IMMUNOBIOLOGICAL STUDIES OF THE SPECIES-SPECIFICITY OF EGG JELLIES OF THE FROG

By

Charles Alex Shivers

AN ABSTRACT

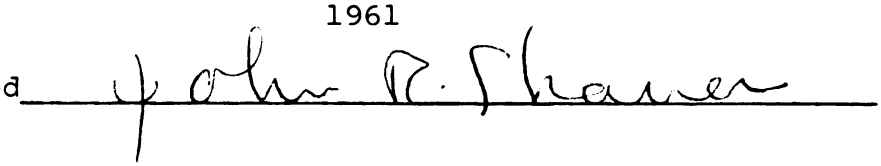
Submitted to the School for Advanced Graduate Studies
of Michigan State University of Agriculture and
Applied Sciences in Partial Fulfillment
of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

1961

Approved 

ABSTRACT

IMMUNOBIOLOGICAL STUDIES OF THE SPECIES-SPECIFICITY OF EGG JELLIES OF THE FROG

By Charles Alex Shivers

Antisera were prepared by injecting lyophilized egg-jelly material from four species of frogs (Rana pipiens, R. clamitans, R. sylvatica, and R. catesbeiana) into rabbits. Using the Ouchterlony techinque it was determined that all four species have common components as well as a number of specific components. By adsorbing anti-jelly serum of each species with jelly from the other species, the specific components of each species were identified. It was also determined that the jelly components of R. pipiens are, in general, restricted to the oviducal tissues and not found in other body tissues of the frog.

Eggs of Rana pipiens were treated with antisera against both homologous and heterologous jellies prior to insemination with normal spermatozoa of R. pipiens. Treatments with both heterologous and homologous antisera resulted in satistically significant inhibition of the fertilizability of the eggs. The inhibitory effect of heterologous antiserum could be removed by treating it with either homologous or heterologous jellies. The inhibitory action of the homologous antiserum

could be removed only by treating it with homologous jelly. These results indicate that the specific components of anti-jelly serum of R. pipiens are inhibitory, as are the common components in the heterologous anti-jelly serum. The specific components of the heterologous sera are non-inhibitory.

The effect of antisera prepared against jelly material of other Amphibian genera has also been tested. In cases (Bufo) where a cross-reaction was observed between these sera and jelly of R. pipiens, inhibition was also observed. No inhibitory action was seen with anti-jelly sera that did not cross-react with the jelly of R. pipiens (Ambystoma).

IMMUNOBIOLOGICAL STUDIES OF THE SPECIES-SPECIFICITY OF EGG JELLIES OF THE FROG

By

Charles Alex Shivers

A THESIS

Submitted to the School for Advanced Graduate Studies
of Michigan State University of Agriculture and
Applied Sciences in Partial Fulfillment
of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

1961

ACKNOWLEDGEMENTS

The author wishes to express his sincere thanks to Dr. J. R. Shaver for his interest, direction and encouragement during the course of this investigation. Grateful thanks are also due to Drs. G. B. Wilson, F. A. Fennell, and P. J. Clark for their able assistance while serving on the author's guidance committee. Thanks are also due to Dr. S. H. Barch for her interest and assistance during the course of this study.

Acknowledgement is made to the National Institutes of Health for the predoctoral fellowship (GF-11,123).

Special recognition is made to my wife, Sara, whose encouragement and sacrifices have made this study possible.

TABLE OF CONTENTS

Page

LIST OF TABLES v

LIST OF PLATES vi

LIST OF TEXT FIGURES vii

INTRODUCTION 1

MATERIALS AND METHODS 11

RESULTS 18

A. Analysis of anti-jelly sera 18

1. Analysis of antisera prepared against jelly of Rana pipiens 18
2. Analysis of antisera prepared against jelly of Rana clamitans 22
3. Analysis of antisera prepared against jelly of Rana sylvatica 24
4. Analysis of antisera prepared against jelly of Rana catesbeiana 24
5. Analysis of antisera prepared against jelly obtained from another genus of Anura and another order of Amphibia (Urodela) 25

B. Effects of antisera on the fertilizability of eggs of Rana pipiens 45

1. Effect of treating eggs with antisera prepared against the jelly of Rana pipiens 46
2. Effect of treating eggs with antisera prepared against the jelly of Rana clamitans 48
3. Effect of treating eggs with antisera prepared against the jelly of Rana sylvatica 50
4. Effect of antisera prepared against egg-jellies of another Anuran genus (Bufo) and of a Urodele species (Ambystoma) 51
5. Effects of antisera prepared against fertilizin of eggs of Arbacia (Echinodermata) 52
6. Effects of treating eggs with antisera prepared against homogenates of organs of adult Rana pipiens 53

Page

C. Effect of treating eggs of Rana pipiens with fractions of anti-jelly sera 60

DISCUSSION

A. Analysis of antigenic components represented in anti-jelly sera by means of agar-diffusion technique 63

B. Analysis of the effect of anti-jelly sera on fertilizability of eggs 72

C. General considerations and speculations 77

SUMMARY 80

LITERATURE CITED 83

LIST OF TABLES

Table		Page
1	Percentages of cleavages in batches of eggs treated with serum dilutions of 1:1 prior to insemination with normal spermatozoa	59
2	Cross-reactions between jellies as determined by the Ouchterlony technique	69
3	The presence of specific components in anti-jelly sera as determined by the technique of adsorption	70
4	Tissue-specificity of Rana pipiens jelly components as determined by the Ouchterlony technique	71

LIST OF PLATES

Plate		Page
1	Representative photographs of Ouchterlony plates and results observed on eggs after treatments with anti-jelly serum	28
2	Diagrams of Ouchterlony plates. Analysis of species-specificity of antisera prepared against Rana pipiens jelly	30
3	Analysis of species-specificity of antisera prepared against Rana clamitans jelly	32
4	Analysis of species-specificity of antisera prepared against Rana sylvatica jelly	34
5	Analysis of species-specificity of antisera prepared against Rana catesbeiana jelly	36
6	Analysis of tissue-specificity of antisera prepared against Rana pipiens jelly I	38
7	Analysis of tissue-specificity of antisera prepared against Rana pipiens jelly II	40
8	Analysis of tissue-specificity of antisera prepared against Rana pipiens jelly III	42
9	Analysis of specificity of antisera prepared against jellies from another Anuran genus and a genus of another Amphibian (Urodela)	44

LIST OF TEXT FIGURES

Figure		Page
1	Species-specificity of inhibitory effect of anti-jelly sera on fertilization of eggs of Rana pipiens	56
2	Tissue-specificity of inhibitory effect of antisera on fertilization of eggs of Rana pipiens	57
3	Table of significant differences between treatments of eggs of Rana pipiens	58
4	Results obtained after treating eggs of Rana pipiens with fractionated serum	62

INTRODUCTION

At fertilization, the egg is incited to undergo a number of changes that lead to the development of a new individual. It has been suggested that the initiation of these changes is due to reactions that occur between the surface of the egg and the spermatozoön which allows the latter to penetrate the egg. The mechanism of activation of the egg by the spermatozoön has been the central problem in the study of fertilization. This problem may be approached experimentally in a number of ways but it seems that the one most likely to produce results would be a study of the rôle of interacting substances of the sperm and egg.

Many attempts have been made to extract interacting substances from eggs and spermatozoa. The first serious study of this nature was done by F. R. Lillie on the isoagglutinins in eggs of the sea urchin, Arbacia, and of the annelid, Nereis.

Lillie (1913) showed that sea water which had been in contact with sea urchin eggs caused the agglutination of spermatozoa of the same species. When eggs were kept for several days in sea water they ceased to produce the agglutinating substance and their capacity for fertilization was

also diminished. Lillie concluded that this agglutinating substance of the egg, which he called "fertilizin," was indispensable for the process of fertilization. It was suggested by Lillie that this agglutination reaction is very similar to an antigen-antibody reaction, and that fertilizin of the egg reacts with "anti-fertilizin" present in the spermatozoön.

More recently, antigen-antibody like reactions have been postulated to account for a number of different kinds of specific intercellular reactions. For example, Spiegel (1954a, b, and 1955) has been able to interfere with the reaggregation of disaggregated cells by the use of species-specific antibodies in a way that would indicate a role of specific surface antigens. This worker found that antiserum prepared against embryos of Rana pipiens inhibits the reaggregation of dissociated ectodermal cells of the homologous species. The antiserum had no effect on the reaggregation of dissociated cells of a species of Triton. Spiegel concluded that the adhesion of homologous cells was due to the presence of surface configurations with the reciprocal structural relationship of antigen and antibody. Tissue incompatibility (Woerdeman, 1955, and Billingham and Sparrow, 1955) and tissue affinity studies (Holtfreter, 1947a, b) also indicate the importance of cellular interactions.

The most thoroughly investigated cells with reference to interacting substances are the spermatozoa and eggs of species of Echinodermata. Studies of these substances have been given considerable attention in the last few years (for recent reviews see Tyler, 1955, and 1959; Rothschild, 1956; Metz, 1957; Runnstrom et. al., 1959). Tyler's work seemingly supports the "fertilizin theory" of F. R. Lillie and further suggests that fertilizin is identical with the jelly-coat material of the sea urchin egg.

Conflicting and divergent interpretations have been suggested for the role of interacting substances of the gametes in fertilization (Perlmann, 1959; Tyler, 1959; Hagstrom, 1956). There can be no doubt at the present time that these substances are operative in the fertilization reaction, and that they occur in the gametes of a number of species of animals, particularly among the Echinoderms (cf. Tyler, 1948, 1957, and 1959; Bishop and Tyler, 1956; Bernstein, 1952).

Before one can understand completely the mechanisms of the process of fertilization, one must know the molecular structure of the surface of both spermatozoön and egg, and the ultrastructual patterns of the interacting molecules.

Chemical analysis of the fertilizin from eggs of the sea urchin has shown it to be composed of mucopolysaccharides

(glycoproteins) with a high sulfur content (Tyler, 1949; Tyler and Fox 1940; Vasseur, 1948). However, very little is known chemically about the groups present in fertilizin molecules which specifically react with antifertilizin. In this connection, studies of specific antibodies used in conjunction with other biochemical and biological techniques, have made possible the analysis of biologically active materials of complex mixtures, which cannot be distinguished by other means at the present time.

The results of a thorough examination of the effects of specific antibodies produced in rabbits against various materials from eggs, spermatozoa, embryos, and adults of various species of Echinoderms, have been reported by Tyler and co-workers (1959) in California, and by Perlmann and co-workers (1959) in Sweden. In general, these investigators have demonstrated that treatment of eggs with antisera produced against eggs and various egg materials of the sea urchin, results in inhibition of fertilization, cleavage, and development of the embryo.

Perlmann and co-workers (for references see Perlmann, 1959) report finding at least four different antigenic components in eggs of Paracentrotus lividus. These workers have attributed certain specific activities such as jelly precipitation, activation, cortical damage, etc., to antisera prepared against

these components, as measured by the effects on eggs treated with the antisera. These components have been designated as (1) J-antigen--that component which is involved in the precipitation of the jelly layer on treatment with antisera against an egg surface antigen. (2) A-antigen--that component which is concerned with the artificial activation of the egg which occurs after treatment with antisera prepared against an egg surface antigen. (3) C-antigen--probably located in the cortex. Cortical damage results when the egg is treated with antibodies against this component. (4) F-antigen--probably the sperm receptor antigen of the egg cortex. Perlmann interprets these results as indicating that these antigenic components have a particular role in connection with the attachment of the sperm and activation of the egg in normal fertilization.

The problems of species- and tissue-specificity of the fertilization reaction has been studied by Lillie (1921), Tyler and co-workers (see Tyler, 1959, for references), and by Perlmann (1959). Tyler (1949) has demonstrated that there is considerable cross-agglutination of spermatozoa of Echinoderm species by antisera prepared against sperm extract of other species. However, antibodies formed against antifertilizin of the sperm react with other tissues from the homologous species but not with the same tissues of foreign species. Considerable cross-reaction was also seen in the case

of the blockage of cleavage of fertilized eggs after treatment with antiserum prepared against fertilizin, in the different species tested (Tyler, 1959). Thus, the specificity of the fertilizin-antifertilizin reaction does not, in itself, account for the specificity of fertilization since a number of other reactions are possibly involved in fertilization. However, Tyler has pointed out that where cross-fertilization occurs, cross-agglutination also generally occurs. Evidence for tissue-specificity of fertilizin comes mainly from work on sperm agglutination, it having been determined that no cellular types other than the sperm or egg yield fertilizin or antifertilizin. The conclusion drawn from these results is that both tissue- and species-specificity of fertilization are based, in part, on the specificity of the fertilizin-antifertilizin reaction.

It is quite surprising that very few studies have been performed on the role of the jelly-coat material of eggs of Amphibians, as compared to the rather extensive studies done on jelly of eggs of sea urchins and other Echinoderms.

There can be no doubt as to the importance of the jelly envelope around eggs of Amphibians, since eggs without their jellies, either as they normally occur before passage down the oviduct, or after removal of jelly from uterine eggs by artificial means, are not fertilizable (Batallion, 1919;

Rugh, 1935; Kambara, 1953; Tchou-Su and Wang, 1956; Shaver and Barch, 1960; Subtelny and Bradt, 1961). The latter workers have shown that coelomic (jellyless) eggs of the frog are capable of normal cleavage before passage down the oviduct, if artificial activation is accomplished by pricking with a glass needle and the subsequent transfer of blastula nuclei. Batallion (1919) demonstrated that unfertilized, jellyless eggs were capable of development after the parthenogenetic inoculation of a cellular element. The fact that the egg is capable of undergoing development prior to the deposition of the jelly, only after artificial stimulation, suggests that the jelly is important in the initial steps in fertilization. Kambara (1953) and Tchou-Su and Wang (1956) have shown that jellyless eggs of the toad are fertilizable when artificially supplied with jelly.

Kambara (1953) noted that eggs of the toad (Bufo vulgaris formosus) become increasingly more fertilizable as they pass down the oviduct, and that the so-called "C layer" (the second layer of jelly to be laid down) was indispensable for fertilization. However, in the results presented by this worker, it was not possible to determine whether a maturation factor is involved, since the eggs possibly do mature during the sojourn in the oviduct. Eggs of Rana pipiens (Shivers, unpublished results) are not fertilizable immediately upon reaching the

uterus but become increasingly more fertilizable after remaining in the uterus for some time, i.e., several hours, suggesting a maturation factor.

It has been well established that unfertilized eggs of the frog become increasingly less fertilizable as they stand in fluid, which has been attributed, in part, to the swelling of the jelly layers which begins immediately upon contact with the fluid (cf. Rugh, 1951). Changes in the surface of the egg (Tyler, 1955) and breakdown of cortical granules (Motomura, 1952), perhaps reflecting initial phases of activation, have been observed in eggs of other Amphibians and other vertebrates which may contribute to the loss in fertilizability. The loss of antigenic components from the jelly material or the egg almost immediately after immersion in water (Shivers, unpublished results) may also account for the decrease in fertilizability.

