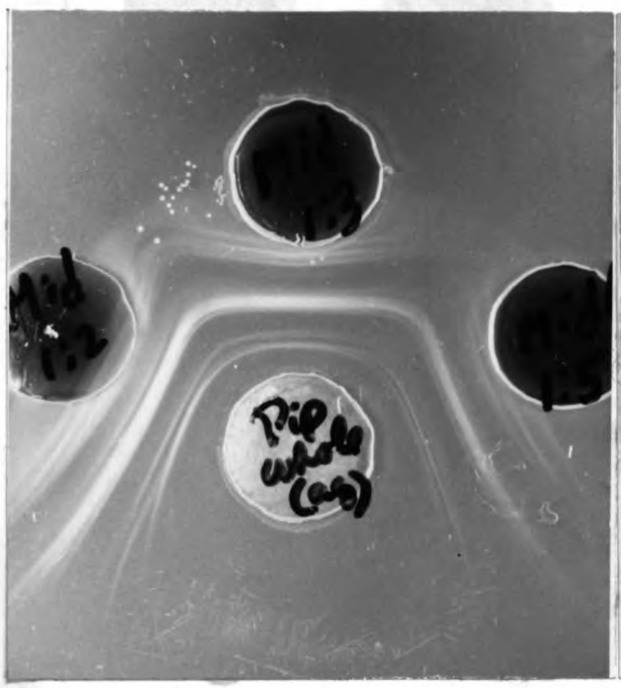


FIGURE 4

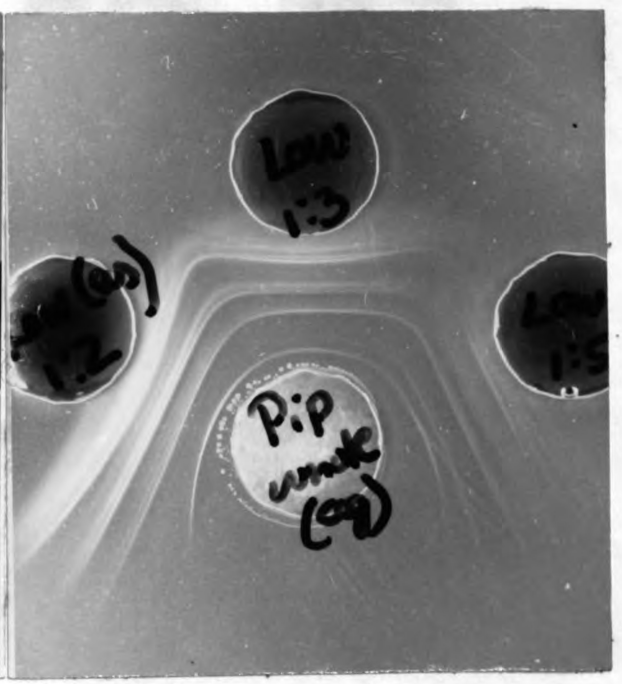
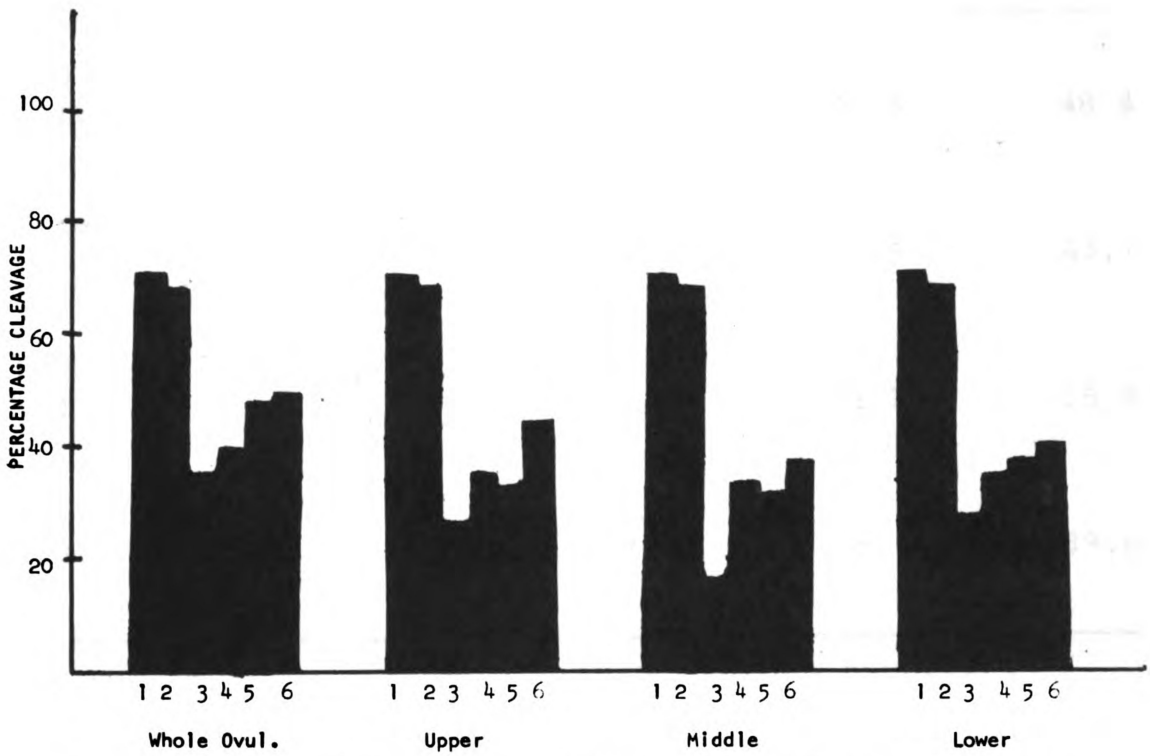


FIGURE 5



TEXT FIGURE 1. CLEAVAGE OF RANA PIPIENS EGGS (INSEMINATED WITH NORMAL SPERMATOZOA) AFTER PREVIOUS TREATMENT WITH: HOLTFRETER'S SOLUTION (BARS 1), NORMAL RABBIT SERUM (BARS 2), FULL STRENGTH ANTI-JELLY SERUM (BARS 3), 1:2 SERUM DILUTION (BARS 4), 1:3 SERUM DILUTION (BARS 5), AND 1:5 SERUM DILUTION (BARS 6).

TABLE 1.--Mean arc sin equivalents of percentages of Rana pipiens eggs reaching cleavage stages after exposure to antisera specific for R. pipiens jellies from different regions of the oviduct.[a,b]

Antiserum Treatment	Full Strength	Dilution		
		1:1	1:2	1:4
Whole jelly	34.4	38.9	47.6	48.4
Upper 1/3 jelly	25.5	34.4	31.6	43.7
Middle 1/3 jelly	16.4	33.1	31.1	36.9
Lower 1/3 jelly	26.7	34.3	36.7	39.6

[a]Values for controls were as follows: 0.1% Holtfreter's soln (normal)--70.01%, non-immune serum (control)--68.2%.

[b]See Appendix [Table II] for analysis of variance of these data.

oviduct, as well as 1:2, 1:3, and 1:5 dilutions of each serum were tested. Unfractionated serum was used in all cases. Bars 1 (Text Figure 1) represent the eggs treated with one-tenth full strength Holtfreter's solution, while Bars 2 represent the eggs treated with non-immune serum. Both treatments served as controls. Each percentage in the bar graph is a mean for the percentage values from 20 different frogs. A one-way analysis of variance of the data (See Appendix Table II) indicated that significant differences observed between any two mean values are significant at the 1% level. The results, as seen from Text Figure 1, are as follows:

(1) The fertilizability of eggs (as expressed by the percentages of eggs that cleaved) after treatment with any of the full-strength anti-jelly sera (Bars 3) was lower by a significant degree from that of the controls (Bars 1 and 2). In other words, all the full-stength antisera tested showed an inhibitory effect on cleavage as shown by comparison with the controls. No significant difference between these 2 types of controls was observed. Therefore, it seems that there is present in these anti-jelly sera a substance or substances which significantly inhibit(s) the

fertilization reaction, or some post-fertilization phenomena, leading to cleavage.

(2) Among the full-strength antisera tested (Bars 3), anti-middle jelly serum is the most inhibitory, as expressed by the lower cleavage percentage in comparison with the other full-strength antisera.

(3) Furthermore, the full-strength anti-whole, anti-upper, and anti-lower sera (See Bars 3) do not differ significantly from each other with respect to their inhibitory effect on cleavage. A similar and consistent effect of these three antisera is observed upon dilution (1:2, 1:3, 1:5). For example, there is no significant difference between the cleavage percentages of eggs treated with whole 1:2, dilution, an upper 1:2 dilution, and a lower 1:2 dilution (Bars 4). The same holds true for the 1:3 dilutions (Bars 5) and the 1:5 dilutions (Bars 6).

However, when one compares the effect of anti-middle serum dilutions with the same dilutions of antisera specific for other jellies, one finds that the middle dilutions differ significantly only from the anti-whole dilutions. For example, middle 1:2 dilution is significantly different from 1:2 whole dilution, but not from an upper 1:2 dilution and lower 1:2 dilution (Bars 4).

In view of such results, further dilutions were done only of these two antisera (anti-whole and anti-middle) to see at what titer their inhibitory effect upon cleavage disappeared. A series of 3-fold dilutions of both antisera was done (starting with a 1:5 serum dilution).

Plate III, Figure 6, shows a double-diffusion plate of whole jelly antigen (in the center well) versus full-strength anti-whole serum, as well as the different dilutions (1:2, 1:3, 1:5, 1:15, 1:45). In this plate immunoprecipitation bands are seen up to 1:15 dilution. Plate III, Figure 7, shows that no bands are obtained with control serum (non-immune serum), as well as with 1:135 and 1:405 dilutions.

In Plate III, Figure 8, whole jelly antigen (in the center well) was tested against full-strength anti-middle serum, as well as with the different dilutions (1:2, 1:3, 1:5, 1:15, 1:45). Immunoprecipitation bands are seen up to 1:45 dilution. Plate III, Figure 9, shows also that no bands are obtained with the non-immune serum, as well as with dilutions of middle antiserum higher than 1:45 (with dilutions 1:135 and 1:405).

PLATE III

Ouchterlony plates showing immunoprecipitation bands obtained when testing Rana pipiens whole oviducal jelly (center well) versus various dilutions of R. pipiens anti-whole and anti-middle oviducal jelly sera (outer wells).

Figures 6 and 7.--Various dilutions of R. pipiens anti-whole oviducal jelly serum (ovj): FS (Full strength), 1:2, 1:3, 1:5, 1:15, 1:45, 1:135, and 1:405 dilutions. Non-immune rabbit serum (Con).

Figure 8.--Various dilutions of R. pipiens anti-middle oviducal jelly serum (Mid): FS (Full strength), 1:2, 1:3, 1:5, 1:15, 1:45 dilutions (Starting at 10 o'clock and reading clockwise).

Figure 9.--Further dilutions of R. pipiens anti-middle oviducal jelly serum (Mid): 1:135, 1:405 dilutions. Non-immune rabbit serum (C), (Read clockwise).

PLATE III

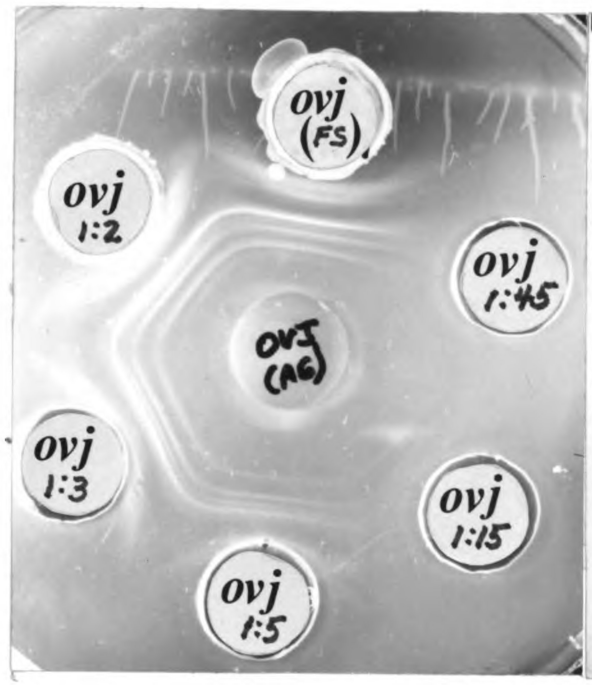


FIGURE 6

FIGURE 7

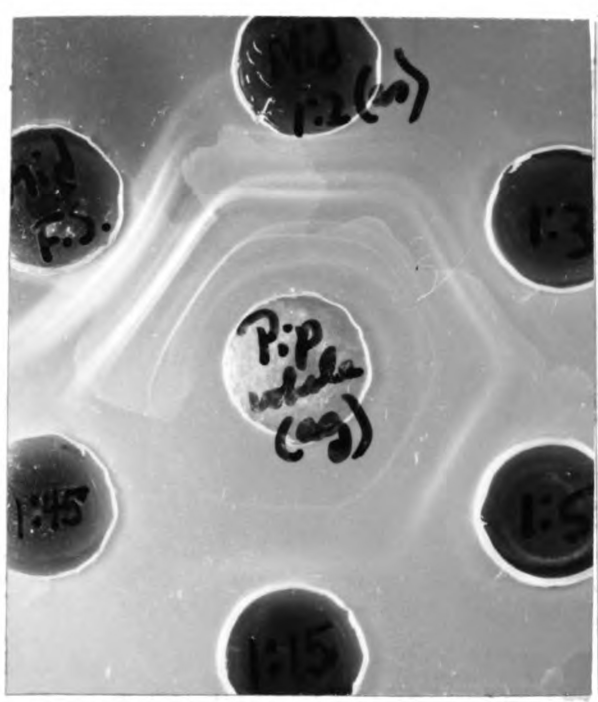


FIGURE 8

FIGURE 9

The effect of anti-whole and anti-middle serum dilutions upon cleavage of eggs was also tested. Percentage cleavage values appear in Table 2 and in Text Figure 2. For individual cleavage percentages see Appendix Table III. A one-way analysis of variance of the data (See Appendix Table IV) indicated that significant differences observed between any two mean values are significant at the one percent level. In this experiment only antisera starting with a 1:5 dilution were used, since the effect of full-strength antisera and 1:2 and 1:3 dilutions had been previously tested (as seen in Text Figure 1). Appropriate controls were used (Bars 1 and 2). The results were as follows:

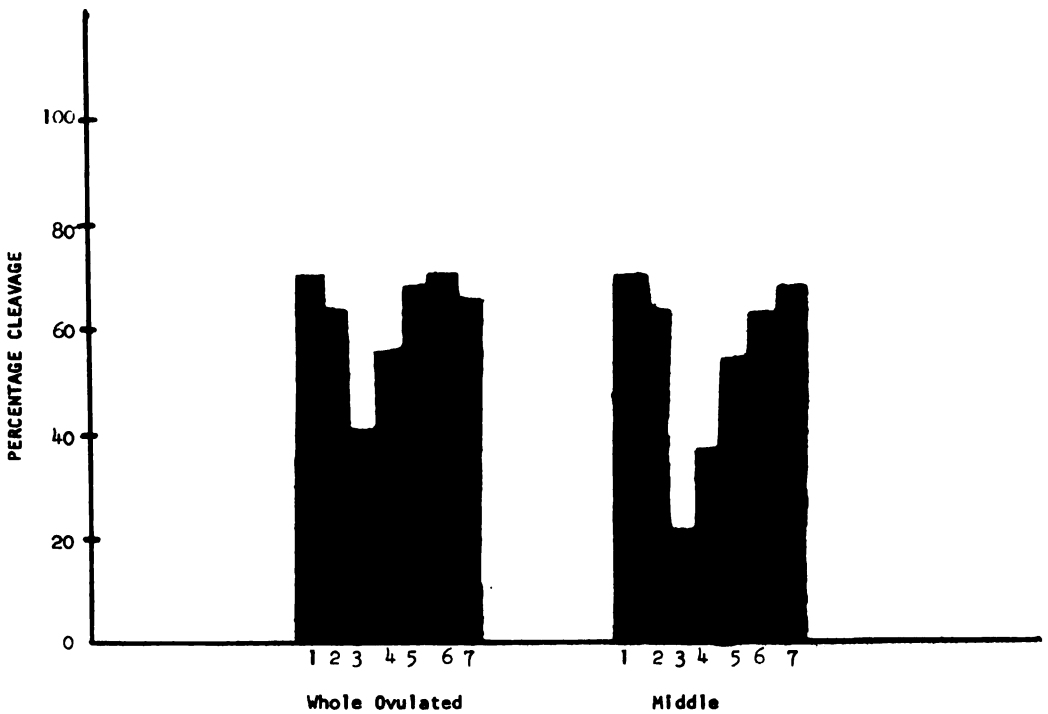
(1) Percentage cleavage values for middle serum dilutions out to and including 1:45 (Bar 5) are significantly lower than both control treatments (Bars 1 and 2). No significant difference between the percentage cleavage values of the two controls was observed. As the serum is diluted out, you get a less inhibitory effect (higher cleavage percentages). In each case, the effects of these dilutions are significantly different from each other, i.e. the 1:5 dilution (Bar 3) has a lower percentage cleavage value (a higher inhibitory effect) than the 1:15 dilution (Bar 4); in turn

TABLE 2.--Mean arc sin equivalents of percentages of Rana pipiens eggs reaching cleavage stages after exposure to various dilutions of whole and middle 1/3 anti-jelly sera obtained from R. pipiens oviduct.[a,b]

Antiserum Treatment	Dilution				
	1:5	1:15	1:45	1:135	1:405
Whole jelly	39.5	54.4	67.3	69.2	64.5
Middle 1/3 jelly	20.1	35.8	53.4	62.3	66.7

[a]Values for controls were as follows: 0.1% Holtfreter's soln (normal)--68.8%, non-immune serum (control)--62.1%.

[b]See Appendix [Table IV] for analysis of variance of these data.



TEXT FIGURE 2. CLEAVAGE OF RANA PIPIENS EGGS (INSEMINATED WITH NORMAL SPERMATOZOA) AFTER PREVIOUS TREATMENT WITH: HOLTFRETER'S SOLUTION (BARS 1), NORMAL RABBIT SERUM (BARS 2), 1:5 SERUM DILUTION (BARS 3), 1:15 SERUM DILUTION (BARS 4), 1:45 SERUM DILUTION (BARS 5), 1:135 SERUM DILUTION (BARS 6), AND 1:405 SERUM DILUTION (BARS 7).

the 1:15 dilution has a lower percentage cleavage value than the 1:45 dilution (Bar 5). Dilutions 1:135 and 1:405 have no inhibitory effect on egg cleavage.

(2) On the other hand, the percentage cleavage values for anti-whole serum dilutions out to and including 1:15 (Bar 4) are significantly lower than the controls, and significantly different from each other. As the serum is diluted out, you get a less inhibitory effect (higher cleavage percentages). Dilutions 1:45, 1:135, and 1:405 have no inhibitory effect on cleavage. It must be said also that for each serum dilution which had significant effect upon cleavage, anti-middle serum always showed a lower percentage cleavage than the corresponding dilution with anti-whole serum.

Thus, a correlation has been observed, in the case of anti-whole jelly serum between the dilution that shows immunoprecipitation bands and the dilution which shows the last significant inhibitory effect on cleavage. Both dilutions coincide at the 1:15 dilution. The same can be said with regard to anti-middle jelly serum. Immunoprecipitation bands were shown up to 1:45 dilution, as well as an inhibitory effect at the same dilution.

The following conclusions can be drawn from these experiments:

(1) Antisera specific for whole, upper, middle, and lower oviducal jellies have an inhibitory effect upon egg cleavage, as compared with the percentage cleavage values of the controls. Such inhibitory effect on the cleavage of Rana pipiens eggs may be due to interactions between antigenic components present in the oviducal jellies and their homologous antibodies present in the antisera specific for such jellies. Such interactions could present a barrier to sperm penetration and thus inhibit fertilization and further cleavage.

(2) These results may be interpreted as indicating that there is more than one antigenic component (surface component) involved in the initial interaction between egg-jelly and sperm. Some of these surface components seem to be present in all oviducal regions, while others seem to be unique to particular regions.

(3) Anti-middle jelly serum has the most inhibitory effect on cleavage, as compared with the effects of sera specific for other oviducal regions. Even at a 1:45 dilution, there is an inhibitory effect on cleavage, while with

anti-whole serum, such an effect lasts only up to 1:15 dilution. An explanation as to the variation in inhibitory effect of the various antisera on egg cleavage will be given later in the discussion.

EFFECTS OF ANTISERA SPECIFIC FOR DIFFERENT OVIDUCAL JELLIES ABSORBED WITH WHOLE JELLIES OF SEVERAL SPECIES OF AMPHIBIANS ON THE FERTILIZABILITY OF *RANA PIPIENS* EGGS

Analysis of the Anti-Oviducal Sera

Analysis of Antisera Specific for Whole Oviducal Jelly of Rana Pipiens

Plate IV, Figure 10, shows an Ouchterlony plate in which *R. pipiens* anti-whole oviducal jelly serum (center well) was tested against whole oviducal jellies of several species of Amphibians, i.e. *Rana pipiens* (Pip OVJ), *Amby-stoma mexicanum* (AX OVJ), *Bufo americanus* (To OVJ), *B. marinus* (MAR OVJ), *R. catesbiana* (Cat OVJ), *R. clamitans* (Clam OVJ). A number of antigenic components (or determinant sites) are shared between *R. pipiens* anti-whole jelly serum and the

PLATE IV

Ouchterlony plates showing antigenic relationships between Rana pipiens anti-whole and anti-upper oviducal jelly sera and the whole oviducal jellies from different species of Amphibians: Pip--R. pipiens, Ax--Ambystoma mexicanum, To--Bufo americanus, Mar--B. marinus, Cat--R. catesbiana, and Clam--R. clamitans.

Figure 10.--R. pipiens anti-whole oviducal jelly serum, i.e., Pip ovj (as)--(center well) tested against various whole oviducal jellies (outer wells).

Figure 11.--R. pipiens whole oviducal jelly (Pip ovj--center well) tested against R. pipiens anti-whole oviducal jelly serum absorbed with various whole oviducal jellies (outer wells).

Figure 12.--R. pipiens anti-upper oviducal jelly serum (Pip uj (as)--(center well) tested against various whole oviducal jellies (outer wells).

Figure 13.--R. pipiens whole oviducal jelly (Pip ovj--center well) tested against R. pipiens anti-upper oviducal jelly serum absorbed with various whole oviducal jellies (outer wells).