Both physical and chemical mechanisms have been used in an attempt to explain the role of the jelly in the process of fertilization of Amphibians. Kambara (1953) came to the conclusion that no chemical substance present in jelly of toad eggs is responsible for fertilization, since eggs deprived of their gelatinous coatings were not fertilizable even after being covered with homogenized jelly. On the other hand, this worker noted that denuded eggs became fertilizable after being

covered with gelatine or agar. Thus, Kambara was led to believe that the jelly layers of the toad egg served as a mechanical foothold for the sperm in the penetration of the egg and that gelatine or agar could serve as a substitute for the jelly in this capacity.

Observations made by other workers have suggested that the jelly coating of eggs of Amphibians operates in the same manner as the fertilizin of the sea urchin egg (Bernstein, 1952). Bernstein (1952) was unable to detect the presence of an agglutinating substance in the egg water of Rana pipiens, but reports the irreversible agglutination of sperm of Rana clamitans by egg water of the homologous species. The fact that sperm agglutinating substances were not observed in non-ovulated, jellyless eggs, suggests that the substances were located in the jelly layers. Sperm agglutinating substances have also been reported in a number of other vertebrates (cf. Bishop and Tyler, 1956).

Chemical studies made on the egg-jelly material of several species of Amphibia have shown it to be very similar to the jelly-coat of the sea urchin egg, e.g., the egg jelly of Rana is composed primarily of mucopolysaccharide or glycoprotein (Folks, Grant, and Jones, 1950). According to Minganti (1955), the principal difference between the egg jelly of Amphibians and the jelly-coat of Echinoderm eggs is the presence of

hexosamine in the former.

Shaver and Barch (1960) have studied the effects of treating eggs and spermatozoa of the frog, Rana pipiens, with antisera prepared against the jelly-coat material of the homologous species. These workers have shown that pre-treatment of gametes with the anti-jelly serum resulted in significant inhibition of cleavage of the egg, implying an interference in the union of the gametes. Also, it was shown that the inhibitory effect of the anti-jelly serum could be completely removed by adsorption of the antiserum with jelly-coat material prior to treating the gametes.

The objectives of the present investigation were: (1) to characterize by serological methods, the components present in egg-jelly of Rana pipiens, both as to tissue- and species-specificity; (2) to test the effects of treating eggs of Rana pipiens, prior to normal fertilization, with antisera prepared against the jelly material from the homologous and heterologous species; (3) to show the effect of treating eggs of Rana pipiens with antisera that had been previously adsorbed with jelly from the heterologous and homologous species; (4) to test the effects of treating eggs of Rana pipiens with antisera prepared against organ extracts of the adult frog; (5) to test the effect of treating eggs of Rana pipiens with antisera prepared against egg-jellies from other taxa of Amphibia.

MATERIALS AND METHODS

Jelly coat material was mechanically removed from mature unfertilized eggs of four species of frogs (Rana pipiens, R. clamitans, R. sylvatica, and R. catesbeiana), after allowing the gelatinous material to hydrate completely in distilled water. The jelly was washed several times with distilled water prior to lyophilization. Antigens were prepared by blending ten mg of the lyophilate with one ml of physiological saline solution, 0.85%, buffered at pH 7.4 with Sorenson's phosphate mixture, to which sodium ehtyl mercurithio-salicylate (Merthiolate, Lilly) was added in a proportion of one part per 10,000.

Tissues that were to be used as antigens were removed from the adult frog, weighed and homogenized, either in a glass homogenizer or a Virtis homogenizer, model 23. The homogenization medium used for these tissues was the buffered saline solution described above. The homogenate was centrifuged at 1200 x g and the supernatant fluid was used as the antigen. Practically all representative tissues have been tested.

Blood for control serum was drawn from the marginal ear vein of large rabbits prior to the injection of the antigen. In no case was a cross-reaction observed between rabbit control

serum and any frog tissues tested. All serum was dialyzed against one-tenth full strength Holtfreter's solution for 48 hours and frozen until ready for use.

Production of antisera has employed the use of the Freund adjuvant technique. The adjuvant mixtures are available in two types, complete and incomplete, from Difco Laboratories, Detroit, Michigan. The complete adjuvant mixture is composed of mannide monooleate, 1.5 ml; parrafin oil, 8.5 ml; and 5 mg of killed and dried Mycobacterium butyricum. The incomplete adjuvant mixture lacks the Mycobacterium butyricum. Each rabbit was injected via the subscapular route with 1.5 ml of emulsion prepared by mixing equal volumes of complete adjuvant mixture and antigen. One week later, a second injection of 1.5 ml of emulsion was given, this time using equal volumes of incomplete adjuvant mixture with the antigen.

Four weeks after the second injection, serum from a bleeding made from the ear vein of the rabbit was tested for the presence of antibodies by the use of the Ouchterlony technique. If the presence of antibodies was indicated, the animal was bled twice more on successive weeks from the ear vein before being exsanguinated by cardiac puncture.

Ouchterlony plates were prepared by heating a 1% solution of "Ionagar" (obtained from Consolidated Laboratories, Inc.) using the buffered physiological saline solution, mentioned

previously, as the solvent. Prior to pouring the heated agar into Petri dishes of 90 mm diameter, strips of filter paper, approximately one inch long and one-half inch wide, are folded over the lip of the "male" half of the dish, the ends of the strips touching the floor of the dish. The strips were moistened thoroughly with distilled water before adding the still warm agar, 20 ml per dish. After the agar was completely cooled, various arrangements of wells were cut and floored with one drop of hot agar. Plates were then covered and stored, bottoms up, in the refrigerator at 4°C until ready for use.

Each well was refilled with antigen or antiserum until a total of 0.75 ml had been given, an attempt being made to refill wells with successive applications before the well dried. Advancing zones of antigens and antisera diffuse into the agar; in the area between the wells where they met, antiserum components which were complementary to antigen components, were indicated by a line of precipitation. When the plate was fully developed, usually after 10 days at room temperature (18 to 20°C), a drawing was made of the plate, with all the lines, and the preparation was photographed for a permanent record.

Adsorption of antisera was accomplished by treating them with various dilutions of the inhibiting antigen in glass tubes for 24 hours at 4°C, care being taken to see that antiserum

sites were completely saturated with complementary antigen sites. An equal volume of antiserum and antigen was sufficient to remove or neutralize complementary sites of the antiserum in each case. (Adsorption with larger amounts of antigen did not noticeably change the patterns of the precipitates.) Plates were then run in the usual way using these adsorbed antisera.

Antisera were prepared against the egg jelly material from species of another Anuran genus (Bufo americanus and Bufo marinus) as well as from another order of Amphibia (Ambystoma maculatum), in the same manner as that described for the species of Rana. An antiserum against a preparation of fertilizin from the eggs of Arbacia punctulata (Eichnodermata), kindly supplied by Dr. C. B. Metz, was also available.

In the experiments to be reported, gametes of Rana pipiens, obtained from commercial dealers in Vermont, were used for egg treatments. The eggs employed were obtained by artificially inducing ovulation by the injection of pituitary glands obtained from adult frogs (Rugh 1934).

Normal sperm suspensions were prepared by macerating whole testes, obtained from previously pithed frogs, in one-tenth full strength Holtfreter's solution. Batches of 30-50 eggs were stripped out on 1 x 3 inch glass slides and flooded with the sperm suspension. Insemination was accomplished by treating

eggs for 10 minutes. After the excess sperm suspension was poured off, eggs were placed in large volumes of aerated tap water and cultured at room temperature (18-20°C). Results were recorded in all cases as the percentages of eggs that cleaved. The number of cleaving eggs was counted immediately after fertilization and again at 8-32 cell stage (Shumway stages 5-7). Results of both the Ouchterlony studies and of treatment of gametes are from experiments in which sera pooled from at least three different rabbits were employed.

Egg treatments: Eggs were treated with serum diluted to 1:1 with one-tenth Holtfreter's solution. After treatments of two minutes, the serum was decanted from the eggs, which were washed with a large volume of Holtfreter's solution to remove any unreacted antibodies. Immediately after washing, the eggs were inseminated with normal spermatozoa. Control batches of eggs were treated with the Holtfreter's solution and washed before insemination. Adsorption of the various sera prior to egg treatment was accomplished by the same method as described in connection with the Ouchterlony plate procedure.

In one experiment an attempt was made to remove a non-specific inhibitory effect of normal rabbit serum on the spermatozoa and eggs of R. pipiens. The technique used in this procedure was the "Rivanol" method of fractionation of

serum as suggested by Horejsi and Smetana (1956). For separation of the gamma globulins and albumins, 3.5 parts of a 0.4% Rivanol solution (ethoxy-diamino acridine lactate) was added to one part serum. The pH was adjusted to 8.0 by the addition of $NaHCO_3$. The bright yellow precipitate which formed (albumin-Rivanol complex) was separated from the supernatant fluid (globulin fraction) by decanting the latter. Unreacted Rivanol was removed from the filtrate by adding an excess of activated charcoal. The charcoal-Rivanol complex was then removed by filtering the solution through Whatman's No. 1 filter paper. The filtrate was lyophilized until dry, resuspended in the original volume of fluid, dialyzed against one-tenth full strength Holtfreter's solution for 48 hours, and frozen until ready for use.

In the spring of 1960, an attempt was made to see if the fertilizability of uterine eggs could be affected by anti-jelly serum treatments prior to the deposition of the jelly-coat material. Female R. pipiens were injected with whole pituitary glands for the induction of ovulation. Several hours later a series of injections, using R. pipiens anti-jelly sera, was started. Eight mls of the sera, diluted 1:1 with Holtfreter's solution, were injected into the body cavity at two hour intervals. Each frog was checked before the injections of the sera were started to make sure that no eggs

were present in the uterus at the time of the first injection. Injections of the anti-jelly sera were continued until ovulation was completed, which was about six hours. Eggs from these injected females were then inseminated with normal spermatozoa.

RESULTS

A. Analysis of Anti-jelly Sera

(1) Analysis of antisera prepared against jelly of R. pipiens.

Species-specificity: Plate 1, Figure 1 shows the result of reacting rabbit control serum, i.e., serum drawn from the rabbit prior to the injection of antigen into the rabbit, with jelly material from three species of frogs (Rana pipiens, R. sylvatica, and R. clamitans). No cross-reactions were observed between rabbit control serum and any of the frog tissues tested.

Plate 1, Figure 2 shows the result of reacting antisera prepared against jelly of R. pipiens with homogenates of jelly material from the same three species of frogs. Drawings made immediately after the completion of plates will be used instead of photographs, since it is very difficult to get lines of precipitation to show up distinctly in photographs. Plate 2, Figure 7 is a drawing made of the Ouchterlony plate using anti-jelly serum of R. pipiens against jelly homogenates of the three species described in Plate 1, Figure 2.

As shown by Plate 1, Figure 2 and Plate 2, Figure 7, the jelly material of R. pipiens contains a number of specific components. These specific components are indicated by

lines of precipitation between the antiserum well and the well containing the jelly material of R. pipiens. A component that is found in jellies of all three species is indicated by a continuous line formed opposite all three antigen wells. The jelly of R. pipiens also contains a component in common with one found in the jelly of R. clamitans that is not present in the jelly of R. sylvatica (Plate 2, Figure 7).

If the anti-jelly serum against R. pipiens is treated with an equal volume of jelly of R. clamitans for 24 hours, the common component between all three species is removed (Plate 2, Figure 8). The component in common with jelly of R. clamitans which is not present in the jelly of R. sylvatica is also removed by treatment with jelly of R. clamitans. The specific components for jelly of R. pipiens are not removed by treatment with jelly of R. clamitans.

Treatment of antiserum against the jelly of R. pipiens with jelly of R. sylvatica removes only the component that is common between all three species (Plate 2, Figure 9). The component of the jelly of R. pipiens that is common with a component found in the jelly of R. clamitans is not removed by treatments with jelly of R. sylvatica. Jelly material of R. catesbeiana, on the other hand, removes a component from anti-jelly serum of R. pipiens that is common to R. pipiens, R. clamitans, and R. catesbeiana, as well as the component shared

between R. pipiens and R. clamitans (Plate 2, Figure 11), leaving only the specific components for the jelly of R. pipiens. All components were removed by treating the anti-jelly sera of R. pipiens with the homologous jelly (Plate 2, Figure 10).

Anti-jelly sera of R. pipiens were also treated with homogenates of various adult tissues from the homologous species prior to the running of the plate. Adsorption with these tissues failed to remove any of the jelly components (see Plate 8, Figure 38).

No cross-reaction was observed between the jelly material of R. pipiens and antiserum against the egg-jelly of Ambystoma maculatum (Plate 9, Figure 42), nor does treatment of anti-jelly serum of R. pipiens with jelly of Ambystoma remove any of the jelly components of R. pipiens (Plate 2, Figure 12). The jelly of a toad species, Bufo americanus, does have a component that is common with a component of the jelly of R. pipiens (Plate 9, Figure 41). Anti-jelly serum of Bufo marinus, on the other hand, does not have components in common with the jelly of R. pipiens (Plate 9, Figure 43). Anti-jelly sera of R. pipiens were treated with the jelly from these species of Bufo and then reacted with the jelly of the species of Rana. The jelly material of Bufo americanus was the only one to remove components from anti-jelly sera of R. pipiens. No cross-reaction was observed between fertilizin prepared from

from eggs of the sea urchin, (Arbacia) and anti-jelly serum of R. pipiens (Plate 9, Figure 44).

Tissue-specificity: A series of reactions was performed to test the tissue-specificity of the jelly components of R. pipiens (see Plates 6, 7, and 8). Anti-jelly sera of R. pipiens were reacted with homogenates of various adult tissues from the homologous species. It was determined by testing practically all representative tissues of the frog that the jelly components were, in general, restricted to the oviducal tissues (Plate 7, Figure 34). Cross-reactions were obtained between the anti-jelly sera and homogenates of mature oviduct (Plate 7, Figure 34), as would be expected since the jelly layers are deposited on the egg during their sojourn in the oviduct. When anti-jelly serum of R. pipiens is adsorbed with homogenized oviduct prior to reaction with jelly of R. pipiens, no lines appear subsequently on plates, indicating that the source of all jelly components must be in the oviduct.

Other components are present in the oviduct homogenate, however, that do not react with the anti-jelly sera. These components may be demonstrated in the oviduct by treating anti-oviducal sera with an equal volume of jelly material and re-acting this treated serum with an homogenate of oviduct (Plate 6, Figure 31). Some of these non-jelly components of the oviduct react with anti-ovary sera (Plate 6, Figures 28 and 31).

Indeed, almost all tissues tested cross-react with anti-ovary sera with the exception of the jelly material. In some instances, however, a very weak reaction has been observed between certain anti-jelly sera and ovarian homogenates. This reaction may be due to contamination of the jelly by egg material which occurs during the process of removing the jelly from the egg.

One component of anti-jelly serum of R. pipiens cross-reacts with a serum component of blood of R. pipiens (Plate 7, Figure 36). This component seems to be species-specific, i.e., the component does not react with serum from the other species of Rana. Anti-jelly serum of R. clamitans on the other hand, contains components that cross-react with blood serum which are not species-specific, i.e., the components react with serum from the homologous species as well as with serum from R. pipiens and R. catesbeiana (Plate 7, Figure 37). Anti-jelly serum of R. catesbeiana reacts with serum from the homologous species but not with serum from R. pipiens or R. clamitans (Plate 7, Figure 38).

(2) Analysis of antisera prepared against jelly of R. clamitans.

Reactions obtained with anti-jelly sera of R. clamitans show that egg-jelly of R. clamitans contains components in common

with components found in the jelly material of the species of Rana tested (Plate 1, Figure 3 and Plate 3, Figure 13). Jelly of R. clamitans has a number of components that are specific for this species and a component in common with one found in jelly of R. pipiens which is not present in the jelly of R. sylvatica (Plate 3, Figure 13). There are also components in common with ones found in jelly of R. catesbeiana, which are not found in either of the jellies of R. pipiens or R. sylvatica (Plate 3, Figure 18).

The configuration in anti-jelly serum of R. clamitans complementary to the component shared by the species of Rana was removed by treating this antiserum with jelly material from any of the species used (Plate 3, Figures 14, 15, 16, and 17). Specific components represented in anti-jelly serum of R. clamitans could be removed only by adsorbing with the homologous jelly material.

Tests for the tissue-specificity of jelly components of R. clamitans have been attempted with the same results being observed as were seen in the comparable situation in R. pipiens, i.e., the jelly components are restricted primarily to the oviducal tissues. However, tissues of R. clamitans have not been tested as thoroughly as have the tissues of R. pipiens.

(3) Analysis of antisera prepared against jelly of R. sylvatica.

Common components were observed between jelly material of all species of Rana tested when the anti-jelly serum of R. sylvatica was used (Plate 4, Figure 19) and these components could be removed by treating the antiserum with jelly material from any of the species of Rana (Plate 4, Figures 20, 21, 22, and 23).

The species-specific components represented in the anti-jelly serum of R. sylvatica were removed only by treating with the homologous jelly material (Plate 4, Figure 22). A component represented in anti-jelly serum of R. sylvatica that was common with one found in jelly of R. pipiens could be removed by treating with the homologous jelly or jelly of R. pipiens.

(4) Analysis of antisera prepared against jelly of R. catesbeiana.

Jelly components common to three species of Rana represented in antiserum against the jelly of R. catesbeiana could be removed by treating with any of the jellies of Rana (Plate 5, Figures 24, 25, 26, and 27). Other components in the jelly of R. catesbeiana were common with components found in R. clamitans, but were not found in R. pipiens or R. sylvatica. Complementary configurations for this component could be

removed from the anti-jelly sera of R. catesbeiana by either of the two former jellies. Jelly of R. sylvatica or R. pipiens would not remove this component. The specific components for R. catesbeiana could be removed only by treating with the jelly of R. catesbeiana (Plate 5, Figure 27).

(5) Analysis of antisera prepared against jelly obtained from another genus of Anura and a genus of another order (Urodela; Ambystoma).

(a) Anti-jelly serum of Bufo americanus contains configurations against a number of components that are specific for this species, as well as against components common with ones found in the jelly material from Bufo marinus. A cross-reaction was also observed between anti-jelly serum of Bufo americanus and jelly material from all the species of Rana (Plate 9, Figure 41).

(b) Anti-jelly serum of Bufo marinus represents a number of components that are specific for this species, as well as components in common with ones found in the jelly of Bufo americanus. No cross-reactions were observed between the anti-jelly serum of Bufo marinus and the jelly from the species of Rana (Plate 9, Figure 43).

(c) Anti-jelly serum of Ambystoma maculatum represents

a number of specific components. No cross-reaction was observed between anti-jelly serum of the Urodele (Ambystoma) and jellies of the Anurans, Rana and Bufo (Plate 9, Figure 42). However, the reciprocal combination (Plate 9, Figure 41) did produce a reaction.

(d) Jelly (fertilizin) of Arbacia punctulata (Echinodermata) contains a specific component. No cross-reaction was observed between antiserum against fertilizin of the sea urchin and jellies of the Urodele or Anuran (Plate 9, Figure 44).

Plate 1

Representative Photographs of Ouchterlony Plates and Results Observed on Eggs after Treatments with Anti-jelly Serum.

Figure 1 Ouchterlony Plate No. 383.

C - control serum drawn from rabbits prior to the injection of antigen.
SJ - R. sylvatica jelly
PJ - R. pipiens jelly
CJ - R. clamitans jelly

Figure 2 Ouchterlony Plate No. 203.

8A - R. pipiens anti-jelly serum
1 - R. sylvatica jelly
2 - R. pipiens jelly
3 - R. clamitans jelly

Figure 3 Ouchterlony Plate No. 200.

29A- R. clamitans anti-jelly serum
1 - R. pipiens jelly
2 - R. clamitans jelly
3 - R. sylvatica jelly

Figure 4 Ouchterlony Plate No. 300.

SA - R. sylvatica anti-jelly serum
1 - R. sylvatica jelly
2 - R. pipiens jelly
3 - R. clamitans jelly

Figure 5 - Eggs treated with rabbit control serum. For explanation see text.

Figure 6 - Eggs treated with anti-jelly serum of R. pipiens in the same dilution as used for results presented in Text Figure 1 & 2, i.e., a dilution of 1:1. For explanation see text.

Plate 1

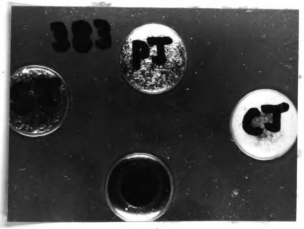


Figure 1

Figure 2

Figure 3

Figure 4

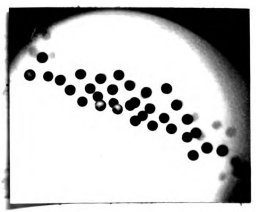


Figure 5

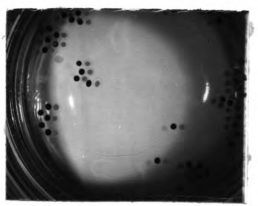


Figure 6

Plate 2

Diagrams of Ouchterlony Plates. Analysis of species-specificity of antisera prepared against R. pipiens jelly. Antigens used were R. pipiens jelly (PJ), R. clamitans jelly (CJ), R. sylvatica jelly (SJ), and R. catesbeiana jelly (FJ).

Figure 7 Ouchterlony Plate No. 203.

PA - Antiserum prepared against R. pipiens jelly.

Figure 8 Ouchterlony Plate No. 208.

PA + CJ - R. pipiens anti-jelly serum inhibited with R. clamitans jelly.

Figure 9 Ouchterlony Plate No. 220.

PA + SJ - R. pipiens anti-jelly serum inhibited with R. sylvatica jelly.

Figure 10 Ouchterlony Plate No. 302.

PA + PJ - R. pipiens anti-jelly serum inhibited with R. pipiens jelly.

Figure 11 Ouchterlony Plate No. 335.

PA + FJ - R. pipiens anti-jelly serum inhibited with R. catesbeiana jelly.

Figure 12 Ouchterlony Plate No. 375.

PA + AJ - R. pipiens anti-jelly serum treated with Ambystoma maculatum jelly.

Plate 2

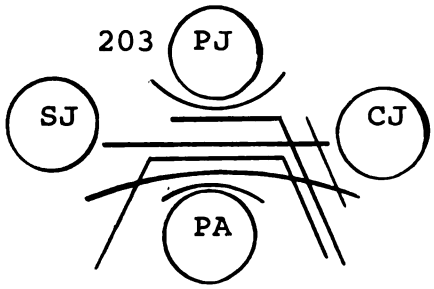


Figure 7

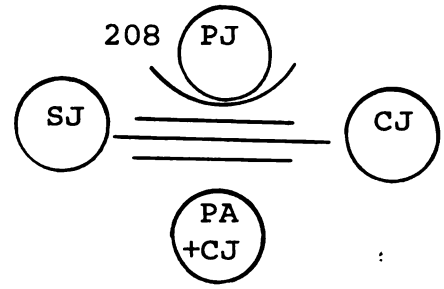


Figure 8

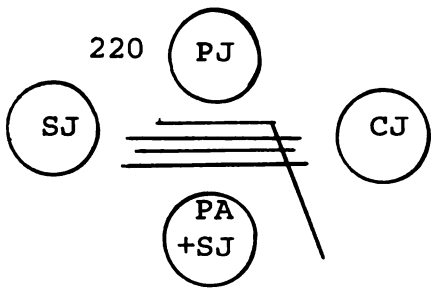


Figure 9

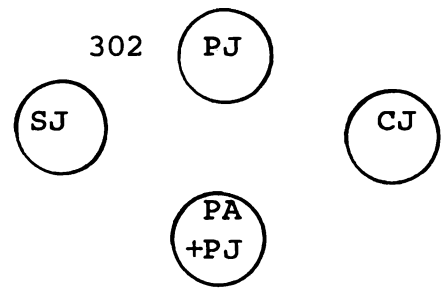


Figure 10

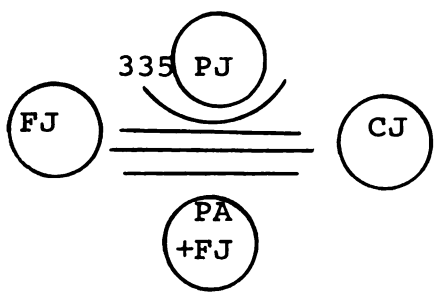


Figure 11

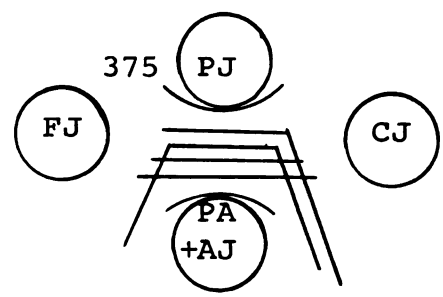


Figure 12

Plate 3

Analysis of species-specificity of antisera prepared against *R. clamitans* jelly. Antigens used were *R. pipiens* jelly (PJ), *R. clamitans* jelly (CJ), *R. sylvatica* (SJ), and *R. catesbeiana* jelly (FJ).

Figure 13 Ouchterlony Plate No. 200.

CA - antiserum prepared against *R. clamitans* jelly.

Figure 14 Oucterlony Plate No. 360.

R. clamitans anti-jelly serum inhibited with *R. pipiens* jelly.

Figure 15 Ouchterlony Plate No. 207.

CA + SJ - *R. clamitans* anti-jelly serum inhibited with *R. sylvatica* jelly.

Figure 16 Ouchterlony Plate No. 324.

CA + CJ - *R. clamitans* anti-jelly serum inhibited with *R. clamitans* jelly.

Figure 17 Ouchterlony Plate No. 373.

CA + FJ - *R. clamitans* anti-jelly serum inhibited with *R. catesbeiana* jelly.

Figure 18 Ouchterlony Plate No. 360a.

CA + PJ - *R. clamitans* anti-jelly serum inhibited with *R. pipiens* jelly.

Plate 3

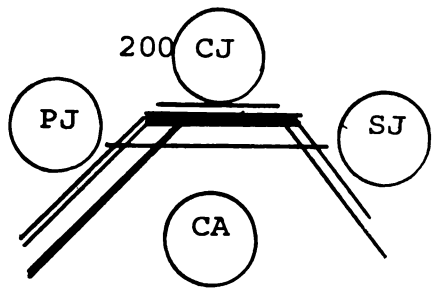


Figure 13

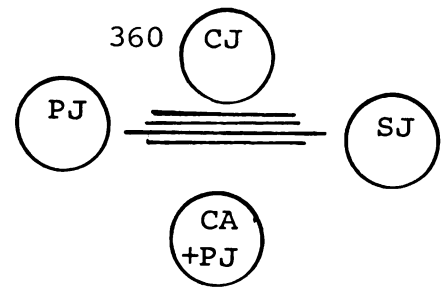


Figure 14

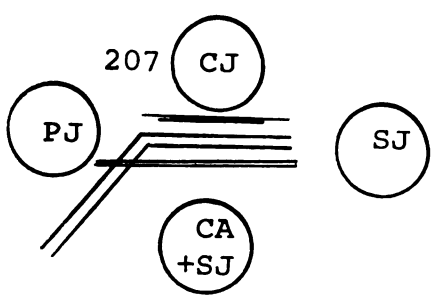


Figure 15

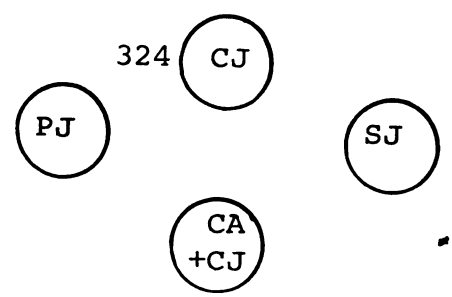


Figure 16

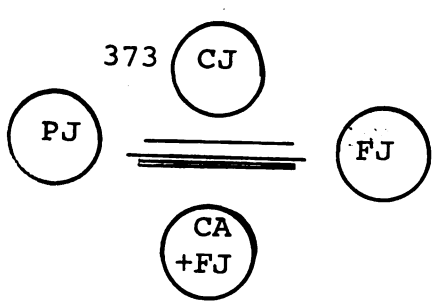


Figure 17

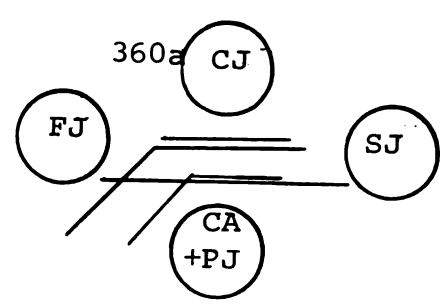


Figure 18

Plate 4

Analysis of species-specificity of antisera prepared against *R. sylvatica* jelly. Antigens used were *R. pipiens* jelly (PJ), *R. clamitans* jelly (CJ), and *R. sylvatica* jelly (SJ).

Figure 19 Ouchterlony Plate No. 300.

SA - antiserum prepared against *R. sylvatica* jelly.

Figure 20 Ouchterlony Plate No. 303.

SA + PJ - *R. sylvatica* anti-jelly serum inhibited with *R. pipiens* jelly.

Figure 21 Ouchterlony Plate No. 322.

SA + CJ - *R. sylvatica* anti-jelly serum inhibited with *R. sylvatica* jelly.

Figure 23 Ouchterlony Plate No. 342.

SA + FJ - *R. sylvatica* anti-jelly serum inhibited with *R. catesbeiana* jelly.