PLATE IV

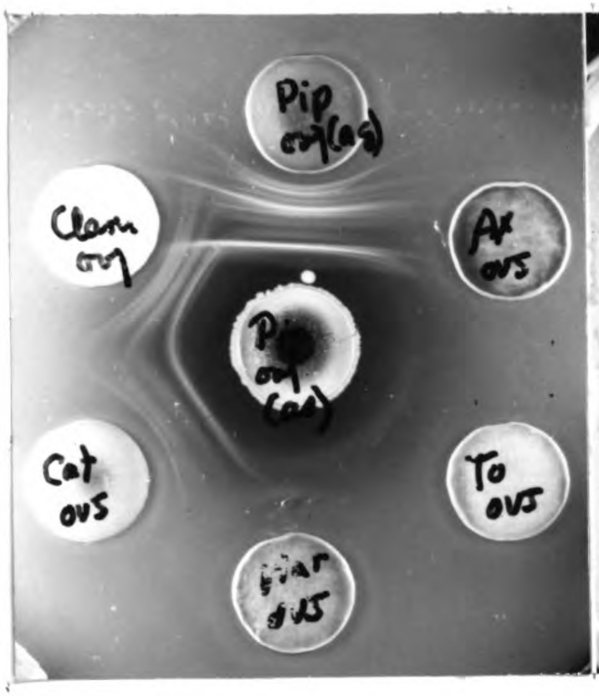


FIGURE 10

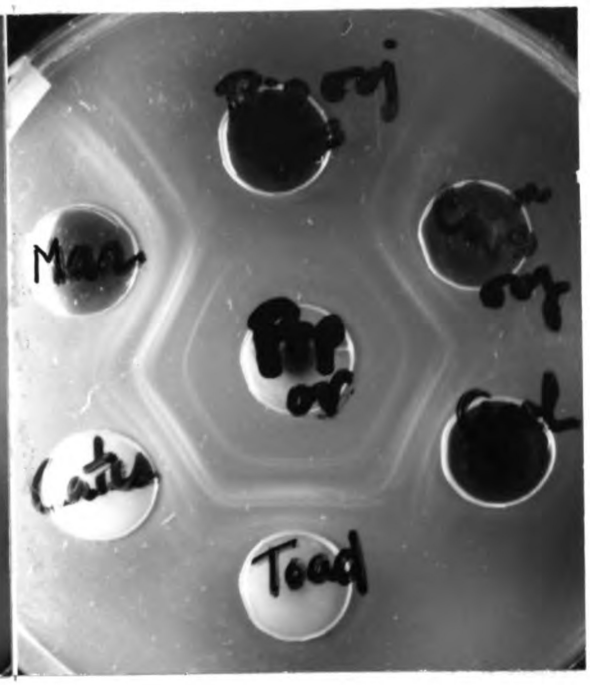


FIGURE 11

FIGURE 12

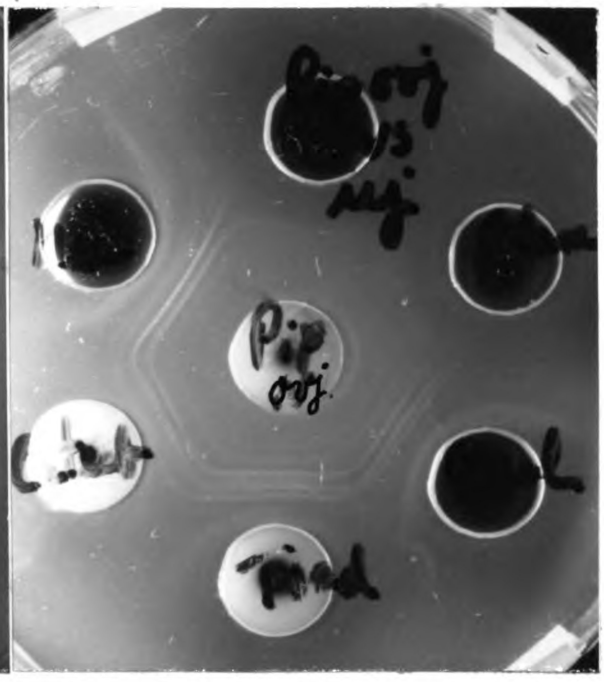


FIGURE 13

whole jellies of homologous and heterologous species. Rana pipiens whole jelly has at least 8 different antigens present, as evidenced by the fact that 8 immunoprecipitation lines were formed between the antigen and its homologous serum. Rana clamitans and R. catesbiana share at least 4 to 5 antigens (or determinant sites) with R. pipiens anti-whole jelly serum. Furthermore, at least 2 of these antigenic components are common to the three species of Rana tested, as evidenced by the confluence of their respective immunoprecipitation lines. Such results are consistent with those of Shivers (1961, 1965), who found that R. pipiens whole egg jelly shared at least 2 antigenic components with the whole jellies of R. clamitans and R. catesbiana. Rana pipiens anti-whole oviducal jelly serum does not share antigenic components with the whole jellies of B. marinus, B. americanus, and A. mexicanum.

Although Rana sylvatica whole jelly was used in the absorptions, which will be discussed later, Ouchterlony plates of this jelly were not run with unabsorbed or absorbed sera specific for any of the oviducal jellies of R. pipiens because of the unavailability of the material at the time. However, it is known from Shivers work (1961, 1965) that

Rana sylvatica whole jelly shares at least 2 antigenic components with anti-whole Rana pipiens egg jelly serum. Furthermore, such common components would be removed or neutralized upon absorption of Rana pipiens anti-oviducal jelly sera with Rana sylvatica whole jelly.

Analysis of antisera prepared against upper, middle, and lower oviducal jellies of Rana pipiens.

Figure 12 (Plate IV), Figure 14 and 16 (Plate V), shows the Ouchterlony plates where Rana pipiens anti-upper, anti-middle and anti-lower oviducal jelly sera (center wells), respectively, were tested against whole oviducal jellies of several homologous and heterologous species of Amphibians (outer wells). At least 8 antigenic components (or determinant sites) are shared between Rana pipiens anti-upper (Plate IV, Figure 12), anti-middle (Plate V, Fig. 14), and anti-lower (Plate V, Figure 16) oviducal jelly serum and its homologous whole oviducal jelly, i.e. Rana pipiens. This is expected since antiserum specific for jelly of any oviducal segment is liable to have determinant sites in common with jelly from the whole oviduct.

PLATE V

Ouchterlony plates showing antigenic relationships between Rana pipiens anti-middle and anti-lower oviducal jelly sera and the whole oviducal jellies from different species of Amphibians: Pip--R. pipiens, Ax--Ambystoma mexicanum, To--Bufo americanus, Mar--B. marinus, Cat--R. catesbiana, and Clam--R. clamitans.

Figure 14.--R. pipiens anti-middle oviducal jelly serum, i.e., Pip mj (as)--(center well) tested against various whole oviducal jellies (outer wells).

Figure 15.--R. pipiens whole oviducal jelly (Pip ovj--center well) tested against R. pipiens anti-middle oviducal jelly serum absorbed with various whole oviducal jellies (outer wells).

Figure 16.--R. pipiens anti-lower oviducal jelly serum, i.e., Pip lj (as)--(center well) tested against various whole oviducal jellies (outer wells).

Figure 17.--R. pipiens whole oviducal jelly (Pip ovj--center well) tested against R. pipiens anti-lower oviducal jelly serum absorbed with various whole oviducal jellies (outer wells).

PLATE V

FIGURE 14

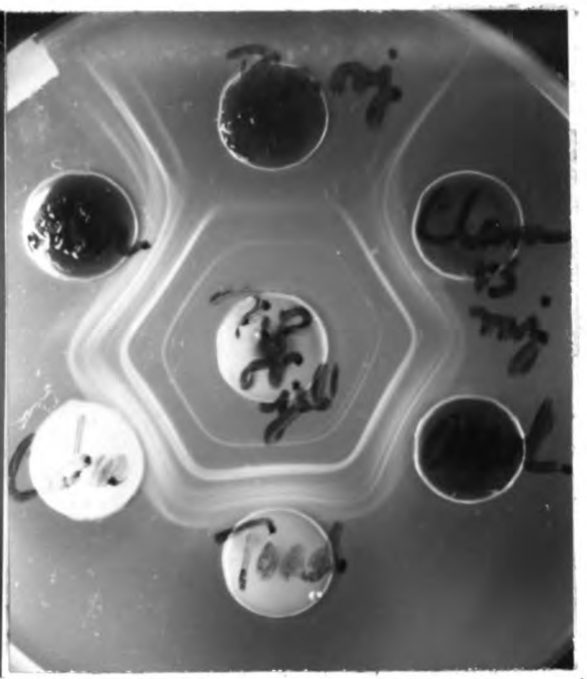


FIGURE 15

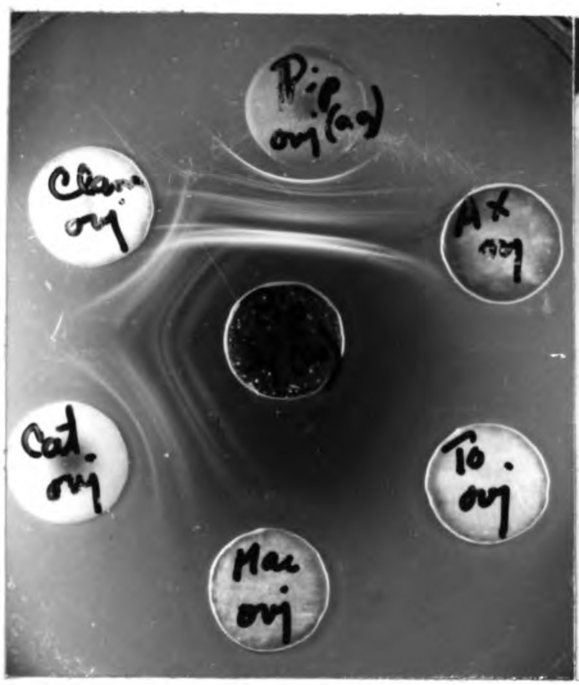


FIGURE 16

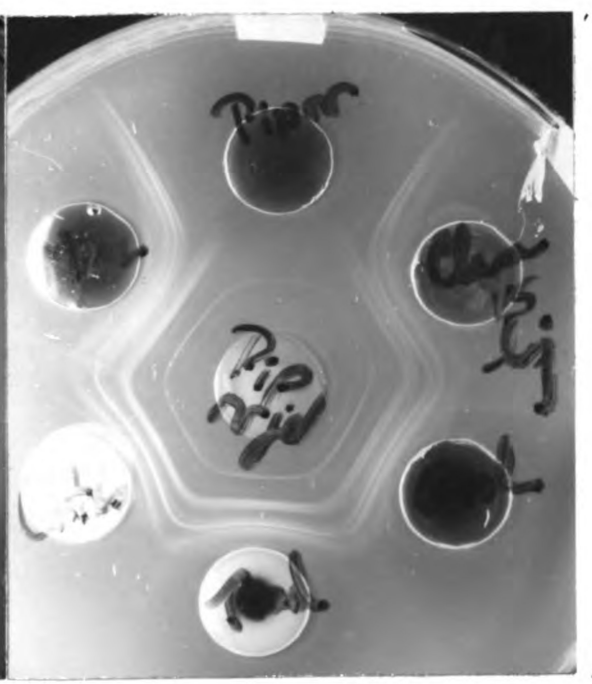


FIGURE 17

It can be seen that antiserum specific for any oviducal jelly of Rana pipiens (upper, middle, and lower) shares antigenic components (or determinant sites) with the whole jellies of Rana clamitans and R. catesbiana. However, the number of these components is far fewer (at most 2) in the reactions between anti-upper jelly serum and the whole jellies of the species tested (Plate IV, Figure 12). These shared antigenic components increase in number as antisera specific for jellies from lower oviducal segments, i.e., middle and lower, are tested against the whole jellies of Rana catesbiana and R. clamitans (Plate V, Figures 14 and 16, respectively).

Furthermore, it is expected that anti-lower and anti-whole jelly sera contain similar antigenic components. However, upon comparison of the immunoprecipitation band patterns of the plates in Figure 16 (Plate V) and Figure 10 (Plate IV), respectively, it is seen that Rana pipiens anti-lower jelly serum shares more antigenic components (or determinant sites) with the whole jellies of Rana catesbiana and R. clamitans (See Plate V, Figure 16) than that of the antiserum specific for jelly from the whole oviduct (See Plate IV, Figure 10). Such differences in immunoprecipitation-band patterns may be only apparent. It seems that the

inner precipitin band between anti-whole R. pipiens jelly serum and the whole jellies of R. clamitans and R. catesbiana observed in this plate is a composite of three different antigenic components (See Plate V, Figure 16) which failed to separate.

Rana pipiens anti-upper, anti-middle, and anti-lower oviducal jelly serum does not share antigenic components with the whole jellies of B. marinus, B. americanus, and Ambystoma mexicanum (See Plate IV, Figure 12, and Plate V, Figure 14, and Figure 16, respectively).

In conclusion, it seems from the analysis of the immunoprecipitation-band patterns that whole oviducal jellies from some species of Amphibians share antigenic components (or determinant sites) with the jellies (or the anti-jelly sera) obtained either from the upper, middle, and lower regions of the oviduct of Rana pipiens.

Effect of Absorbed Antisera on the Fertilizability of R. pipiens Eggs

In an attempt to further characterize these oviducal jelly antigenic components, the following experiments were done. Antisera specific for the different oviducal jellies

of Rana pipiens, i.e., anti-whole, anti-upper, anti-middle, and anti-lower sera, were absorbed with the whole oviducal jellies of homologous and heterologous Amphibian species (Rana pipiens, R. catesbiana, R. clamitans, R. sylvatica, Bufo marinus, B. americanus, and Ambystoma mexicanum). The effect of such absorbed sera on the fertilizability of Rana pipiens eggs was tested, as measured by the inhibition of cleavage of eggs. The purpose of this experiment was to determine which of the different antigenic components found in oviducal jellies were shared (common) with other Amphibian species and their localization along the oviduct. This might give an idea as to the relative importance of these antigenic surface components in the initial egg jelly-sperm interactions and in the specificity of fertilization.

The following criteria were used for determining whether the whole jellies of heterologous and homologous Amphibian species had antigenic components in common with the jellies obtained from the different oviducal levels of Rana pipiens:

(1) If upon absorption of an anti-jelly serum with a certain jelly, and subsequent treatment of the eggs with such absorbed serum, their cleavage percentage values do not

differ significantly from that of controls (N and C), this suggests that antigenic components (or determinant sites) are shared by the two jellies, because inhibitory substances (jelly antibodies) present in the serum are removed or neutralized by reacting with complementary antigenic components (or sites) in the absorption jellies.

(2) If upon absorption of an anti-jelly serum with a certain jelly, and subsequent treatment of eggs with such absorbed serum, their cleavage percentage values do not differ significantly from that of the respective unabsorbed serum, it may be concluded that the two jellies probably do not share antigenic components (or determinant sites), since the absorbed jelly did not remove the inhibitory substances (jelly antibodies) present in the serum. The inhibitory effect of such absorbed sera upon cleavage of eggs would be due to the presence of common antigenic components (or complementary sites) between the jelly coat of the treated eggs and the anti-jelly serum used.

The results of testing the effect of these absorbed sera upon the fertilizability of Rana pipiens eggs are summarized in Table 3 and in Text Figures 3, 4, 5, 6. For individual cleavage percentages see Appendix Table V. A two-way

TABLE 3.--Mean arc sin equivalents of percentages of Rana pipiens eggs reaching early cleavage stages after exposure to R. pipiens antisera specific for different oviducal jellies absorbed with whole oviducal jellies of heterologous Amphibian species.[a,b]

Antiserum against:	R. pipiens	R. catesbiana	R. sylvatica	R. clamitans	B. marinus	B. americanus	A. mexicanum
Whole oviducal jelly							
absorbed	59.3	46.3	47.7	43.3	36.9	39.1	34.3
unabsorbed	35.4	-	-	-	-	-	-
Upper 1/3 oviducal jelly							
absorbed	54.7	39.3	41.6	48.4	31.5	32.3	30.2
unabsorbed	38.3	-	-	-	-	-	-
Middle 1/3 oviducal jelly							
absorbed	53.4	38.1	38.6	27.2	24.9	24.9	27.0
unabsorbed	26.9	-	-	-	-	-	-
Lower 1/3 oviducal jelly							
absorbed	51.2	54.0	43.7	31.5	30.2	24.9	26.6
unabsorbed	32.4	-	-	-	-	-	-

[a]Values for Controls were as follows: 0.1% full strength Holtfreter's solution (Normal) = 71.7; non-immune rabbit serum (Control) = 55.9

[b]See Appendix Table VI for analysis of variance of these data.

Legend for symbols used in Text Figure 3: various treatments of Rana pipiens eggs prior to insemination.

C --non-immune rabbit serum.

N --one-tenth percent full-strength Holtfreter's solution.

W --R. pipiens anti-whole oviducal jelly serum.

W + P --R. pipiens anti-whole oviducal jelly serum absorbed with R. pipiens whole oviducal jelly.

W + Cl --R. pipiens anti-whole oviducal jelly serum absorbed with R. clamitans whole oviducal jelly.

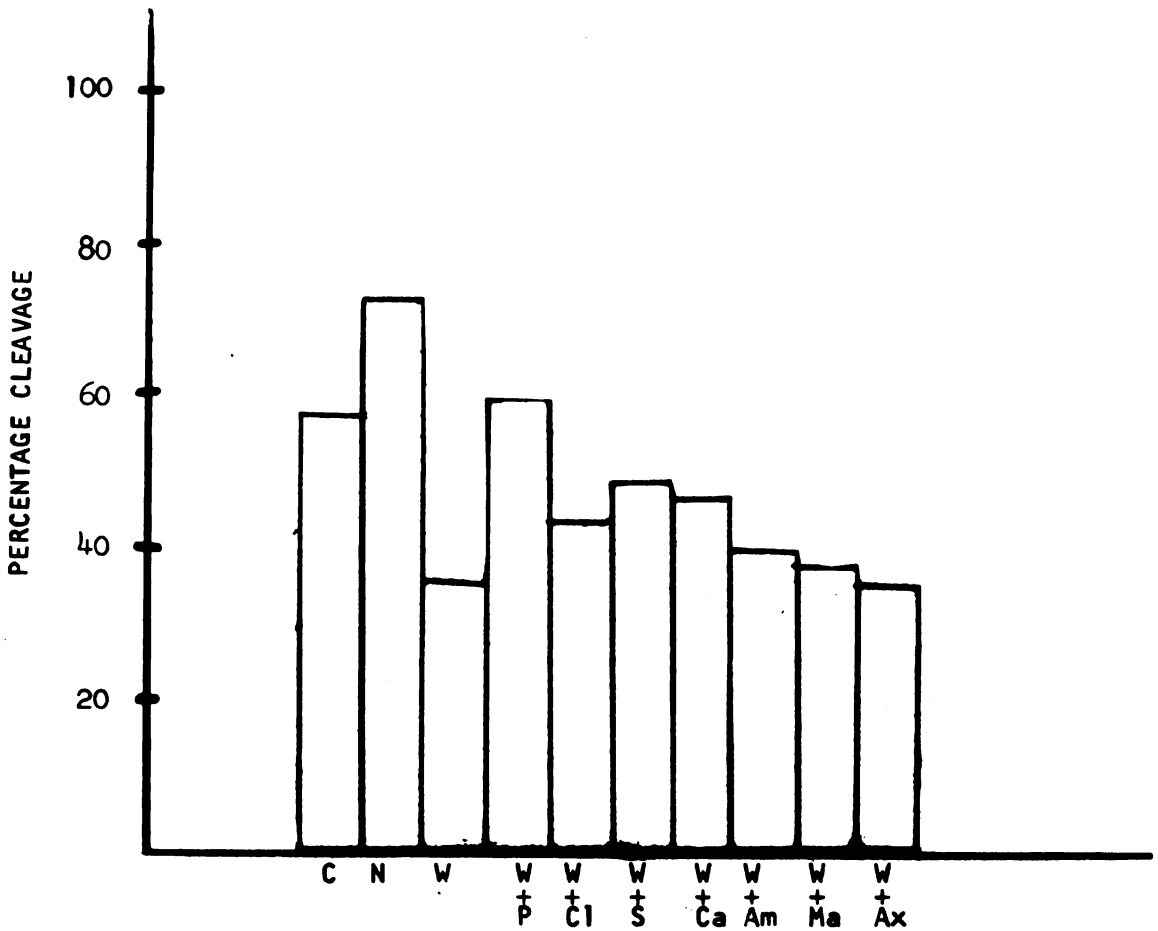
W + S --R. pipiens anti-whole oviducal jelly serum absorbed with R. sylvatica whole oviducal jelly.

W + Ca --R. pipiens anti-whole oviducal jelly serum absorbed with R. catesbiana whole oviducal jelly.

W + Ma --R. pipiens whole oviducal jelly serum absorbed with Bufo marinus whole oviducal jelly.

W + Am --R. pipiens whole oviducal jelly serum absorbed with B. americanus whole oviducal jelly.

W + Ax --R. pipiens whole oviducal jelly serum absorbed with Ambystoma mexicanum whole oviducal jelly.



TEXT FIGURE 3. CLEAVAGE OF RANA PIPIENS EGGS TREATED WITH ANTI-RANA PIPIENS WHOLE OVIDUCAL JELLY SERA ABSORBED WITH WHOLE OVIDUCAL JELLIES OF SEVERAL SPECIES OF AMPHIBIANS.

Legend for symbols used in Text Figure 4: various treatments of Rana pipiens eggs prior to insemination.

C --non-immune rabbit serum.

N --one-tenth percent full-strength Holtfreter's solution.

U --R. pipiens anti-upper oviducal jelly serum.

U + P --R. pipiens anti-upper oviducal jelly serum absorbed with R. pipiens whole oviducal jelly.

U + Cl --R. pipiens anti-upper oviducal jelly serum absorbed with R. clamitans whole oviducal jelly.

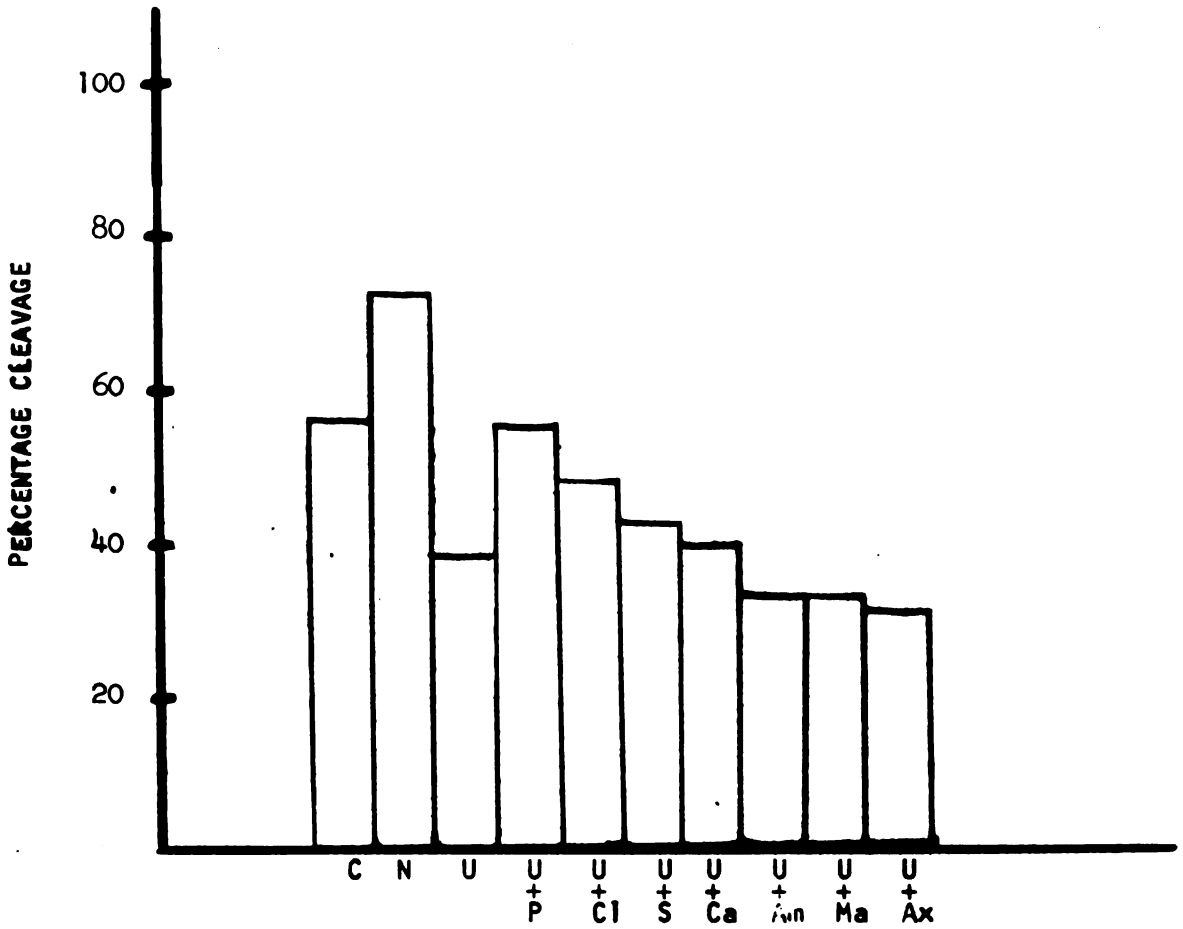
U + S --R. pipiens anti-upper oviducal jelly serum absorbed with R. sylvatica whole oviducal jelly.