Plate 4

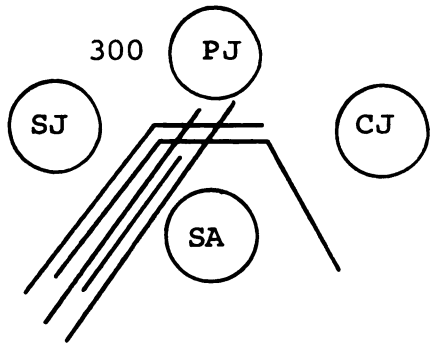


Figure 19

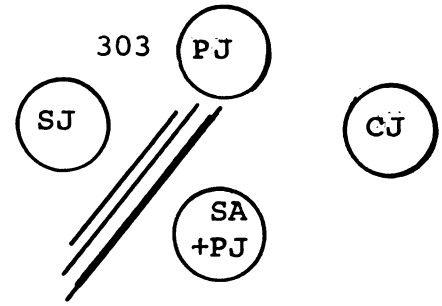


Figure 20

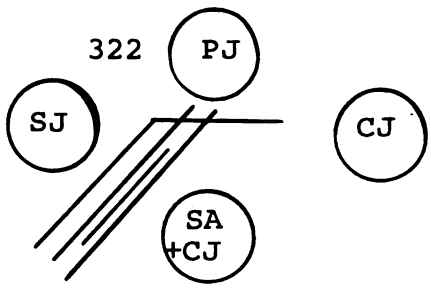


Figure 21

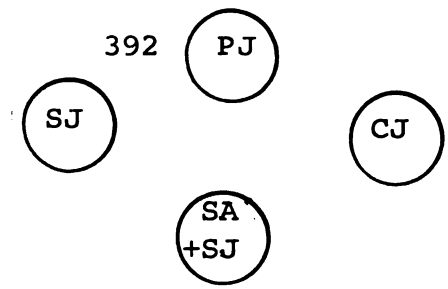


Figure 22

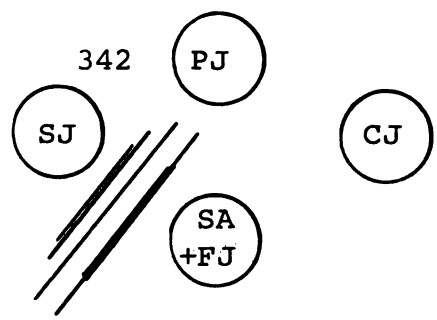


Figure 23

Plate 5

Analysis of species-specificity of antisera prepared against *R. catesbeiana* jelly. Antigens used were *R. pipiens* jelly (PJ), *R. clamitans* jelly (CJ), and *R. catesbeiana* jelly (FJ).

Figure 24 Ouchterlony Plate No. 361.

FA - antiserum prepared against *R. catesbeiana* jelly.

Figure 25 Ouchterlony Plate No. 370.

FA + PJ - *R. catesbeiana* anti-jelly serum inhibited with *R. pipiens* jelly.

Figure 26 Ouchterlony Plate No. 371.

FA + CJ - *R. catesbeiana* anti-jelly serum inhibited with *R. clamitans* jelly.

Figure 27 Ouchterlony Plate No. 369.

FA + FJ - *R. catesbeiana* anti-jelly serum inhibited with *R. catesbeiana* jelly.

Plate 5

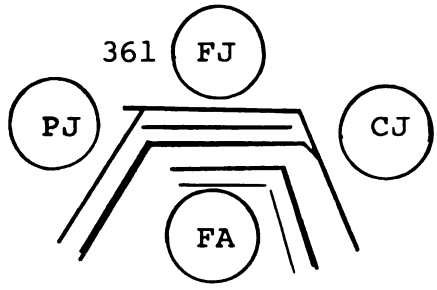


Figure 24

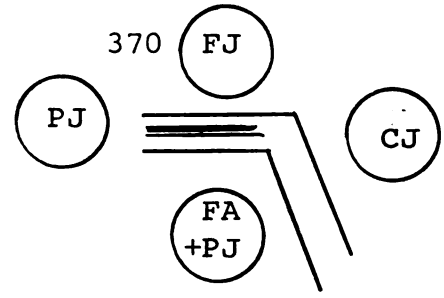


Figure 25

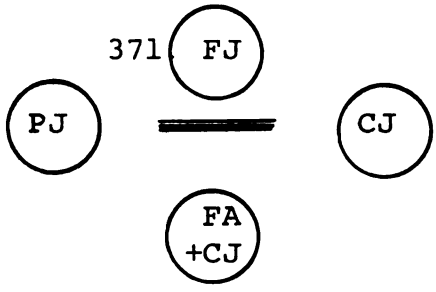


Figure 26

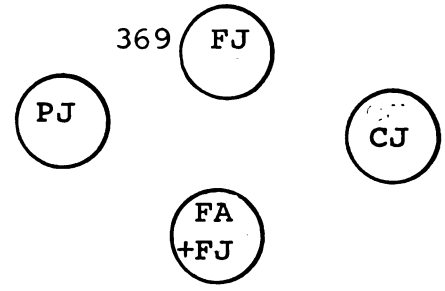


Figure 27

Plate 6

Analysis of tissue-specificity of antisera prepared against

R. pipiens jelly I.

Figure 28 Ouchterlony Plate No. 285.
1 - Ovary homogenate
2 - R. pipiens jelly
3 - Oviduct homogenate
4 - Anti-ovary serum

Figure 29 Ouchterlony Plate No. 286.
1 - Ovary homogenate
2 - R. pipiens jelly
3 - Oviduct homogenate
4 - Anti-oviduct serum

Figure 30 Ouchterlony Plate No. 287.
1 - R. pipiens jelly
2 - Ovary homogenate
3 - Oviduct homogenate
4 - Anti-ovarian serum inhibited with R. pipiens jelly

Figure 31 Ouchterlony Plate No. 309.
1 - Ovary homogenate
2 - R. pipiens jelly
3 - Oviduct homogenate
4 - Anti-oviduct serum inhibited with R. pipiens jelly

Figure 32 Ouchterlony Plate No. 310.
1 - Ovary homogenate
2 - R. pipiens jelly
3 - Oviduct homogenate
4 - Anti-oviduct serum inhibited with ovary homogenate

Figure 33 Ouchterlony Plate No. 374.
1 - Ovary homogenate
2 - R. pipiens jelly
3 - Oviduct homogenate
4 - R. pipiens anti-jelly serum inhibited with ovary homogenate

Plate 6

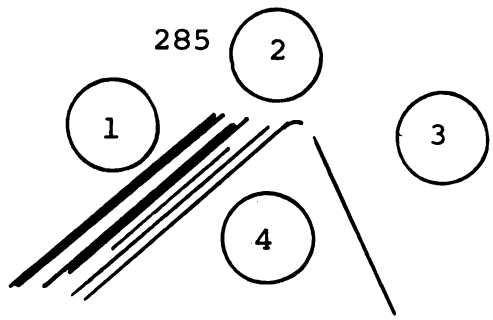


Figure 28

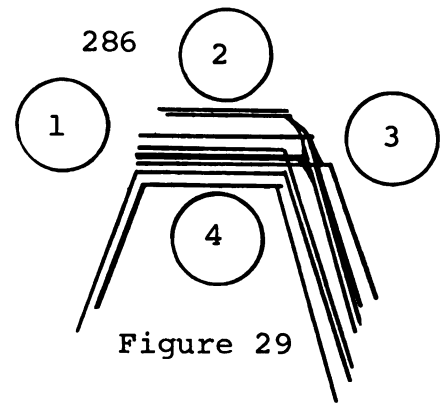


Figure 29

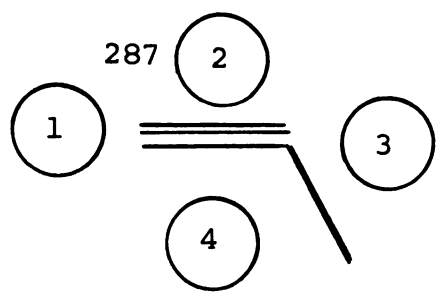


Figure 30

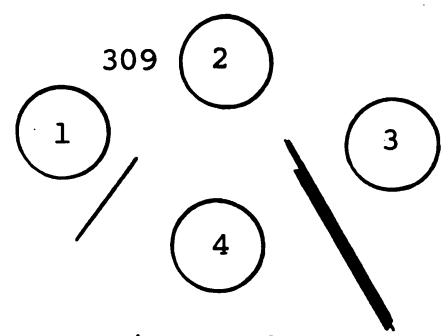


Figure 31

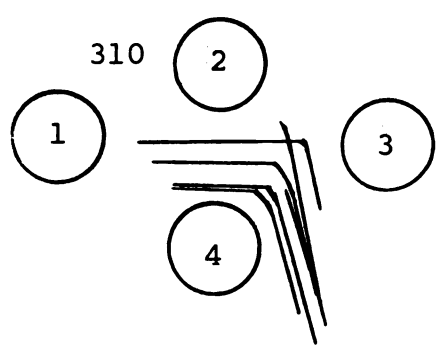


Figure 32

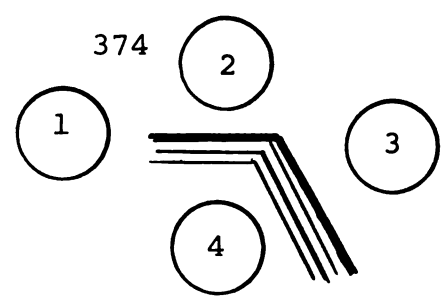


Figure 33

Plate 7

Analysis of tissue-specificity of antisera prepared against

R. pipiens jelly II.

Figure 34 Ouchterlony Plate No. 284.

1 - Ovary homogenate
2 - R. pipiens jelly
3 - Oviduct homogenate
4 - R. pipiens anti-jelly serum

Figure 35 Ouchterlony Plate No. 386.

PA - R. pipiens anti-jelly serum
PJ - R. pipiens jelly
O - Ovary homogenate
M - Muscle homogenate
B - Brain homogenate
K - Kidney homogenate
H - Heart homogenate

Figure 36 Ouchterlony Plate No. 387.

PA - R. pipiens anti-jelly serum
FS - R. catesbeiana serum
CS - R. clamitans serum
PS - R. pipiens serum
PJ - R. pipiens jelly

Figure 37 Ouchterlony Plate No. 388.

CA - R. clamitans anti-jelly serum
FS - R. catesbeiana serum
PS - R. pipiens serum
CS - R. clamitans serum
CJ - R. clamitans jelly

Figure 38 Ouchterlony Plate No. 389.

Same as Plate No. 387 (Figure 36) except R. catesbeiana anti-jelly serum and R. catesbeiana jelly were used.

Plate 7

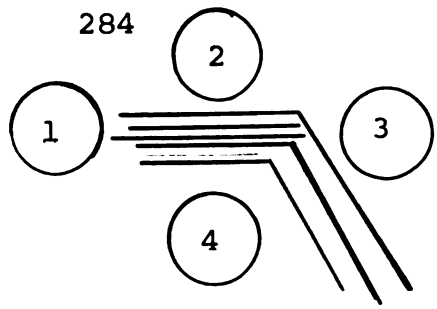


Figure 34

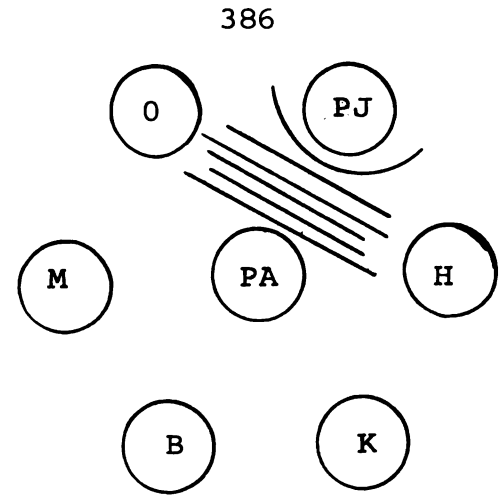


Figure 35

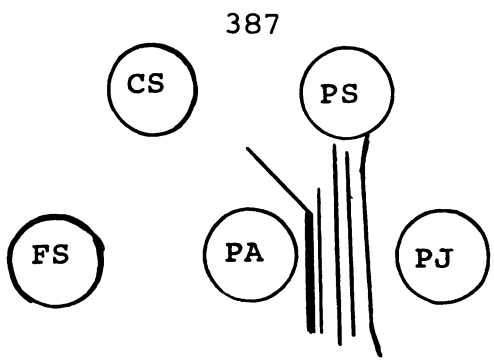


Figure 36

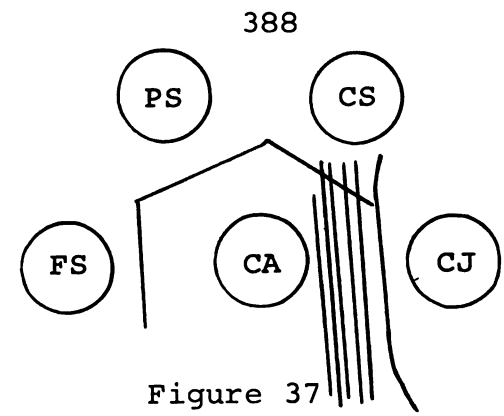


Figure 37

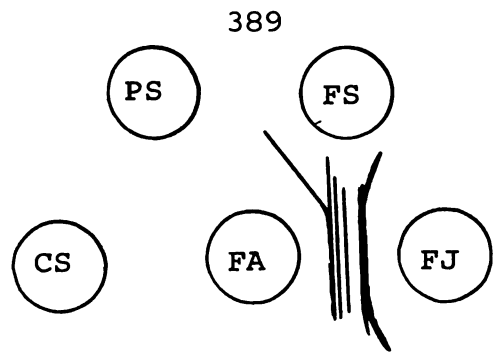


Figure 38

Plate 8

Analysis of tissue-specificity of antisera prepared against

R. pipiens jelly III.

Figure 38 Ouchterlony Plate No. 376.

PA + H - *R. pipens* anti-jelly serum inhibited with heart homogenate.
FJ - *R. catesbeiana* jelly
PJ - *R. pipiens* jelly
CJ - *R. clamitans* jelly

Figure 39 Ouchterlony Plate No. 378.

1 - Oviduct homogenate
2 - Heart homogenate
3 - Ovary homogenate
4 - Anti-heart serum

Figure 40 Ouchterlony Plate No. 377.

1 - Oviduct homogenate
2 - Heart homogenate
3 - Ovary homogenate
4 - Anti-heart serum inhibited with *R. pipiens* jelly

Plate 8

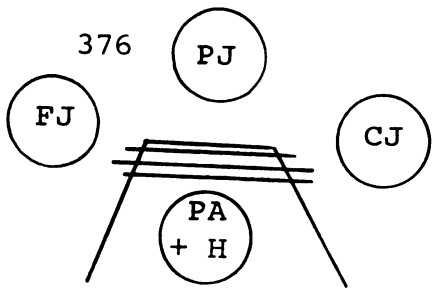


Figure 38

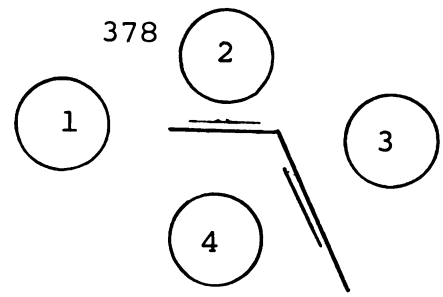


Figure 39

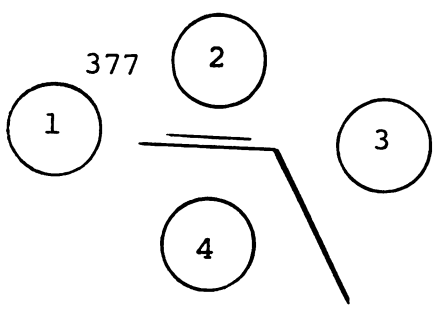


Figure 40

Plate 9

Analysis of specificity of antisera prepared against jellies from another Anuran genus and a genus of another Amphibian (Urodela).

Figure 41 Ouchterlony Plate No. 346.

BA - Bufo americanus anti-jelly serum
BJ - Bufo americanus jelly
MJ - Bufo marinus jelly
AJ - Ambystoma maculatum jelly
SJ - R. sylvatica jelly
PJ - R. pipiens jelly
CJ - R. clamitans jelly

Figure 42 Ouchterlony Plate No. 347.

Same as Plate No. 346 (Figure 41) except Ambystoma maculatum anti-jelly serum was used.

Figure 43 Ouchterlony Plate No. 382.

Same as Plate No. 346 (Figure 41) except Bufo marinus anti-jelly serum was used.

Figure 44 Ouchterlony Plate No. 351.

Same as Plate No. 346 (Figure 41) except Arbacia jelly and Arbacia anti-jelly serum were used.

Plate 9

346

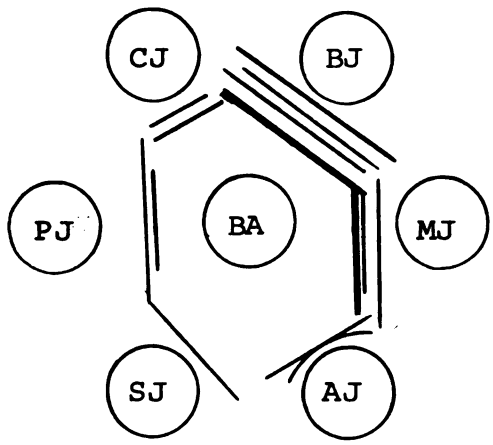


Figure 41

347

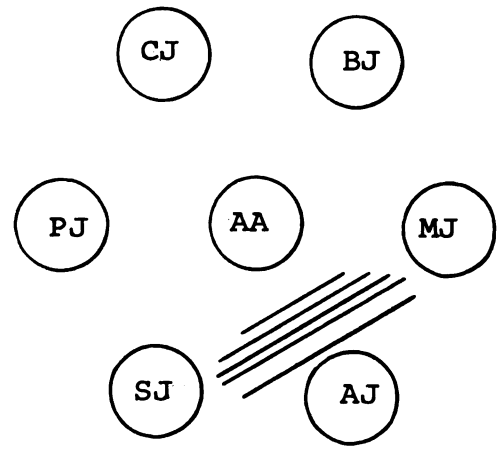


Figure 42

382

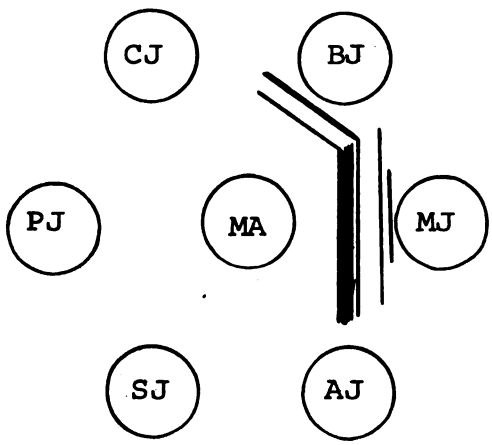


Figure 43

351

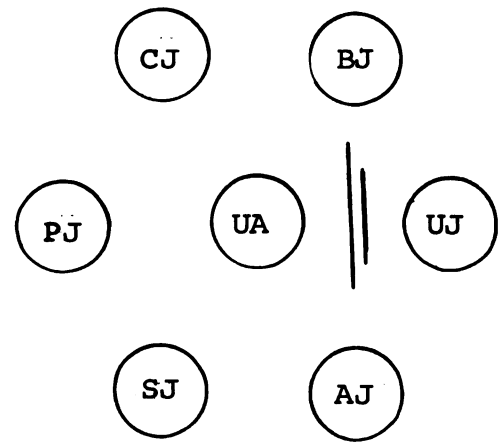


Figure 44

B. The Effect of Antisera on the Fertilization of Eggs of R. pipiens.

When eggs were treated with anti-jelly serum, two effects were observed: (1) A visible precipitation layer forms around the outer layer of jelly (Plate 1, Figure 6); the extent of the jelly precipitation was dependent on the concentration of antiserum used. (2) Eggs, which normally adhere together in a mass (Plate 1, Figure 5), became separated.

The results obtained after inseminating eggs that had been previously treated with antisera in 1:1 dilutions are presented in Table 1 (summarized in Text Figures 1 and 2). All together, the eggs of 25 females were treated with antisera pooled from at least three different rabbits. The eggs from a female were not used more than once in testing each series of treatments.

Three sets of controls were employed in each series of treatments. (a) A test of the quality of the eggs by inseminating untreated eggs with normal spermatozoa (Column Nnsp, Table 1, and Bar Nnsp, Text Figure 1). (b) A test of the effect of treating eggs for two minutes with one-tenth full strength Holtfreter's solution and washing with a large volume of the Holtfreter's solution prior to insemination with normal spermatozoa (Column N, Table 1, and Bar N, Text Figure 1).

In all subsequent treatments with antisera, eggs were washed with a large volume of the Holtfreter's solution prior to insemination to remove any unreacted antibodies. (c) A test of the effect of treating eggs with control rabbit serum in the same dilution as antiserum, prior to inseminating with normal spermatozoa (Column C, Table 1, and Bar C, Text Figure 1).

Percentages of cleaving eggs were calculated and these values were transformed into arcsin equivalents in accordance with the statistical test employed, i.e., an analysis of variance. The values obtained were compared by means of a sequential Q procedure as described in Statistical Methods by G. W. Snedecor, Sections 10 and 11 (see Text Figure 3 for table of significant differences). Although variations in response of the eggs of different females were significant, the statistical design renders this difference irrelevant. There was no significant difference between the fertilizability of untreated eggs and those treated with the Holtfreter's solution or with control serum, i.e., no significant difference was observed between any of the control treatments (Bar Nnsp, N, and C, Text Figure 1).

(1) Effect of treating eggs with antisera prepared against the jelly of R. pipiens.

The fertilizability of eggs after treatment with antiserum

prepared against jelly of R. pipiens was lower by a very significant degree from that of eggs treated with control serum (Bar PA, Text Figure 1). Therefore, there is a substance in the anti-jelly serum which significantly inhibits the fertilization reaction, or some post-fertilization phenomena leading to cleavage.

In order to determine if the inhibiting substances in anti-jelly serum of R. pipiens could be removed by adsorbing with the homologous jelly material, experiments were performed in which an equal volume of the jelly material was reacted with an equal volume of the anti-jelly serum. Adsorption was continued for 24 hours at 4°C. After removal of the jelly by centrifugation, the antiserum was used to treat eggs in the same manner as with the control serum and the unadsorbed antiserum, described above. Bar PA + PJ, Text Figure 1, represents the values obtained after insemination of eggs, treated with antiserum against the jelly of R. pipiens adsorbed with the homologous jelly. It may be concluded that the inhibitory material in the non-adsorbed anti-jelly serum is removed or neutralized by the jelly material.

Experiments were also made in which anti-jelly serum of R. pipiens was adsorbed with the jelly material of R. clamitans and R. sylvatica in the same manner as that described above. Bars PA + CJ and PA + SJ, Text Figure 1, represent the values

obtained after treating eggs with anti-jelly serum of R. pipiens which had been adsorbed with the jelly material from these other species. Since no significant differences were observed between treatments with anti-jelly serum of R. pipiens adsorbed with these heterologous jelly materials and the unadsorbed antiserum of R. pipiens, it may be concluded that the heterologous jelly material did not remove the inhibitory substance. These results suggest the presence of species-specific inhibitory substances in anti-jelly serum of R. pipiens.

The anti-jelly serum of R. pipiens was also adsorbed with the jelly material from Ambystoma maculatum, which also failed to remove the inhibitory material (Bar PA + AJ, Text Figure 1).

(2) Effect of treating eggs with antisera prepared against the jelly of R. clamitans.

Bar CA, Text Figure 1 represents the values obtained after treating eggs of R. pipiens with antiserum against the jelly of R. clamitans in the same manner as described for treatments with the anti-jelly serum of R. pipiens. These results are significantly lower than the values obtained in treatments with the control serum, which indicates the presence of inhibitory substances in this heterologous anti-jelly serum. The values obtained in treatments with anti-serum against the jelly of

R. clamitans were significantly higher than the values obtained in treatments with antiserum against the jelly of R. pipiens; however, this difference may be explained either by assuming that the components represented in the anti-jelly serum of R. clamitans, which are common to this species and to R. pipiens, were less concentrated in the antiserum of R. clamitans than in the antiserum of R. pipiens, or by assuming that more inhibition is produced by the species-specific components represented in the anti-jelly serum of R. pipiens, or by both possibilities.

Adsorption of anti-jelly serum of R. clamitans was performed with the jelly material of R. clamitans, R. pipiens, and R. sylvatica (Bars CA + CJ, CA + PJ, and CA + SJ, Text Figure 1). Values obtained by treating eggs prior to normal insemination with antiserum against the jelly of R. clamitans adsorbed with these three jelly materials were not significantly different from that obtained by treating eggs with control serum. Thus, the inhibitory substances found in anti-jelly serum of R. clamitans were removed by treating with the homologous as well as the heterologous jelly material. If the anti-jelly serum of R. clamitans contains species-specific inhibiting components, it may be concluded that these components have no effect on the eggs of R. pipiens. The Ouchterlony plate studies do indeed indicate that the anti-jelly serum of R. clamitans does contain species-specific components.

(3) Effect of treating eggs with antisera prepared against the jelly of R. sylvatica.

Bar SA, Text Figure 1, represents the values obtained after treating eggs of R. pipiens with anti-jelly serum of R. sylvatica. These values are significantly lower than those obtained by treating eggs with the control serum, and significantly higher than obtained after treatment with anti-jelly serum of R. pipiens. These results demonstrate that the anti-jelly serum of R. sylvatica contains inhibitory substances, as did the anti-jelly serum of R. clamitans, but that these inhibitory substances differ qualitatively and/or quantitatively from those found in the anti-jelly serum of R. pipiens, since there is a significant difference between the effect observed due to treatment with anti-jelly sera of R. sylvatica and of R. pipiens.

Adsorption of the anti-jelly serum of R. sylvatica was performed with jelly materials of R. sylvatica, R. pipiens, and R. clamitans (Bars SA + SJ, SA + PJ, and SA + CJ, Text Figure 1). Values obtained by treating eggs with anti-jelly serum of R. sylvatica adsorbed with these three jelly materials, were not significantly different from those obtained after treating eggs with control serum. Thus, the inhibitory substance found in anti-jelly serum of R. sylvatica was removed by treating the serum with either the homologous or heterologous

jelly materials. Again Ouchterlony plate studies have shown that anti-jelly serum of R. sylvatica contains species-specific components; however, these components have no effect on the fertilizability of eggs of R. pipiens. Also these results, plus the fact that the values obtained by treatments with anti-jelly sera of R. sylvatica and of R. clamitans of eggs of R. pipiens do not differ significantly may be interpreted as indicating that the inhibitory substance of these two anti-jelly sera are the same. Indeed the Ouchterlony plate studies have demonstrated that these two anti-jelly sera contain common components.

(4) Effect of Antisera prepared against egg-jellies of another Anuran genus (Bufo) and of a Urodele species (Ambystoma).

Bar AA, Text Figure 1, represents the values obtained by treating eggs with antiserum against egg-jelly of Ambystoma maculatum. These values are not significantly different from the results obtained by treating eggs with control serum, which demonstrates that anti-jelly serum of Ambystoma contains no components that are inhibitory to fertilization of eggs of R. pipiens.

Eggs were also treated with anti-jelly serum of R. pipiens that had been previously adsorbed with the jelly material of

Ambystoma in the same manner as described for the adsorption with the jelly material of species of Rana. Results obtained were not significantly different from those obtained by treating eggs with unadsorbed anti-jelly serum of R. pipiens (PA + AJ, Text Figure 1). Thus, none of the inhibiting material of the anti-jelly serum of R. pipiens was removed by adsorbing with the jelly material of Ambystoma. In the Ouchterlony plate studies, no cross-reaction was observed between these two jelly materials.

Results obtained by treating eggs with anti-jelly serum of Bufo americanus were significantly lower than the results of treatments with control serum and significantly higher than the results of treatments with antisera prepared against jelly of the species of Rana (Bar BA, Text Figure 1). Cross-reactions were observed between anti-jelly sera of Bufo americanus and jelly material of R. pipiens in the Ouchterlony plate studies.

(5) Effects of antisera prepared against fertilizin of eggs of Arbacia (Echinodermata).

Experiments employing anti-jelly serum against fertilizin of eggs of Arbacia punctulata have indicated that this serum does not cause any inhibitory effect on eggs of R. pipiens. Owing to the fact that insufficient numbers of egg samples from

different females were tested for statistical analysis, the effects of antiserum against fertilizin are not included in Text Figure 1.

(6) Effect of treating eggs with antisera prepared against homogenates of organs of adult R. pipiens.

Bar HA, Text Figure 2, represents the values obtained after treating eggs with antisera prepared against heart. These values are significantly lower than those obtained after treatment with control sera, but higher than after treatment with anti-jelly sera. However, in Ouchterlony tests, these antisera do not display any components in common with the jelly material. Pretreatment of the anti-heart serum with heart material did not completely remove all of the inhibiting substances from the serum (Bar PA + H Text Figure 2). However, pretreatment with the jelly material removed the inhibitory substances present in anti-heart serum (Bar HA + PJ, Text Figure 2). The heart material, on the other hand, does not remove the inhibitory substance from the anti-jelly serum (Bar PA + H, Text Figure 2).

Experiments employing anti-ovary serum (Bar OA, Text Figure 2) were performed, with no inhibitory effect being observed, since these values are not significantly lower than

those obtained by treating with control serum. Also, the ovary material did not remove the inhibitory substance of the anti-jelly serum (Bar PA + O, Text Figure 2). Cross-reactions were not usually observed between the anti-heart or anti-ovary sera and jelly on the Ouchterlony plates.

Legend for symbols used in test figures 1, 2, and 3; table 1; treatments of eggs of Rana pipiens prior to insemination.