U + Ca --R. pipiens anti-upper oviducal jelly serum absorbed with R. catesbiana whole oviducal jelly.

U + Am --R. pipiens anti-upper oviducal jelly serum absorbed with Bufo americanus whole oviducal jelly.

U + Ma --R. pipiens anti-upper oviducal jelly serum absorbed with B. marinus whole oviducal jelly.

U + Ax --R. pipiens anti-upper oviducal jelly serum absorbed with Ambystoma mexicanum whole oviducal jelly.



TEXT FIGURE 4. CLEAVAGE OF RANA PIPIENS EGGS TREATED WITH ANTI-R. PIPIENS UPPER OVIDUCAL JELLY SERA ABSORBED WITH WHOLE OVIDUCAL JELLIES OF SEVERAL SPECIES OF AMPHIBIANS.

Legend for symbols used in Text Figure 5; various treatments of Rana pipiens eggs prior to insemination.

C --non-immune rabbit serum.

N --one-tenth percent full-strength Holtfreter's solution.

M --R. pipiens anti-middle oviducal jelly serum.

M + P --R. pipiens anti-middle oviducal jelly serum absorbed with R. pipiens whole oviducal jelly.

M + Cl --R. pipiens anti-middle oviducal jelly serum absorbed with R. clamitans whole oviducal jelly.

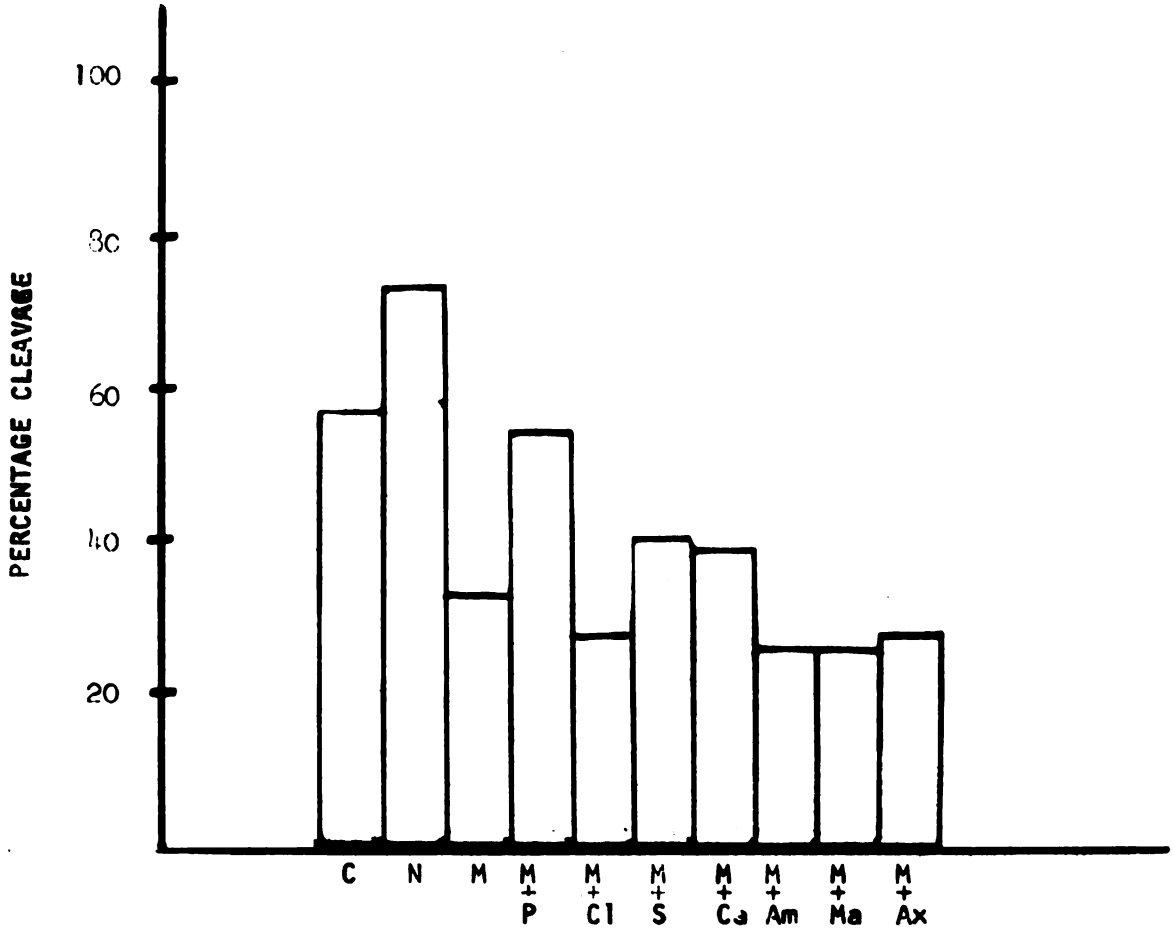
M + S --R. pipiens anti-middle oviducal jelly serum absorbed with R. sylvatica whole oviducal jelly.

M + Ca --R. pipiens anti-middle oviducal jelly serum absorbed with R. catesbiana whole oviducal jelly.

M + Am --R. pipiens anti-middle oviducal jelly serum absorbed with Bufo americanus whole oviducal jelly.

M + Ma --R. pipiens anti-middle oviducal jelly serum absorbed with B. marinus whole oviducal jelly.

M + Ax --R. pipiens anti-middle oviducal jelly serum absorbed with Ambystoma mexicanum whole oviducal jelly.



TEXT FIGURE 5. CLEAVAGE OF RANA PIPIENS EGGS TREATED WITH ANTI-R. PIPIENS MIDDLE OVIDUCAL JELLY SERA ABSORBED WITH WHOLE OVIDUCAL JELLIES OF SEVERAL SPECIES OF AMPHIBIANS.

Legend for symbols used in Text Figure 6: various treatments of Rana pipiens eggs prior to insemination.

C --non-immune rabbit serum.

N --one-tenth percent full-strength Holtfreter's solution.

L --R. pipiens anti-lower oviducal jelly serum.

L + P --R. pipiens anti-lower oviducal jelly serum absorbed with R. pipiens whole oviducal jelly.

L + Cl --R. pipiens anti-lower oviducal jelly serum absorbed with R. clamitans whole oviducal jelly.

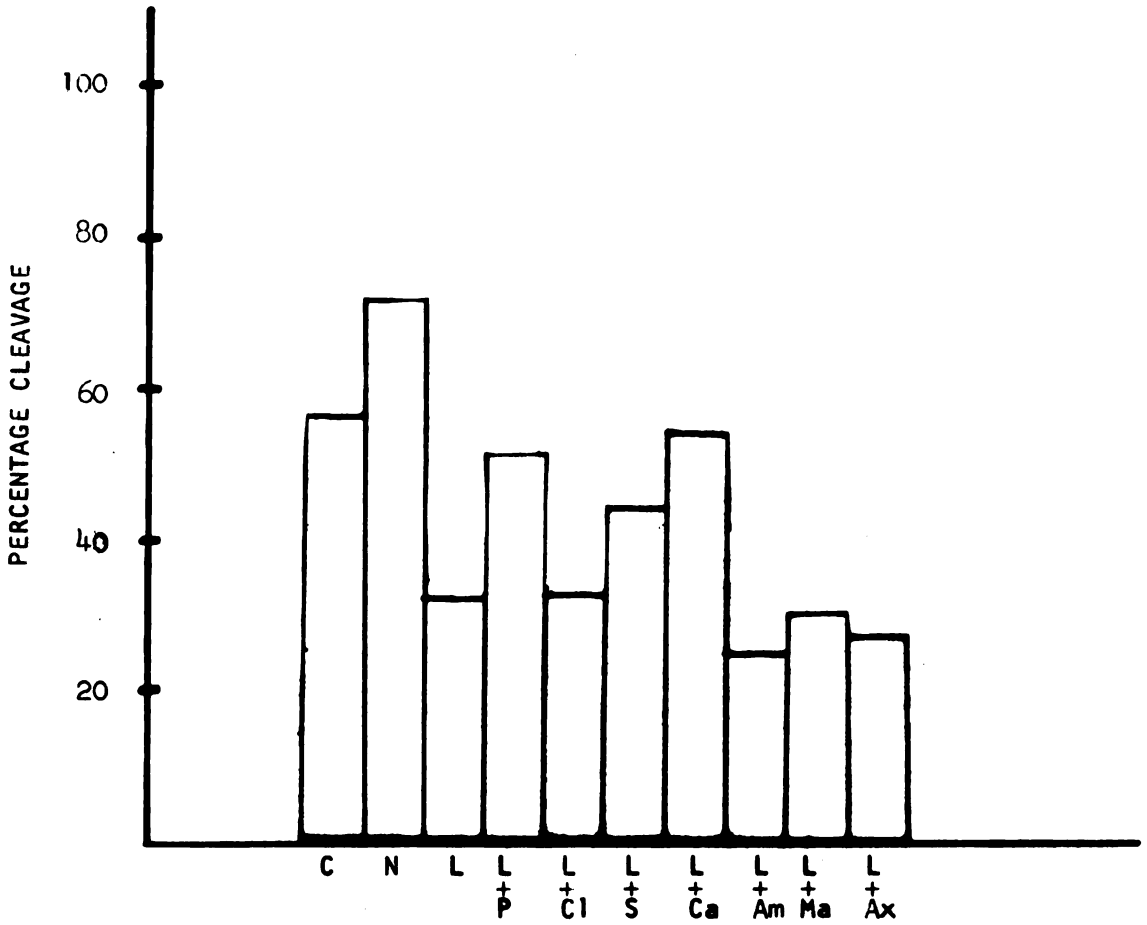
L + S --R. pipiens anti-lower oviducal jelly serum absorbed with R. sylvatica whole oviducal jelly.

L + Ca --R. pipiens anti-lower oviducal jelly serum absorbed with R. catesbiana whole oviducal jelly.

L + Am --R. pipiens anti-lower oviducal jelly serum absorbed with Bufo americanus whole oviducal jelly.

L + Ma --R. pipiens anti-lower oviducal jelly serum absorbed with B. marinus whole oviducal jelly.

L + Ax --R. pipiens anti-lower oviducal jelly serum absorbed with Ambystoma mexicanum whole oviducal jelly.



TEXT FIGURE 6. CLEAVAGE OF RANA PIPIENS EGGS TREATED WITH ANTI-R. PIPIENS LOWER OVIDUCAL JELLY SERA ABSORBED WITH WHOLE OVIDUCAL JELLIES OF SEVERAL SPECIES OF AMPHIBIANS.

analysis of the data (see Appendix Table VI) indicated that significant differences observed between any two mean values are significant at the 1% level.

Several types of controls were used in these experiments (See Table 3): Eggs treated with non-immune serum (C), those treated with one-tenth percent full strength Holtfreter's solution (N), those treated with unabsorbed sera: anti-whole oviducal jelly (W), anti-upper oviducal jelly (U), anti-middle oviducal jelly (M), and anti-lower oviducal jelly (L).

Effect of treating eggs with antisera specific for lower oviducal jelly of R. pipiens, absorbed with whole oviducal jellies of several species of Amphibians (See Text Figure 6).

Treatment of eggs with anti-lower oviducal jelly serum absorbed with the homologous jelly, i.e., Rana pipiens whole jelly (Bar L + P), prior to insemination, shows no significant difference from the percentage cleavage values of the controls (Bars N + C). This suggests that the inhibitory component(s) in the anti-jelly serum was (were) removed or neutralized by the jelly material, due to the presence of complementary sites in both substances (See Plate V, Figure 17), as seen by the absence of immunoprecipitation lines.

Treatment of eggs with anti-lower oviducal jelly serum of R. pipiens absorbed with heterologous jelly, i.e., Rana catesbiana whole jelly, (Bar L + Ca) shows no significant difference from the controls (Bars N and C). This indicates that R. catesbiana whole jelly may have common antigenic components (or determinant sites) with R. pipiens lower oviducal jelly.

Treatment of anti-lower oviducal jelly of R. pipiens serum absorbed with heterologous whole jellies (Rana sylvatica (Bar L + S), R. clamitans (Bar L + Cl), Bufo marinus (Bar L + Ma), B. americanus (Bar L + Am), and Ambystoma mexicanum (Bar L + Ax) shows no significant difference from the percentage cleavage values obtained with unabsorbed lower antiserum (Bar L). This might imply the absence of common antigenic components between Rana pipiens lower jelly and the whole jellies of these various species. These jellies did not remove the inhibitory substances normally present in unabsorbed anti-lower jelly serum, as evidenced by the fact that similar immunoprecipitation band patterns were obtained when using unabsorbed serum (Plate V, Figure 16) and absorbed serum (Plate V, Figure 17), and the fact that the percentage cleavage values for such absorptions are significantly lower than

the controls. Finally, the possibility exists that the presence or absence of immunoprecipitation lines may not be directly connected with effect on fertilizability.

Effect of treating eggs with antisera specific for upper oviducal jelly of Rana pipiens absorbed with whole oviducal jellies of several species of Amphibians. (See Text Figure 4)

Treatment of eggs with anti-upper oviducal jelly serum absorbed with the homologous jelly Rana pipiens whole jelly (Bar U + P) shows no significant difference from the control (Bars N and C) percentage cleavage values. This suggests that the inhibitory component(s) in the unabsorbed anti-jelly serum was (were) removed or neutralized by the jelly material, due to the presence of complementary sites in both substances, as seen by the absence of immunoprecipitin lines (See Plate IV, Figure 13).

Treatment of eggs with anti-upper oviducal jelly serum (R. pipiens) absorbed with Rana clamitans whole oviducal jelly (Bar U + Cl) shows no significant difference from the control percentage cleavage values (Bars N and C). This indicates that Rana clamitans whole jelly may have common antigenic components (or determinant sites) with Rana

pipiens upper oviducal jelly. This is evidenced by the fact that no precipitin bands appeared when R. pipiens anti-upper oviducal jelly serum which had been absorbed with R. clamitans whole jelly (outer well) was tested against R. pipiens whole jelly (outer well) was tested against R. pipiens whole oviducal jelly (center well) (see Plate IV, Figure 13).

Treatment of eggs with anti-upper oviducal jelly serum (R. pipiens) absorbed with heterologous whole jellies of Rana sylvatica (Bar U + S), R. catesbiana (Bar U + Ca), Bufo marinus (Bar U + M), B. americanus (Bar U + AM) and Ambystoma mexicanum (Bar U + Ax) shows no significant difference from the percentage cleavage values obtained with unabsorbed upper antiserum (Bar U). This might imply the absence of common antigenic components between Rana pipiens upper oviducal jelly and the whole jellies of these various species. These jellies did not remove the inhibitory substances normally present in anti-upper jelly serum, as evidenced by the fact that similar immunoprecipitation band patterns were obtained when using unabsorbed serum (Plate IV, Fig. 12) and absorbed serum (Plate IV, Figure 13), and that the percentage cleavage values for the above mentioned absorptions are significantly lower than that of the controls (see Text Figure 4).

Effect of treating eggs with antisera specific for middle oviducal jelly of Rana pipiens absorbed with whole oviducal jellies of several species of Amphibians (See Text Figure 5).

Treatment of eggs with anti-middle oviducal jelly serum absorbed with the homologous jelly (Rana pipiens whole jelly, Bars M + P), prior to insemination, shows no significant difference from the controls (Bars N and C) percentage cleavage values. This suggests that the inhibitory components in the unabsorbed anti-jelly serum were removed or neutralized by the homologous jelly material, due to the presence of complementary sites on both substances, as seen by the almost complete absence of immunoprecipitation lines (see Plate V, Figure 15).

Treatment of eggs with anti-middle oviducal jelly serum absorbed with heterologous whole jellies of Rana catesbiana (Bar M + Ca), R. sylvatica (Bar M + S), Rana clamitans (Bar M + Cl), Bufo marinus (Bar M + Ma), B. americanus (Bar M + AM), and Ambystoma mexicanum (Bar M + AX), shows no significant difference from that of the unabsorbed middle jelly antiserum (Bar M). Thus, these whole jellies do not seem to have common antigenic component(s) (or determinant sites) with Rana pipiens middle oviducal

jelly since they did not remove the inhibitory substances normally present in anti-middle serum. The fact that similar immunoprecipitation band patterns were obtained when testing unabsorbed middle jelly serum (Plate V, Figure 14) and absorbed middle jelly serum (Plate V, Figure 15), and that the percentage cleavage values for such absorptions are significantly lower than the controls, are evidence for this.

Effect of treating eggs with antisera specific for whole oviducal jelly of Rana pipiens absorbed with whole oviducal jellies of several species of Amphibians (see Text Figure 3).

Treatment of eggs with anti-whole oviducal jelly serum absorbed with the homologous jelly (Rana pipiens whole jelly--W + P), prior to insemination, shows no significant difference from the controls cleavage percentage values (Bars N and C). This suggests that the inhibitory components (anti-jelly antibodies) in the anti-jelly serum were removed or neutralized by the homologous jelly material (antigens), due to the presence of complementary sites on both substances, as seen by the absence of immunoprecipitation lines (See Plate IV, Figure 11).

Treatment of eggs with anti-whole oviducal jelly serum absorbed with heterologous whole jellies of Rana catesbiana (Bar W + Ca), R. sylvatica (Bar W + S), R. clamitans (Bar W + Cl), Bufo marinus (Bar W + Ma), B. americanus (Bar B + AM), and Ambystoma mexicanum (Bar W + AX), shows no significant difference from that of the unabsorbed anti-whole jelly serum (Bar W) cleavage percentage values. Thus, the whole jellies did not remove the inhibitory substances normally present in the anti-whole serum. This does not imply that common antigenic components (or determinant sites) are not present in these jellies, but if present, they are not present in sufficient amounts to neutralize the inhibitory effects of antibodies present in the antiserum. Absorptions of anti-whole R. pipiens serum with whole jellies from Rana catesbiana and R. clamitans should have removed some of the inhibitory effect of anti-whole serum, since they share antigenic components (determinant sites) with Rana pipiens anti-lower jelly serum and anti-upper jelly serum, respectively.

The following conclusions can be drawn from these experiments:

(1) Rana catesbiana whole jelly shares common antigenic components (or sites) with Rana pipiens lower anti-jelly serum, i.e. components present in lower oviducal jellies.

(2) Rana clamitans whole jelly shares common antigenic components (or combining sites) with Rana pipiens upper anti-jelly serum, i.e., with components present in upper oviducal jellies.

(3) The other whole jellies tested (Rana sylvatica, Bufo marinus, B. americanus, and Ambystoma mexicanum) appear not to share common antigenic components or determinant sites with any of the different Rana pipiens oviducal jellies. It can not be said with absolute certainty that any two jellies do not share antigenic components. The possibility always exists that these components may be present in the jellies of the other species in such small quantities that they would not be visible as precipitates on agar plates, or manifested in the percentage cleavage values of eggs treated with such jellies.

DISCUSSION

Analyses of the Ouchterlony double-diffusion plates indicated the presence of several antigenic components in the jellies obtained from different oviducal regions of Rana pipiens (whole, upper, middle, and lower). Furthermore, it was demonstrated that antisera specific for such jellies have an inhibitory effect on the fertilizability of Rana pipiens eggs. Such an inhibitory effect was manifested by the inability of such eggs to undergo cleavage in significant numbers. Among the various antisera tested, that specific for middle oviducal jelly was the most inhibitory compared to whole, upper, and lower oviducal jelly specific antisera. Furthermore, these latter antisera did not differ significantly from each other with respect to their inhibitory effect on cleavage, i.e., eggs treated with such antisera showed similar cleavage percentages. Dilutions of 1:45 of anti-middle oviducal jelly serum had an inhibitory effect on cleavage. On the other hand,

the inhibitory effect was not present in greater than a 1:15 dilution of anti-whole oviducal jelly serum.

By absorption of the oviducal jellies with homologous anti-jelly sera, the inhibitory effect was removed and immunoprecipitation bands were not formed when the absorbed sera were tested against Rana pipiens whole oviducal jellies. Thus, a close correlation has been observed between the precipitation reaction of the oviducal jelly antigens with complementary configurations in the antiserum, observed on the Ouchterlony plates, and inhibitory effects observed in treatments of eggs. This fact suggests that the antibody component(s) of the precipitate on the agar plates is actually responsible for the inhibitory effect. However, it should be pointed out that the absence of an immunoprecipitation line does not necessarily preclude an effect of the anti-serum on fertilization.

The inhibitory effects of oviducal anti-jelly sera may result from blocking antigenic sites essential for fertilization. It has been proposed that inhibition of cleavage of Rana pipiens eggs is due to interactions between antigenic components present in the oviducal jellies and their homologous antibodies present in the antisera specific for

such jellies. Such interactions could present a barrier to sperm penetration and thus inhibit fertilization and subsequent cleavage (Shaver and Barch, 1960). Furthermore, these interactions might be analogous to the "fertilizin-antifertilizin" system, which has been suggested in the fertilization of sea urchin eggs (Tyler, 1957, 1959, 1963). The antibodies in the sera specific for fertilizin would react with complementary substances present in the egg jellies. These complexes would inhibit the interaction of jelly-coat materials with complementary sites, i.e., anti-fertilizin molecules, on the surface of the spermatozoon. Thus, union of sperm and egg cannot be accomplished, and consequently egg cleavage is inhibited.

Jelly from the middle third of Rana pipiens oviduct may be the most potent in inhibiting cleavage of Rana pipiens eggs due to the following reason. It has been shown that there is an overlap of antigenic components in the middle segments of the oviduct, i.e., a component is present in the upper and middle segments which is not present in the lower oviduct, and another is found in middle and lower segments not found in the upper segments (Barch and Shaver, 1963). Therefore, anti-middle jelly serum shares more combining or

determinant sites with the egg jelly surrounding the treated eggs than upper or lower antisera. Interaction of these components with more complementary sites of the egg-jelly could result in a greater inhibitory effect on egg cleavage.

Whole oviducal jelly, since it contains antigenic components from the middle portion of the oviduct, would be expected to show higher inhibitory effects than upper and lower jellies. In these experiments, however, whole, upper, and lower antigenic components, showed inhibitory effects not significantly different from each other. This could be due to the fact that middle antigenic components, although more inhibitory, are present in lower amounts in the whole oviducal jelly. If the amount of antigen is a limiting factor for the production of antibodies specific for middle oviducal antigens in rabbits, then whole jellies, having less middle antigenic components than the isolated middle jellies, would not be so effective in eliciting the production of antibodies that will inhibit egg cleavage.

It was noted in the introduction that previous investigators (Barch and Shaver, 1963) when testing for the effect of antisera specific for whole homogenates (thus including jelly material present in them) of the different

parts of the oviduct, the cleavage percentage values were different than the ones presented here. The percentage cleavage of eggs treated with antiserum specific for the lower oviducal segment was significantly lower than that of eggs treated with antibodies specific for the upper segment. Furthermore, antiserum specific for the middle oviduct was least inhibitory to fertilization, as compared with the effect of antisera specific for either whole, upper, or lower oviduct. In the experiments reported here, however, anti-middle jelly serum has the greatest inhibitory effect on egg cleavage, while the other anti-oviducal jelly sera (whole, upper, and lower) had inhibitory effects considerably lower than that of middle anti-sera. The inhibitory effects of the whole, upper, and lower antisera were similar. The differences between the results could be due to differences in the methods employed in the two cases. In the former experiments, antisera specific for whole homogenates (including jelly material present in them) of the different parts of oviduct were used. In the experiments reported here the antisera used were only specific for the jelly material obtained from such oviducal regions. Antibodies produced against antigenic components derived from the non-jelly secreting oviducal tissues of

Rana pipiens could account for the variation in inhibitory effects observed between the antisera in the two cases. It has been shown that antisera specific for various adult tissues of the frog, e.g. kidney, ovary, testis, and heart have inhibitory effects on cleavage (Shaver & Barch, 1966). Thus, there is the possibility of the existence of antigenic components present in the oviducal tissue, which can inhibit cleavage.