Nnsp - untreated eggs

N - one-tenth full strength Holtfreter's solution

C - rabbit control serum

PA - anti-jelly serum of R. pipiens
PA + PJ - anti-jelly serum of R. pipiens + jelly of R. pipiens
PA + CJ - anit-jelly serum of R. pipiens + jelly of R. clamitans
PA + SJ - anti-jelly serum of R. pipiens + jelly of R. sylvatica
PA + H - anti-jelly serum of R. pipiens + heart
PA + AJ - anti-jelly serum of R. pipiens + jelly of Ambystoma maculatum
PA + O - anti-jelly serum of R. pipiens + ovary

CA - anti-jelly serum of R. clamitans
CA + CJ - anti-jelly serum of R. clamitans + jelly of R. clamitans
CA + PJ - anti-jelly serum of R. clamitans + jelly of R. pipiens
CA + SJ - anti-jelly serum of R. clamitans + jelly of R. sylvatica

SA - anti-jelly serum of R. sylvatica
SA + SJ - anti-jelly serum of R. sylvatica + jelly of R. sylvatica
SA + PJ - anti-jelly serum of R. sylvatica + jelly of R. pipiens
SA + CJ - anti-jelly serum of R. sylvatica + jelly of R. clamitans

AA - anti-jelly serum of Ambystoma maculatum

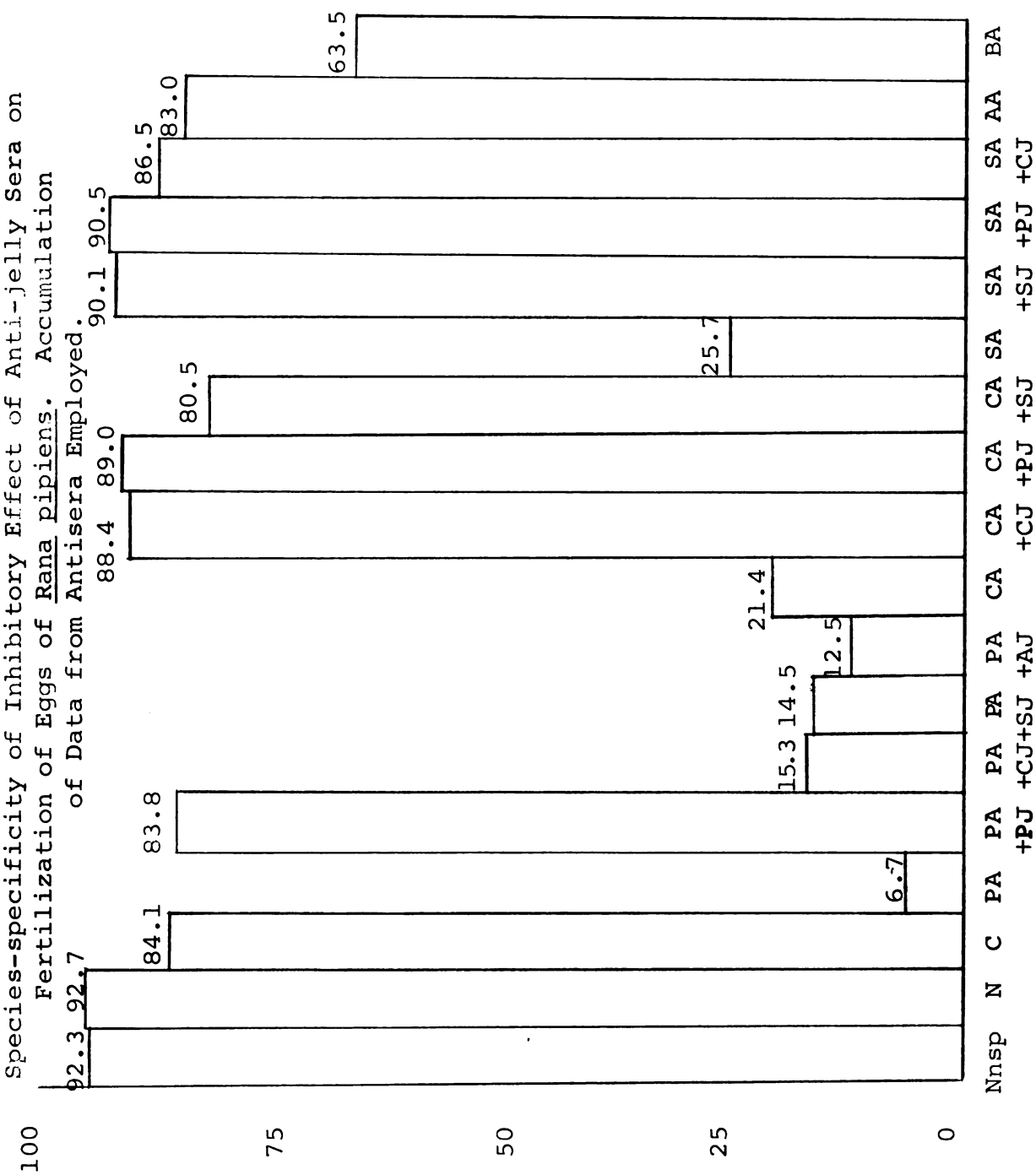
HA - antiserum against heart homogenate
HA + H - antiserum against heart adsorbed with heart
HA + PJ - antiserum against heart adsorbed with jelly of R. pipiens

OA - antiserum against ovary
OA + O - antiserum against ovary + ovary
OA + PJ - antiserum against ovary adsorbed with jelly of R. pipiens

BA - antiserum against jelly of Bufo americanus

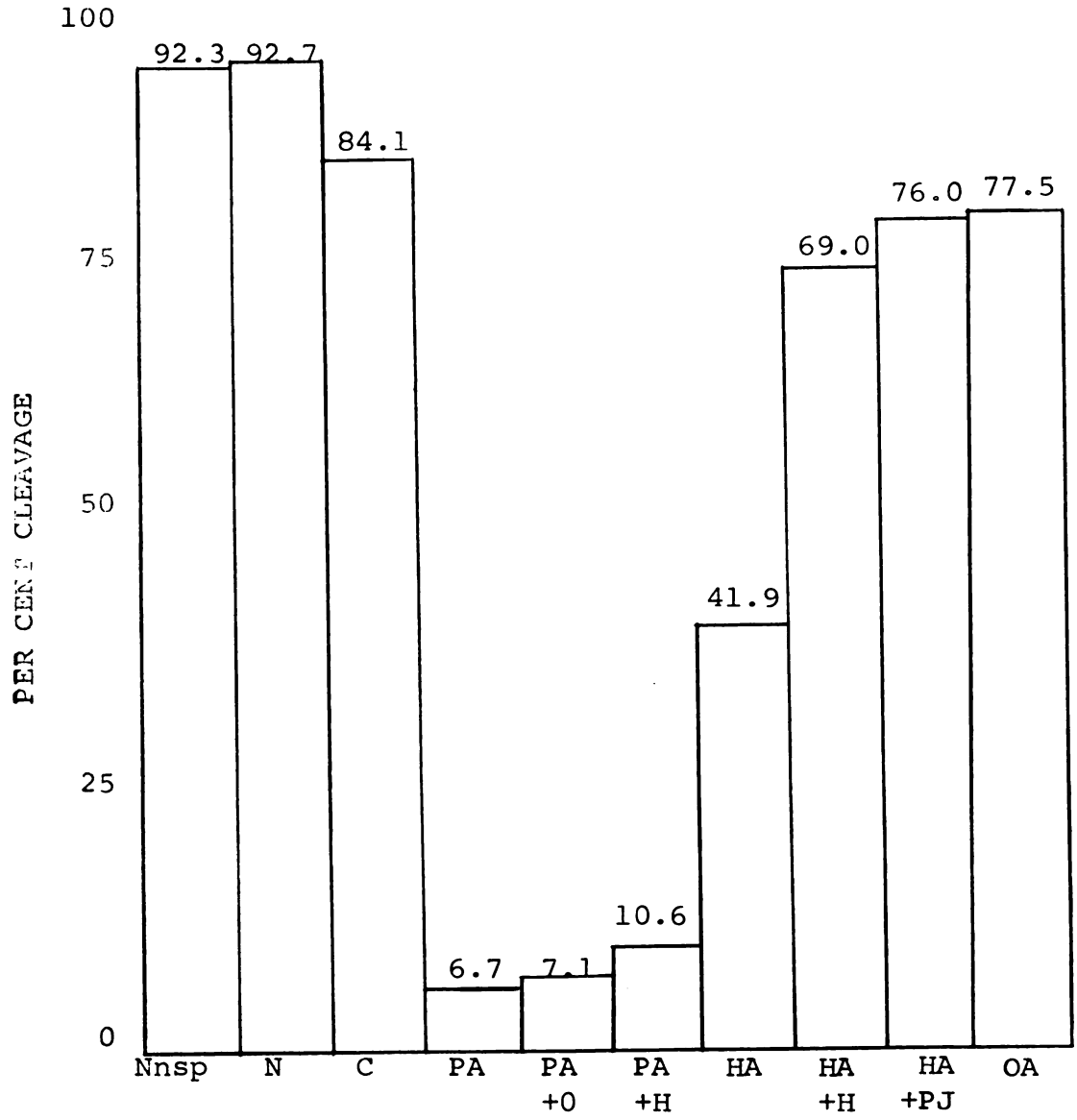
Text Figure 1

Species-specificity of Inhibitory Effect of Anti-jelly Sera on Fertilization of Eggs of Rana pipiens. Accumulation of Data from Antisera Employed.



Text Figure 2

Tissue-specificity of Inhibitory Effect of Antisera on Fertilization of Eggs of Rana pipiens. Accumulation of Data from All Antisera Employed.*



*Values for Nnsp, N, C, and PA are the same as on Text Figure 1.

Text Figure 3

Table of Significant Differences Between Treatments of Eggs of Rana pipiens.

	Nnsp	N	C	PA	PA+PJ	PA+CJ	PA+SJ	PA+H	PA+AJ	PA+O	CA	CA+CJ	CA+PJ	CA+SJ	SA	SA+SJ	SA+PJ	SA+CJ	AA	HA	HA+H	HA+PJ	OA	OA+O	OA+PJ	BA
Nnsp				X	X	X	X	X	X	X	X			X	X				X	X	X	X	X	X	X	X
N				X	X	X	X	X	X	X	X			X	X				X	X	X	X	X			X
C				X		X	X	X	X	X	X				X					X	X					X
PA	X	X	X		X						X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
PA +PJ	X	X		X		X	X	X	X	X	X				X					X	X					X
PA +CJ	X	X	X		X							X	X	X		X	X	X	X	X	X	X	X	X	X	X
PA +SJ	X	X	X		X							X	X	X		X	X	X	X	X	X	X	X	X	X	X
PA +H	X	X	X		X						X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
PA +AJ	X	X	X		X							X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
PA +O	X	X	X		X						X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
CA	X	X	X	X	X			X		X		X	X	X		X	X	X	X	X	X	X	X	X	X	X
CA +CJ				X		X	X	X	X	X	X				X					X	X	X	X			X
CA +PJ				X		X	X	X	X	X	X				X					X	X	X	X			X
CA +SJ	X	X		X		X	X	X	X	X	X				X		X			X						X
SA	X	X	X	X	X			X	X	X		X	X	X		X	X	X	X	X	X	X	X	X	X	X
SA +SJ				X		X	X	X	X	X	X				X					X	X	X	X			X
SA +PJ				X		X	X	X	X	X	X			X	X					X	X	X	X			X
SA +CJ				X		X	X	X	X	X	X				X					X	X					X
AA	X	X		X		X	X	X	X	X	X				X					X	X					X
HA	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X
HA +H	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X				X	X	
HA +PJ	X	X		X		X	X	X	X	X	X	X	X		X	X	X			X						X
OA	X	X		X		X	X	X	X	X	X	X	X		X	X	X			X						X
OA +O	X			X		X	X	X	X	X	X				X					X	X					X
OA +PJ	X			X		X	X	X	X	X	X				X					X	X					X
BA	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	

X = Significant differences at 0.05 level.

Table 1

Percentages of Cleavages in Batches of Eggs Treated with Serum Dilutions of 1:1 Prior to Insemination with Normal Spermatozoa

Female No.	Nnsp	N	C	PA	PA + PJ	PA + CJ	PA +SJ	PA + H	PA + AJ	PA + O	CA	CA + CJ	CA + PJ	CA + SJ	SA	SA + SJ	SA + PJ	SA + CJ	AA	HA	HA + H	HA + PJ	OA	OA + O	OA + PJ	BA
14	100.0	88.0	85.0	5.7	77.7	10.5	12.7	5.8	19.5	6.0	7.5	75.0	89.3	84.2	8.3	85.7	64.8	75.5	77.5	72.7	88.5	84.2	75.6	86.2	77.7	37.9
16	89.8	96.6	91.4	0.0	93.4	5.7	4.2	13.6	13.6	2.5	7.8	73.8	82.1	77.2	14.2	81.8	71.4	46.1	88.2	64.5	76.9	77.0	84.6	75.6	75.0	27.2
18	97.7	100.0	100.0	16.2	93.6	8.8	4.7	11.6	9.7	15.3	10.0	75.0	96.9	78.5	25.0	100.0	94.1	90.0	87.0	48.1	87.0	93.1	84.6	86.2	96.2	61.9
19	100.0	96.4	94.1	6.0	70.5	2.8	19.5	0.0	7.4	6.6	17.1	100.0	94.5	84.3	27.0	93.5	97.2	86.1	73.8	25.8	85.7	66.6	85.2	96.4	84.3	88.0
20	97.5	100.0	96.0	0.0	86.9	13.3	8.0	4.6	6.5	8.0	23.2	92.1	74.4	73.6	22.2	89.2	94.2	84.0	93.6	22.9	91.8	81.0	70.4	80.0	89.1	67.7
21	76.9	95.3	92.3	5.4	69.2	14.2	8.3	13.7	12.8	3.3	32.1	88.2	89.1	72.9	32.3	91.4	76.1	83.3	70.5	34.6	74.3	62.8	78.9	75.4	67.3	74.0
22	96.0	100.0	72.9	7.3	75.0	5.2	10.7	5.2	6.5	12.1	25.0	77.5	90.0	65.2	28.5	79.0	88.2	82.3	84.8	29.7	55.8	66.6	63.6	85.0	84.4	75.0
23	100.0	84.0	74.3	5.0	75.4	6.5	9.6	23.0	14.8	6.2	10.2	89.0	87.7	94.8	52.0	84.6	93.3	68.7	86.8	39.5	65.1	80.4	85.2	90.4	76.4	64.2
24	100.0	97.7	97.4	2.3	95.3	2.2	18.7	9.5	15.9	12.8	11.9	95.1	100.0	81.4	22.6	90.1	87.8	90.6	97.8	39.6	88.0	93.3	79.1	97.9	85.4	85.1
25	97.9	95.1	96.0	3.6	80.9	6.3	25.5	3.2	22.6	21.6	72.2	90.2	91.6	95.3	33.3	100.0	94.2	86.2	97.9	53.0	92.1	84.2	80.3	96.4	95.6	64.9
26	93.9	96.9	97.7	8.1	87.1	6.0	27.0	13.3	13.3	0.0	16.6	93.7	87.5	82.1	45.4	96.5	96.4	100.0	96.9	44.4	84.8	88.8	76.0	95.8	71.4	66.6
28	96.8	94.7	46.1	0.0	79.1	40.0	15.3	37.5	3.4	0.0	0.0	91.8	75.7	66.6	12.1	80.0	94.9	82.0	62.2	38.2	43.9	71.0	52.6	80.9	74.5	56.4
29	75.9	77.8	64.0	13.3	70.0	3.4	0.0	4.7	0.0	0.0	12.1	65.8	85.0	72.9	7.3	86.5	91.5	76.0	69.4	21.8	16.1	93.1	51.2	76.7	86.8	43.9
31	45.7	73.3	70.5	3.3	68.7	2.9	5.7	0.0	0.0	5.7	9.1	90.3	83.3	67.8	0.0	86.6	84.6	94.2	92.0	42.6	42.0	82.0	80.0	100.0	90.7	78.0
32	100.0	100.0	64.3	0.0	71.7	6.7	7.3	5.7	2.3	0.0	9.1	79.5	76.3	69.2	7.5	91.9	90.6	93.3	78.8	40.0	46.0	84.2	66.6	83.3	86.5	53.6
34	100.0	92.5	77.1	3.1	91.9	33.3	19.0	12.2	9.1	6.8	31.9	93.0	97.5	87.8	26.9	91.1	90.2	88.1	90.7	59.2	76.5	86.1	66.6	87.9	90.2	55.9
36	92.4	91.3	95.8	3.8	97.8	18.6	5.3	8.5	18.5	1.9	35.5	95.4	91.1	80.7	16.9	92.8	97.6	95.4	83.3	75.7	76.3	69.0	81.2	77.7	82.7	68.4
37	83.8	88.0	81.2	11.1	77.3	22.2	18.6	15.9	22.8	3.0	41.3	100.0	90.3	91.8	41.9	82.7	87.5	83.9	77.5	59.1	71.1	65.8	78.7	81.2	71.0	76.0
38	100.0	92.1	77.1	4.4	94.0	36.6	7.6	1.9	23.1	6.3	35.4	98.2	96.0	86.8	33.3	94.1	92.8	95.3	90.6	41.5	82.5	88.3	82.0	76.3	86.0	71.0
39	89.4	100.0	94.7	27.2	94.6	48.7	45.8	10.9	29.4	21.8	26.8	93.3	100.0	86.2	48.0	97.0	94.6	100.0	89.5	32.4	82.8	76.9	97.0	89.5	94.1	73.0
40	97.7	79.4	92.5	6.6	88.6	20.0	17.5	9.7	6.1	0.0	25.8	93.7	95.2	97.8	21.2	97.3	100.0	97.2	73.3	16.6	57.1	76.7	93.7	74.1	100.0	62.2
41	94.1	97.7	85.3	10.4	88.0	18.7	15.2	15.2	22.9	14.9	20.4	97.4	87.7	86.9	28.8	91.9	94.1	82.2	93.2	37.1	57.9	75.0	85.7	86.6	90.6	70.8
42	100.0	100.0	89.5	7.3	97.5	18.0	25.6	17.9	21.2	14.5	12.5	97.7	91.5	87.5	22.2	92.5	97.5	97.8	87.1	38.5	76.3	93.3	82.5	84.3	87.9	60.0
43	87.1	89.4	70.8	0.0	88.4	16.0	14.2	11.7	5.0	5.1	28.2	82.9	84.8	65.1	12.8	92.1	96.9	92.5	88.9	32.2	57.1	87.9	78.8	88.2	96.5	21.9
44	96.2	90.9	97.4	16.6	83.9	17.5	16.7	10.6	6.6	4.8	13.3	82.0	88.2	76.9	54.2	84.3	95.1	91.7	43.7	40.0	50.0	33.3	77.4	84.4	97.0	83.7
	$\bar{X}$ = 92.3	92.7	84.1	6.7	83.8	15.3	14.5	10.6	12.5	7.1	21.4	88.4	89.0	80.5	25.7	90.1	90.5	86.5	83.0	41.9	69.0	76.0	84.0	85.4	85.5	63.5

C. Effect of Treating Eggs of R. pipiens with Fractions of Anti-jelly Sera

Experiments employing serum fractionated by the "Rivanol" method have been made to test the effect of the globulin fraction on eggs of R. pipiens. Text Figure 4 represents the values obtained by treating eggs with the globulin fraction of serum after separation of albumins by the Rivanol technique. Eggs from seven females were used in the same manner as that described for treatments with whole serum. No statistical analysis was performed on the results; therefore, conclusions drawn are based strictly on percentages of cleavages. The results obtained by treating eggs with fractionated control serum (Bar PCR, Text Figure 4) are considerably higher than the values obtained with the unfractionated control serum (PC). Thus, it may be concluded that the difference observed between fractionated and unfractionated serum is due to albumins present in the serum.

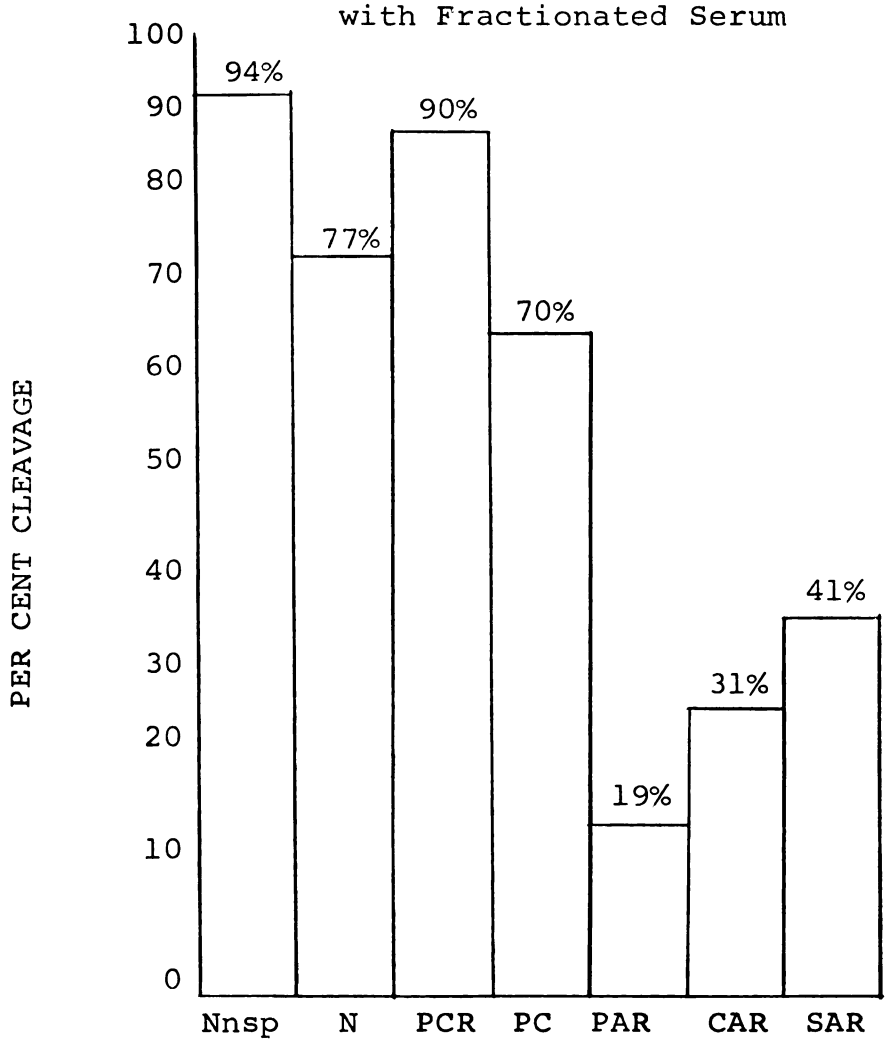
Results obtained by treating eggs with fractionated anti-serum prepared against the Rana jelly materials are presented in Text Figure 4: PAR = Globulin fraction of anti-jelly serum of R. pipiens, CAR = Globulin fraction of anti-jelly serum of R. clamitans, and SAR = Globulin fraction of anti-jelly serum of R. sylvatica. These results indicate that inhibitory

materials are still present in the globulin fractions of the anti-jelly sera.

Comparisons of fractionated and unfractionated anti-jelly sera by agar diffusion studies have demonstrated that some antibodies are lost during the fractionation procedure. For this reason whole serum was used instead of the Rivanol fractionated serum.

Text Figure 4

Results Obtained after Treating Eggs of R. pipiens with Fractionated Serum



Nnsp - untreated eggs inseminated with normal spermatozoa.

N - eggs treated with one-tenth Holtfreter's solution prior to insemination.

PCR - eggs treated with globulin fraction of control serum prior to insemination.

PC - eggs treated with unfractionated control serum prior to insemination

PAR - eggs treated with globulin fraction of anti-jelly serum of R. pipiens prior to insemination.

CAR - eggs treated with globulin fraction of anti-jelly serum of R. clamitans prior to insemination.

SAR - eggs treated with globulin fraction of anti-jelly serum of R. sylvatica prior to insemination.

DISCUSSION

A. Analysis of Antigenic Components Represented in Anti-jelly Sera by Means of Agar-diffusion Technique.

Results obtained by the modified agar-diffusion technique by Ouchterlony have been presented for determining antigenic components of frog egg-jelly materials. It became apparent rather quickly that components of the jelly materials were quite strong antigenically and that the jelly material of each species of Rana contained a number of antigenic components. For a summary of cross-reacting components in jellies of different species of Amphibia, as determined by serological methods, see Tables 2 and 3, pages 69 and 70.

One of the difficulties encountered with this type of immunological method is the different responses of individual rabbits to complex antigen preparations, especially tissue preparations. However, this differential response of different rabbits to the jelly antigens is not nearly as great as that for antigens represented in other tissues of the frog. A strong titer of antibodies was obtained in each rabbit injected with egg jelly.

The results of injecting some fifty rabbits with jelly materials and of running several hundred Ouchterlony plates

on the resulting antisera make it appear that each species of Amphibia tested has a number of components in the egg jelly which are species-specific. In addition to species-specific components, it has been demonstrated that the jelly from the eggs of each of the species of Rana contains a component that is common with a component found in the jelly of the eggs of each of the other species of Rana tested (R. pipiens, R. sylvatica, R. clamitans, and R. catesbeiana).

Results have been presented (Table 3, page 70) which indicate that jelly of eggs of R. pipiens contains a component in common with a component found in jelly of eggs of R. clamitans that is not present in jelly of eggs of R. sylvatica; jelly of eggs of R. clamitans contains a component in common with a component found in the jelly of eggs of R. catesbeiana that is not present in the jelly of either R. pipiens or R. sylvatica eggs; jelly of R. clamitans also contains a different component that is common to one found in the jelly of R. pipiens which is not found in the jelly of R. sylvatica; jelly of R. sylvatica contains a component that is common with one found in the jelly of R. pipiens that is not present in jelly of R. clamitans. The fact that components could be demonstrated to be common between the jellies of two different species and not present in a third species, does not rule out the possibility of the difference being quantitative and not

qualitative. 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 the agar plates.

The jelly of another species of Anuran, Bufo americanus, was analyzed and found to contain components in common with components present in the jelly of each of the species of Rana. However, the jelly material of Bufo marinus does not contain components in common with the species of Rana but does contain components in common with Bufo americanus.

The exact meaning of the species-specificity of jelly antigens as determined serologically, in connection with the biological role of these components in the process of fertilization, is limited to speculation at the present time, as will be suggested in the next part of the discussion. What few chemical studies that have been performed on amphibian egg jellies (Folkes, Grant & Jones, 1950; Minganti, 1955) have indicated a close chemical similarity between them and the fertilizin of the sea urchin egg, which is rather well analyzed. If the jelly material of the frog egg plays a role in fertilization similar to that postulated for fertilizin of the sea urchin egg, then this might be the basis for the rather high degree of specificity of fertilization, in Amphibia.

The specificity of fertilization leads one to believe that there are specific molecular patterns on the surface of gametes which interact in order for fertilization to occur. Since this

specificity in frogs is not absolute but allows a certain degree of cross-fertilization between species, it would seem that the union of gametes of different species would depend on the degree of similarity between surface layers of gametes of species which are capable of cross-fertilization. One cannot assume, however, that the identification of common components in the jelly-coatings of eggs of different species necessarily indicates the capability of cross-fertilization between these species. For example, a cross-reaction between the anti-jelly serum of Bufo americanus and the jelly of R. pipiens does not necessarily mean that these Anurans are capable of hybridization. Cross-fertilization tests employing European species of Bufo and Rana, were done by G. Hertwig (cited by Bataillon and Tchou-Su, 1929), in which sperm penetration occurred. It should be pointed out that where cross-fertilization occurs between the different species of Rana tested in this experiment, common components have also been observed between the jellies. It is quite possible that the spermatozoa of one of two or more species which have egg-jelly antigens in common would actually be capable of penetrating the surface of the egg of the other species without causing rotation or cleavage of the egg. However, the specificity of fertilization may indeed be greater than the specificity of serological cross-reactions.

It has been suggested that a relationship might exist between

the composition of the jelly and the ecological conditions where the eggs develop (Moore, 1940; Minganti, 1955). No attempt was made to compare the jellies of Amphibians of the same species, from different geographical areas.

In connection with cross-fertilization between the species of Rana, an observation made during this study should be pointed out here. According to Rugh (1948), Moore (1955) and others, R. clamitans eggs do not undergo cleavage when inseminated with R. pipiens spermatozoa. In the spring of 1960, it was noted that eggs of R. clamitans inseminated with spermatozoa of R. pipiens (both obtained from a commercial dealer in Wisconsin) did undergo cleavage up to the blastula stage. The reason for the discrepancy between this observation and that made by other workers is not clear at the moment.

The tissue-specificity of the egg-jelly components of Amphibia closely parallels the tissue-specificity of fertilizin of the sea urchin egg, i.e., the jelly components of the species of Rana are in general restricted to the oviducal tissue. (For summary of reactions showing the tissue-specificity of anti-jelly sera of R. pipiens see Table 4, page 71.) Results have been presented in which anti-jelly serum reacted with a component present in frog blood serum. Unless special attention is given to the washing of tissues in order to remove all blood, a cross-reaction is always possible between the anti-jelly serum and the

body tissue being tested.

The question arises as to whether the specific and common components of the jelly visualized by the reaction on the agar plates with the complementary configuration in the antiserum against the jelly, are actually different molecules present in the jelly materials, or different reactive sites on a single large molecule. Immunochemical research has not provided much information about size, number, or structure of substances eliciting the immune response in rabbits (cf. Tyler, 1957). Since very little is known about the chemical nature of the jelly, it is not easy to decide whether the components represent different molecules or merely different reactive sites on a large complex molecule. However, adsorption techniques employed in which anti-jelly sera of different species were adsorbed with heterologous jellies present evidence which would suggest that more than one molecule is involved.

Table 2 Cross-reactions between Jellies as Determined by the Ouchterlony Technique.