Differences in results could also be due to quantitative and qualitative differences in the titers of antibodies elicited by the egg jelly components. In these experiments, all of the antisera used were pooled before testing on eggs in order to render insignificant the variations in antibody titer of sera from different rabbits with the same treatment and the number of bleedings performed. It is a well known fact that antibody titer elicited from a certain antigen can vary between individuals, and also, with the number of bleedings performed on an animal (Carpenter, 1965). The possibility should be discarded that the greatest inhibitory effect of anti-middle oviducal jelly serum on fertilization is a reflection of a higher antibody titer of the antiserum employed, instead of indicating that

the higher activity of this antiserum is due to differences in the role in fertilization of the middle oviducal antigen(s).

A possible implication of the fact that anti-middle jelly sera are the most inhibitory of egg cleavage may be that the most essential components for the initial specific adherence of sperm to egg jelly are localized in the jellies of this oviducal region.

ABSORPTION EXPERIMENTS

The absorption of antisera specific for the different oviducal jellies of Rana pipiens with whole oviducal jellies of heterologous origin indicated that some antigenic components were shared between some of the jellies, i.e., antigenic components present in the heterologous whole jellies had combining or determinant sites identical or closely related to those in the different oviducal jellies of Rana pipiens. Rana catesbiana whole oviducal jelly seems to share common determinant sites with Rana pipiens lower oviducal jelly, while R. clamitans whole oviducal jelly shares them with R. pipiens upper oviducal jelly. The inhibitory antibodies normally present in unabsorbed serum

were removed or neutralized by absorption with heterologous jelly sufficiently that the percentage cleavage values of such absorptions did not differ significantly from that of the controls (N and C), i.e., no inhibitory effect was observed after absorption.

The percentage cleavage values for some anti-Rana pipiens oviducal jelly sera absorbed with heterologous whole jellies did not differ significantly from that of the respective unabsorbed sera. This can be interpreted in two ways: either the inhibitory antibodies present in the unabsorbed sera were not removed or neutralized by correspondingly complementary molecules (determinant sites) on the jellies, due to the absence of common determinant sites between the two substances; or determinant sites between the two substances were in common, but in too small amounts to neutralize the inhibitory effects of antibodies on cleavage of eggs. In any case, the inhibition of cleavage observed for such absorbed sera was due to the reaction of antibodies present in the antisera specific for R. pipiens oviducal jellies with complementary sites in the jelly coat of the treated eggs. This may be interpreted as indicating the presence of species-specific jelly components which might

also be involved in the initial interactions of sperm and egg jellies.

In conclusion, both common generic and species-specific antigens are involved directly or indirectly in the initial interactions of egg-jelly with sperm. Similar results were observed and conclusions made by Shivers (1961, 1965) while working with egg jellies obtained from uterine eggs.

With regard to the localization of the common generic antigens in the oviducal jellies of Amphibian species, it can only be said that antigenic components shared by Rana pipiens oviducal jellies and that of heterologous species, may either be localized in the upper and lower oviducal regions (consequently in the inner and outermost jelly layers of the egg, respectively). With fluorescein-conjugated antibodies, Shivers (1962) found that the whole jellies of Rana clamitans and R. sylvatica contained antigens common with the innermost jelly layer of Rana pipiens eggs. He concluded that common antigens among egg jellies are confined in the innermost jelly layers of the eggs. The results presented here show that common antigenic components need not be confined only to this layer, since it

was shown that Rana catesbiana contains antigens (or determinant sites) common to Rana pipiens lower oviducal jelly, and consequently, the outermost layer deposited on the egg. Similar results with fluorescein-tagged antibodies would corroborate these results.

These common antigens might be involved in the hybridization of Amphibian species. As Shivers (1965) pointed out, crosses between the species of Rana which he studied are, in general, able to develop in some degree, and in no case where such hybrid development occurs are jelly antigens common to the two species found lacking.

The role of the essential jelly components in fertilization is not yet understood. Further studies are needed, such as absorption of anti-Rana pipiens sera specific for the jellies obtained from different oviducal regions with jellies of heterologous species; but with jellies obtained from the different oviducal regions of heterologous species (upper, middle, and lower). Such studies may elucidate the correct localization of common and species-specific antigenic components associated with the initial egg jelly-sperm interactions in Anurans.

SUMMARY

Considerable literature exists concerning the role of surface (antigenic) components of the jelly coats of Anuran eggs with respect to their possible role in fertilization. Among these is the role of these components in the initial specific adherence of spermatozoon to egg during fertilization. Since it is known that different antigenic components are localized in the jellies obtained from different levels of the oviducts, and that these are deposited around the egg during its passage down the oviduct, experiments were designed to test for the possible roles and relative importance of these components in the initial egg-jelly sperm interactions during fertilization of Rana pipiens eggs.

Antisera specific for jellies obtained from different oviducal regions (whole, upper, middle, and lower) of R. pipiens were tested for their effects on the fertilizability of R. pipiens eggs. A one-way analysis of variance of the data showed that treatment of eggs with these antisera had an inhibitory effect on fertilization, as indicated

by the low percentages of cleavage of eggs treated with such antisera.

Anti-middle oviducal jelly serum had the most inhibitory effect on cleavage of eggs, in comparison with anti-whole, anti-upper, and anti-lower oviducal jelly sera. The three latter sera did not differ significantly from each other with respect to their inhibitory effect on cleavage.

Analysis of antigenic components present in the different oviducal jellies was done by a modification of the Ouchterlony double-diffusion plate technique.

Dilutions of anti-whole and anti-middle oviducal jelly sera were done to determine at which titer their inhibitory effects upon cleavage would disappear. Even at a 1:45 dilution, anti-middle jelly serum showed a significant inhibitory effect on cleavage of eggs. With anti-whole jelly serum, such effects lasted only up to a 1:15 dilution. A correlation was observed in both cases between the dilution that showed immunoprecipitation bands on double-diffusion plates, and the dilution which showed the last significant inhibitory effect on cleavage.

The inhibitory effects of the anti-oviducal jelly sera on cleavage of R. pipiens eggs were interpreted as being due to interactions between antigenic components present in the jellies surrounding the treated eggs, and their homologous antibodies present in the antisera specific for such jellies. An explanation as to why anti-middle jelly serum is the most inhibitory of cleavage of eggs is also suggested.

Rana pipiens anti-whole, anti-upper, anti-lower oviducal jelly sera were absorbed with whole oviducal jellies of different species of Amphibians. Such absorbed sera were tested for effects on cleavage of R. pipiens eggs. The results of such absorptions indicated that both common generic, as well as species-specific antigenic components are present in oviducal jellies. Both seem to be involved in the initial interactions between sperm and egg jelly.

Common generic components are present in R. catesbiana whole oviducal jelly and R. pipiens lower oviducal jelly, and in R. clamitans whole oviducal jelly and R. pipiens upper oviducal jelly. In other words, the whole oviducal jellies of these two species of Rana seem to share common generic components with the lower and upper oviducal

jellies of R. pipiens, respectively, and consequently with the outer and inner egg-jelly layers.

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APPENDIX

TABLE I.--Individual cleavage percentage values of eggs treated with antisera specific for different oviducal jellies

Animal \ Treatments	N	C	OVUL	OVUL 1:2	OVUL 1:3	OVUL 1:5	UPPER	UPPER 1:2	UPPER 1:3	UPPER 1:5	MID	MID 1:2	MID 1:3	MID 1:5	LOW	LOW 1:2	LOW 1:3	LOW 1:5
1	59.7	63.4	18.9	28.6	40.7	52.4	7.0	31.8	25.9	46.4	0	30.7	36.9	18.4	8.9	14.8	40.9	42.7
2	72.2	57.4	51.2	26.9	61.0	30.0	47.2	25.6	26.6	25.8	24.9	31.3	29.0	38.5	31.5	34.7	34.6	54.1
3	64.2	63.9	54.8	48.2	21.6	31.8	39.5	24.1	23.6	24.8	12.3	33.2	21.4	62.1	26.6	64.8	48.4	48.2
4	79.9	77.3	40.7	57.0	61.9	74.1	57.4	25.9	45.0	46.8	17.6	55.4	59.5	30.5	28.4	34.6	39.6	44.1
5	69.0	66.3	64.2	46.2	51.3	47.9	46.0	32.8	40.2	59.3	35.2	56.9	38.9	41.5	39.5	55.9	31.1	32.1
6	60.0	56.1	24.6	43.3	28.7	44.7	20.4	35.2	29.7	39.9	15.7	33.8	24.5	45.9	20.5	41.7	28.7	28.6
7	79.1	74.7	41.1	49.5	47.5	44.5	19.5	45.0	40.6	42.2	0	37.8	20.7	38.6	36.3	44.4	31.1	47.7
8	50.8	58.5	45.9	36.8	53.5	51.9	17.2	35.2	54.8	47.5	30.9	34.6	23.1	21.4	41.6	35.2	53.4	36.8
9	78.9	80.7	37.9	45.0	71.8	60.8	31.1	45.0	47.4	54.8	0	42.3	48.2	38.6	17.1	31.5	32.5	37.1
10	73.3	50.8	11.1	47.6	48.9	28.0	25.8	36.5	25.0	49.3	0	34.8	40.5	23.1	32.1	18.2	36.0	19.5
11	78.0	61.6	40.3	31.8	63.9	67.4	25.8	36.8	42.0	41.1	15.8	13.1	15.0	31.6	24.1	35.2	27.6	31.7
12	68.0	77.8	31.7	0	48.9	49.5	22.2	29.0	31.1	51.4	11.2	42.5	19.3	45.0	26.6	25.9	49.8	27.6
13	67.5	73.7	17.9	47.2	48.0	59.0	13.1	27.1	10.9	34.8	0	24.1	22.2	21.0	28.6	35.2	34.8	30.9
14	72.2	74.1	26.3	23.0	27.1	44.4	0	29.5	23.9	35.9	31.6	17.1	27.0	43.7	33.0	52.8	55.3	32.0
15	74.8	66.3	38.9	55.3	68.4	56.8	43.1	52.7	27.7	47.8	30.5	38.5	40.3	43.2	37.1	48.6	34.8	53.4
16	72.6	78.8	47.4	22.6	69.9	40.7	19.5	25.3	25.4	36.5	19.2	21.4	14.5	10.1	14.8	26.6	23.0	21.6
17	78.2	64.9	21.1	24.1	39.5	46.4	14.8	54.8	47.8	63.9	15.0	51.7	56.0	59.2	39.9	39.5	38.1	45.9
18	70.8	82.1	24.1	48.2	28.3	52.2	14.5	26.9	13.7	45.0	15.5	18.9	20.7	20.1	8.9	9.8	0	50.1
19	70.5	74.7	38.4	52.7	39.9	31.8	28.7	43.6	43.6	37.4	18.7	26.1	34.3	52.2	20.4	38.8	38.8	50.8
20	70.7	65.9	29.0	45.0	43.3	54.8	20.4	41.8	8.9	45.0	35.2	35.7	27.4	28.9	35.7	32.8	54.1	54.2
21	62.2	62.6	17.1	37.5	35.8	48.0	21.4	18.8	30.4	42.4	14.3	15.7	34.2	60.7	8.9	0	37.2	43.3
$\bar{X}$	70.1	68.2	34.4	38.9	47.6	48.4	25.5	34.4	31.6	43.7	16.4	33.1	31.1	36.9	26.7	34.3	36.7	39.6

TABLE II.--One-way analysis of variance of effect of exposing eggs of Rana pipiens to antisera specific for R. pipiens jellies from different regions of the oviduct.

Source of Variation	Degree of Freedom	Sum of Squares	Mean Square	F
Treatment	17	64603.9	3800.2	29.8**
Animal	20	11148.9	557.4	4.4**
Error	340	43348.3	127.4	
Total	377	119101.1	315.9	

**Significant to the 1% probability level.

TABLE III.--Individual cleavage percentage values of eggs treated with various dilutions of R. pipiens anti-whole and anti-middle jelly sera.

Animals \ Treatments	N	C	OV 1.5	OV 1:15	OV 1:45	OV 1:135	OV 1:405	MID 1:5	MID 1:15	MID 1:45	MID 1:135	MID 1:405
1	70.5	60.9	49.3	69.5	99.2	76.3	51.6	24.8	53.1	65.4	59.2	81.3
2	61.1	63.4	33.6	43.2	72.1	71.6	54.8	14.1	10.5	42.8	58.0	62.5
3	51.4	48.5	11.5	38.9	47.8	45.0	47.6	9.8	9.6	36.6	50.4	10.3
4	63.8	53.9	23.0	27.7	53.0	66.3	61.8	0.0	21.0	40.3	45.0	52.5
5	75.0	73.9	37.0	55.8	57.7	73.7	58.9	20.7	31.4	60.9	58.5	67.2
6	90.0	73.0	44.4	61.1	67.5	73.9	71.3	18.4	64.8	56.0	78.8	65.6
7	69.3	73.4	36.2	66.9	74.9	63.4	70.1	33.4	34.0	55.1	70.7	65.6
8	72.8	66.3	47.1	62.0	74.6	69.9	70.3	12.0	30.4	50.2	67.1	70.3
9	67.8	45.5	38.1	34.3	45.7	62.1	42.9	8.5	29.0	49.3	45.8	54.3
10	75.7	80.9	62.3	73.7	76.3	74.3	80.7	50.5	47.2	59.5	71.6	43.5
11	81.5	66.6	39.6	75.9	71.6	80.2	80.7	0.0	48.5	50.8	61.6	73.3
12	81.3	59.5	57.1	63.4	77.1	81.3	65.9	56.4	45.8	63.0	73.9	80.4
13	76.7	68.4	45.8	71.6	74.7	77.5	77.5	36.6	46.2	62.0	69.3	69.9
14	39.8	52.1	22.5	56.2	67.1	65.2	57.7	12.0	20.4	29.4	51.1	72.1
15	70.3	47.6	37.9	53.5	59.6	64.1	62.7	16.7	41.5	51.4	60.9	58.1
16	72.4	57.7	35.8	39.7	63.4	77.1	63.8	21.6	38.4	59.3	63.7	62.1
17	59.7	53.6	22.8	32.1	80.2	40.9	58.1	9.1	32.3	50.8	71.1	77.8
18	66.5	48.9	56.8	53.9	70.5	73.5	67.5	14.3	29.0	56.9	65.9	81.3
19	57.5	67.5	51.0	52.5	68.0	70.3	73.9	22.0	46.3	57.2	61.9	79.9
20	73.9	72.4	42.7	52.9	72.4	80.7	68.1	20.7	38.6	60.0	65.6	65.3
21	67.5	69.9	35.2	56.7	62.1	66.3	69.3	19.7	33.7	64.8	57.5	76.7
$\bar{X}$	68.8	62.1	39.5	54.4	67.3	69.2	64.5	20.1	35.8	53.4	62.3	66.7

TABLE IV.--One-way analysis of variance of effect of exposing eggs of Rana pipiens to various dilutions of whole and middle anti-jelly sera obtained from R. pipiens oviduct.

Source of Variation	Degree of Freedom	Sum of Squares	Mean Square	F
Treatments	11	56802.2	5163.8	74.2**
Animals	20	18701.2	935.1	13.4**
Error	220	15313.3	69.6	
Total	251	90816.7	361.8	

**Significant to the 1% probability level.

TABLE V.--Individual cleavage percentage values of eggs treated with absorbed sera.

Animals \ Treatments	C	N	Whole l:l	Upper l:l	Mid l:l	Low l:l	Sylv ovj vs ovj	Cat ovj vs ovj	Mar ovj vs ovj	Ax ovj vs ovj	To ovj vs ovj	Pip ovj vs ovj	Clam ovj vs ovj	Sylv ovj vs uj	Cat ovj vs uj	Mar ovj vs uj	Ax ovj vs uj	To ovj vs uj	Pip ovj vs uj	Clam ovj vs uj	Sylv ovj vs mj	Cat ovj vs mj	Mar ovj vs mj	Ax ovj vs mj	To ovj vs mj	Pip ovj vs mj	Clam ovj vs mj	Sylv ovj vs lj	Cat ovj vs lj	Mar ovj vs lj	Ax ovj vs lj	To ovj vs lj	Pip ovj vs lj	Clam ovj vs lj
1	58.7	65.9	14.8	33.7	47.1	22.6	71.6	51.8	24.5	41.8	42.3	75.2	56.8	52.7	39.5	41.1	37.5	48.0	75.2	59.4	38.2	48.2	35.2	46.0	41.2	57.0	52.8	49.7	55.5	39.2	37.4	26.6	49.1	30.0
2	64.4	71.9	45.0	33.7	40.1	24.1	48.0	33.4	37.5	16.7	35.2	61.6	55.4	25.3	55.6	21.8	17.6	38.6	59.2	51.5	37.3	31.7	20.7	48.0	31.1	65.9	31.1	56.4	71.9	24.7	28.9	19.5	79.7	34.6
3	65.2	90.0	27.7	53.1	10.6	36.6	62.3	45.0	54.8	53.1	34.7	45.0	72.8	61.9	45.0	46.7	17.6	22.2	47.2	39.2	38.9	29.3	28.4	28.9	18.4	61.3	30.0	45.0	55.5	33.0	24.7	39.2	59.2	28.9
4	61.3	60.0	0.0	25.3	0.0	22.8	58.0	66.9	48.4	25.5	42.3	58.2	58.3	69.7	45.8	45.0	44.1	58.2	74.6	71.1	37.5	34.0	17.1	39.2	18.2	67.5	10.0	68.6	77.2	32.2	29.5	33.0	66.4	41.8
5	63.4	81.3	64.4	62.1	56.4	72.7	70.2	55.4	45.0	65.2	33.2	79.7	77.2	90.0	14.3	53.6	46.9	49.7	90.0	72.8	73.7	70.3	53.4	16.7	44.1	90.0	33.6	61.1	79.9	63.4	35.2	53.4	78.9	69.0
6	90.0	78.0	30.0	37.5	10.3	18.2	48.9	50.4	27.9	30.5	52.2	58.9	52.7	54.2	51.5	26.6	45.0	29.0	73.0	43.1	37.4	14.8	36.3	45.0	0.0	67.5	31.0	37.5	58.8	25.3	10.1	0.0	48.5	43.1
7	42.4	59.6	33.2	31.3	0.0	18.7	42.9	56.6	34.5	17.9	26.6	57.4	25.0	56.6	0.0	0.0	20.1	11.1	55.5	60.8	50.0	49.4	46.9	24.5	28.1	72.2	38.5	39.9	58.8	34.2	41.6	18.4	60.0	26.6
8	69.3	90.0	57.4	37.5	35.2	26.6	68.2	69.0	35.2	30.0	49.4	75.6	33.8	65.9	74.2	37.8	29.0	24.4	65.9	64.8	27.9	50.8	28.4	26.6	32.0	48.0	40.6	46.4	53.4	47.5	33.0	27.0	55.8	42.5
9	56.8	74.2	46.7	45.8	17.9	29.0	27.9	42.0	31.0	28.9	45.9	49.7	17.6	27.1	45.9	30.5	23.3	23.6	45.0	55.4	26.1	14.5	9.5	22.2	49.9	56.4	10.5	29.5	47.2	23.7	23.6	45.9	79.1	56.0
10	69.3	75.0	50.8	44.0	26.1	34.5	62.2	73.3	58.0	53.1	45.0	67.5	70.8	58.1	65.0	49.7	55.3	43.4	74.6	56.3	51.5	28.5	35.2	21.0	37.9	43.9	38.9	59.5	79.1	60.0	22.6	0.0	56.2	38.8
11	45.0	61.4	9.6	14.5	18.4	20.4	30.4	11.2	17.6	24.8	24.4	52.7	26.0	28.6	24.4	10.3	36.9	25.0	20.7	27.4	26.6	18.4	14.3	13.8	11.5	49.3	18.9	27.1	54.1	18.2	17.1	27.6	56.2	50.8
12	46.4	63.4	42.3	36.9	37.9	40.5	37.5	30.5	38.4	24.1	18.0	53.6	33.2	29.4	41.2	55.8	22.8	22.2	62.6	63.9	26.9	30.7	10.9	37.8	15.0	42.5	30.6	38.3	50.2	11.1	19.8	16.7	43.5	0.0
13	48.9	64.2	17.9	15.0	17.6	14.5	23.6	28.1	24.7	15.0	22.0	38.2	14.1	23.7	42.0	30.0	33.8	17.6	34.6	47.1	25.3	18.4	10.3	29.5	17.3	46.7	10.1	10.6	21.4	15.5	0.0	10.5	29.0	23.4
14	17.1	69.6	48.0	41.2	14.1	36.6	15.5	13.4	23.7	27.8	33.0	38.5	20.4	12.0	10.1	32.0	18.2	20.7	54.8	41.2	23.3	34.6	0.0	15.0	0.0	48.6	17.6	38.2	27.1	15.7	30.6	35.2	41.2	13.4
15	56.3	65.9	24.1	35.2	17.9	27.8	31.1	57.4	21.1	18.4	48.5	40.2	53.3	13.8	33.6	18.4	25.3	48.7	52.7	47.8	19.8	39.2	24.1	0.0	17.2	38.2	20.3	33.2	43.1	21.1	34.5	10.0	26.6	35.2
16	53.1	61.4	0.0	0.0	28.1	0.0	27.1	25.7	19.5	14.8	21.6	44.3	24.1	13.1	23.7	0.0	10.5	0.0	0.0	24.8	21.4	40.5	26.1	24.1	0.0	15.8	29.1	31.8	30.0	0.0	17.6	0.0	0.0	0.0
17	28.0	63.7	47.8	34.1	19.5	34.6	55.4	36.3	45.0	30.0	48.2	57.4	40.6	42.9	38.4	25.7	0.0	45.9	45.0	16.1	50.2	49.7	15.8	23.6	18.2	33.4	20.7	32.8	23.3	11.1	26.1	25.3	34.2	15.9
18	74.2	58.5	38.1	53.4	25.5	60.0	65.5	68.6	33.0	45.0	33.7	80.5	39.6	33.5	58.0	24.5	38.0	13.7	56.8	33.5	59.5	31.5	30.0	19.1	50.8	76.7	23.4	55.5	77.3	48.5	48.9	26.0	68.9	36.0
19	37.3	90.0	46.7	63.1	59.5	63.4	48.4	59.5	52.7	56.3	63.4	72.1	49.4	28.9	37.8	39.9	30.0	41.6	47.4	39.2	50.8	56.6	20.7	34.0	46.1	61.0	14.3	48.7	50.1	39.2	27.1	46.1	47.4	15.0
20	71.1	90.0	63.9	68.6	56.4	43.9	58.9	51.5	60.6	67.5	62.6	78.8	44.1	45.0	40.3	40.1	52.2	62.9	60.0	52.7	49.7	70.3	34.7	24.1	21.1	26.6	42.3	63.4	55.4	36.3	24.4	38.2	43.2	28.4
$\bar{X}$	55.9	71.7	35.4	38.3	26.9	32.4	47.7	46.3	36.9	34.3	39.1	59.3	43.3	41.6	39.3	31.5	30.2	32.3	54.7	48.4	38.6	38.1	24.9	27.0	24.9	53.4	27.2	43.7	54.0	30.2	26.6	24.9	51.2	31.5

TABLE VI.--Two-way analysis of variance (replicated) of effect of exposing eggs of Rana pipiens to various anti-R. pipiens oviducal jelly sera absorbed with whole oviducal jellies of heterologous Amphibian species.

Source of Variation	Degree of Freedom	Sum of Squares	Mean Square	F
Antisera	3	7932.3	2644.1	15.4**
Jellies	6	41700.5	6950.1	40.5**
Interaction	18	57393.8	3188.5	18.6**
Error	532	91229.1	171.5	
Total	559	198255.7		

**Results significant to the 1% probability level.