Anti-jelly Sera	Jelly Materials: *R. pipiens*	*R. clamitans*	*R. sylvatica*	*R. catesbiana*	*Ambystoma maculatum*	*Bufo americanus*	*Bufo marinus*	*Arbacia punctulata*
R. pipiens	+	+	+	+	-	+	-	-
R. clamitans	+	+	+	+	-	-	-	-
R. sylvatica	+	+	+	+	+	+	-	-
R. catesbeiana	+	+	+	+	-	-	-	-
Ambystoma maculatum	-	-	-	+	+	-	-	-
Bufo americanus	+	+	+	+	+	+	+	+
Bufo marinus	-	-	-	-	-	+	+	-
Arbacia punctulata	-	-	-	-	-	-	-	+

\+ = presence of line (or lines) in Ouchterlony plates.

\- = absence of line in Ouchterlony plate.

Table 3 The Presence of Specific Components in Anti-jelly Sera as Determined by the Technique of Adsorption.

Anti-jelly Sera	Jellies Used for adsorption	Jelly Materials: *R. pipiens*	*R. clamitans*	*R. sylvatica*	*R. catesbeiana*
R. pipiens	*R. pipiens*	-	-	-	0
R. pipiens	*R. clamitans*	+	-	-	-
R. pipiens	*R. sylvatica*	+	+	-	0
R. pipiens	*R. catesbeiana*	+	-	0	-
R. clamitans	*R. clamitans*	-	-	-	-
R. clamitans	*R. pipiens*	-	+	-	+
R. clamitans	*R. sylvatica*	+	+	-	0
R. clamitans	*R. catesbeiana*	-	+	-	-
R. sylvatica	*R. sylvatica*	-	-	-	0
R. sylvatica	*R. pipiens*	-	-	+	-
R. sylvatica	*R. clamitans*	+	-	+	-
R. sylvatica	*R. catesbeiana*	-	-	+	0
R. catesbeiana	*R. catesbeiana*	-	-	0	-
R. catesbeiana	*R. pipiens*	-	+	0	+
R. catesbeiana	*R. clamitans*	-	-	0	+

\+ = presence of lines in the Ouchterlony plate which appeared specific.

\- = absence of specific lines in the Ouchterlony plate.

0 = no test made.

Table 4 Tissue-specificity of R. pipiens Jelly Components as Determined by the Ouchterlony Technique.

		Antigens					
Antisera	Adsorption	Jelly	Ovary	Oviduct	Heart	Kidney	Brain
Jelly	-	+	+?	+	-	-	-
Ovary	-	-	+	+	+	+	+
Oviduct	-	+	+	+	0	0	0
Heart	-	-	+	+	+	+	+
Kidney	-	-	+	0	+	+	+
Jelly	Ovary	+	-	+	0	0	0
Jelly	Oviduct	-	-	-	0	0	0
Ovary	Jelly	-	+	+	0	0	0
Ovary	Oviduct	-	+	-	0	0	0
Oviduct	Jelly	-	+	+	0	0	0
Oviduct	Ovary	+	-	+	0	0	0

+ = presence of lines in Ouchterlony plates.

- = absence of lines in Ouchterlony plates.

0 = no test made.

B. Analysis of the Effect of Anti-jelly Sera on Fertilizability of Eggs.

It has been demonstrated that antisera prepared against the jelly material of each of the species of Rana has a significant inhibitory effect on the fertilizability of eggs of R. pipiens. However, the adsorption of antiserum, prior to treatment of the egg, with jelly of heterologous origin, indicates that the specific components represented in anti-jelly serum of R. pipiens decrease the fertilizability of eggs of R. pipiens, whereas the specific components of egg-jellies of heterologous species do not elicit production of inhibitory antibodies. Consequently, it may be concluded that the inhibition observed in treating eggs of R. pipiens with the unadsorbed heterologous anti-jelly sera was due to components common to all three species of Rana. Subsequent experiments, employing treatments of eggs of R. sylvatica with adsorbed and unadsorbed homologous and heterologous anti-jelly sera have produced similar results.

Obviously it would be desirable to know specificially what is being affected by these anti-jelly sera treatments. As pointed out previously in this paper, Shaver and Barch (1960) have demonstrated that the antiserum against jelly of R. pipiens has an inhibitory effect on the fertilizing capacity of spermatozoa and the fertilizability of eggs of the homologous

species. Due to the technique employed by these workers, they were unable to determine whether the inhibition observed, resulted from an effect on the spermatozoön or egg, or both, in the treatment of the gametes. In the experiments reported here, eggs were washed subsequent to the antiserum treatments to remove any unreacted antiserum. Thus, it may be concluded that the inhibition observed was due to a block in fertilization produced by a reaction between the jelly or the egg and the anti-jelly serum and not to an effect directly on the spermatozoa.

An attempt was made to determine if the effect of anti-jelly serum treatments was on the jelly layers or on the surface of the egg by injecting anti-jelly serum of R. pipiens into the body cavity of female frogs during the process of ovulation. By this procedure eggs were in contact with the anti-jelly serum prior to the deposition of the jelly coat. A total of 24 mls of antiserum was injected into each of six female frogs. At the completion of ovulation, eggs fertilized and cultured in the normal way, were observed for cleavage. No inhibition of fertilization was observed in any of the eggs tested. Also, if the inhibition is due to a reaction between the egg or the surface of the egg and the anti-jelly serum, one would expect anti-ovary serum to have a greater effect than the anti-jelly serum since the jelly components

are restricted to the oviducal tissues. However, no inhibition was observed due to the treatments with anti-ovary sera. These results further implicate a reaction between the jelly and anti-jelly serum as the blocking mechanism.

In conclusion it would seem that the inhibition observed may be attributed to a reaction between the jelly and complementary configurations produced in the globulin fraction of rabbit serum against antigenic components present in the jelly-coat material, since treatments with control serum show no inhibition. Also the inhibitory effect could be completely removed by adsorbing the anti-jelly serum with the homologous antigen, prior to treatment of eggs.

The question arises as to whether the reactions observed on the Ouchterlony plates actually represent the inhibitory substances involved in the egg treatments. The fact that a close correspondence exists between the precipitation reaction of the jelly antigens with the complementary configurations in the antiserum observed on the agar plates, and inhibitory effects observed in egg treatments, indicates that the antibody component of the precipitate on the agar plates is actually responsible for the inhibitory effect. In other words, where a precipitation was observed between an antiserum and the egg-jelly of R. pipiens, an inhibition was also observed. Another experiment which demonstrates this fact is

a case where antisera prepared against the jelly of another genus of Amphibia (Ambystoma) failed to react with jelly of R. pipiens, inhibition was not observed. In cases where an anti-jelly serum prepared against the jelly of another Anuran (Bufo) did react with the jelly of R. pipiens, inhibition was also observed.

The inhibitory effect observed in treating eggs of R. pipiens with antiserum prepared against a homogenate of heart of the adult frog cannot be explained on this basis since no cross-reaction was observed between the anti-heart serum and the jelly on the agar plates. Also, since treatments with anti-ovary serum fail to inhibit fertilization, it is not likely that the effect of the anti-heart serum is on the surface of the egg. A resolution of the uncertainty as to the location of the reactive sites might be accomplished if jelly-less, uterine eggs were available, but the technical difficulties of doing this makes the procedure unfeasible. Experiments employing antisera prepared against various other adult tissues of the frog (kidney, brain, testis, and liver) have shown that these antisera also result in slight inhibitory effects. The fact that practically all the inhibitory effect of the anti-heart serum could be removed by adsorbing the antiserum with either a heart or jelly homogenate, suggests that the inhibitory effect may be due to non-precipitating

components present in the anti-heart serum, or to a component common between jelly and blood serum represented in the anti-serum.

Although the problem of tissue-specificity of the inhibitory effect is not completely clear at the moment, it is believed that a study employing antibodies labeled with fluorescent dyes would be helpful in demonstrating what reactions account for the inhibition observed in treatments with antisera employed in this study and where the reactions occur.

Recent observations of Hathaway and Metz (1961) suggest the possibility of isotopic labeling of surface components in order to identify the sites of interaction of complementary configurations of the spermatozoa and eggs.

C. General Considerations and Speculations

The purpose of this section is to try to fit obervations presented here into the broader framework of cellular interactions, from a speculative point of view. Two possible consequences of reaction systems such as have been demonstrated in this study might be: (1) a species-isolating mechanism representing a barrier to fertilization between species such as the patterns of antigen-antibody reactions analyzed above; (2) the origin of tissue-specificity of cells on both phylogenetic and ontogenetic levels.

(1). Interacting substances between cells have been shown to exist by a number of workers. The most thoroughly investigated cells exhibiting such substances are gametes of species of Echinoderms and Amphibians. It has been shown that these substances are mucopolysaccharide in nature and that they have some relationship to the process of fertilization. Workers have long suspected that the tissue-and species-specificity of fertilization is due to specific components which are present on the surface of the gametes. Indirect evidence for this specificity has come mainly from work on gametes of the Echinoderms, by studies of the effect of "egg water" on the spermatozoa. The presence of species-specific components in egg-jellies of Amphibians has been directly demonstrated in

this study, and offers concrete evidence for what has been postulated to occur in gametes of Echinoderms. These specific components could serve as the basis for an isolating mechanism at the time of fertilization. Such a mechanism would be necessary to prevent hybridization and gene-transfer between species whose breeding areas and periods coincide.

(2). Considering the similarity of the chemical make-up and immunological characteristics of interacting substances of gametes and of surface components of cells making up tissues, it is interesting to speculate on the phylogenetic and ontogenetic origins of tissue-specificity.

For example, a well-known case of specificity of cellular types is that observed in Pneumococcus, where genetically different strains exist which are marked by the presence or absence of a polysaccharide sheath. It is also quite common to find colonies of certain algae which are enclosed in a gelatinous matrix which is probably mucopolysaccharide in nature (for example Volvox and Anaboena). One could speculate that in the course of evolution, cells of similar structure and function became enclosed or bound together by such a matrix or gelatinous sheath. In this respect, Volvox would represent a primitive tissue differentiation, inasmuch as "somatic" cells may be distinguished from "reproductive" cells. It is very interesting that wherever cells are bound

together as a unit, the intercellular matrix is often represented by a gelatinous or hyaline material.

From an ontogenetic point of view, the first evidence one sees of cells being bound together by a matrix (in the Amphibian, for example) occurs during cleavage of the egg. At cytokinesis the hyaline layer, beginning with first cleavage, follows the contours of the daughter cells, so that each cell is enclosed within this layer. It is interesting to analogize the dividing egg with a colony of cells bound together by hyaline intercellular matrices. Holtfreter (1943 and 1948) and Lewis (1949) have emphasized the importance of the surface gel layer (matrix) in gastrulative movements of cells in the Amphibian and fish embryos. Grobstein (1955) has suggested that intercellular matrices may play an important part in induction and that one way in which these matrices might interact is by molecular complementariness. The rather high degree of specificity which governs the aggregative properties of cells (see Holtfreter, 1943 and 1948; Spiegel, 1954a, b, and 1955) suggests a role for interacting substances in cellular adhesion and the latter worker suggested that the adhesion of these cells is due to an antigen-antibody like reaction which is similar to that postulated to occur in the egg-jellies of the Amphibian.

Thus, one is struck by the fundamental similarity of the mechanisms which, in development, first insure species-specificity, and later are responsible for tissue-specificity

SUMMARY

1. Antisera were prepared against jelly-coat material of eggs of several species of Rana (R. pipiens, R. clamitans, R. sylvatica, and R. catesbeiana) and other species of Amphibia (Bufo americanus, Bufo marinus, and Ambystoma maculatum). Serological characterizations as to species- and tissue-specificity of antigenic components found in these jellies have been presented.

2. Analysis of the jelly material tested showed that the jellies of each species contain a number of species-specific components. Common components were observed in the jelly of species belonging to the same genus (either Rana or Bufo). In certain cases common components were observed between species of different genera (Bufo americanus and each of the species of Rana).

3. Experiments employing antisera prepared against homogenates of adult frog organs of R. pipiens have shown that the jelly components are restricted in general to the oviducal tissues, which demonstrates the tissue-specificity of the jelly components.

4. Results of treating unfertilized eggs of R. pipiens with both heterologous and homologous anti-jelly sera, prior to insemination with normal spermatozoa, have been presented. Treatments of eggs of R. pipiens with anti-jelly sera of each

of the species of Rana resulted in a significant decrease in the fertilizability of the eggs. In cases where a cross-reaction was observed between the jelly of R. pipiens and the anti-jelly sera of a different genus (Bufo), inhibition of fertilization was also observed. No inhibition was observed in cases where a cross-reaction failed to take place between the jelly of R. pipiens and anti-jelly sera of a genus of a different order (Ambystoma).

5. By the technique of adsorption it was demonstrated that treatments of eggs of R. pipiens with specific components represented in the anti-jelly serum of R. pipiens and with the common components represented in the anti-jelly serum of each of the species of Rana, decrease the fertilizability of the eggs. Treatments with species-specific components represented in anti-jelly sera of heterologous species (either Rana, Bufo, or Ambystoma) did not decrease the fertilizability of the eggs.

6. Treatments of eggs of R. pipiens with anti-organ sera of the adult frog resulted in significant inhibitory effects in the fertilizability of the eggs. The possibility of this effect being due to non-precipitating components in the jelly has been presented.

7. The results of these studies of specificity of antigenic components of the jellies are discussed in connection

with the possible role of these components in the process of normal fertilization. Some possibilities concerning the nature of the inhibitory effect observed in treatment of eggs with anti-jelly sera are discussed.

LITERATURE CITED

Bataillon, E. 1919 Analyse de l'activation par la technique des oeufs nus et la polyspermie expérimentale chez les Batraciens. Ann. Sci. Nat. et Zool. Série I 8, pp. 1-40 (Cited in Shaver and Barch, 1960, p. 180.)

Bataillon, E., and Tchou-Su. 1929 Analyse de la Fécondatien chez les Batraiens par l'hybridisation et la polyspermie physiologique. Roux' Archiv. F. Entwick. Mech. 115:779-824.

Bernstein, G. S. 1952 Sperm agglutinins in the egg jelly of the frogs, Rana pipiens Schreber and Rana clamitans Latreille. Biol. Bull., 103:285.

Billingham, R. E., and Sparrow, Elizabeth M. 1955 The effect of prior intravenous injections of dissociated epidermal cells and blood on the survival of skin homographs in rabbits. J. Embryol. Exptl. Morphol., 3:265-285.

Bishop, D. W., and Tyler, Albert. 1956 Fertilizin of mamalian eggs. J. Exptl. Zool., 132:575-601.

Folkes, B. F., Grant, R. A., and Jones, J. K. N. 1950 Frog spawn mucin. J. Chem. Soc., No. 8., 2136.

Grobstein, C. 1955 Tissue-interaction in the morphogenesis of mouse embryonic rudiments in vitro. In Aspects of Synthesis and Order in Growth, D. Rudnick, editor. Princeton University Press, Princeton, N. J , pp. 233-256.

Hagstrom, B. E. 1956 The effect of removal of the jelly coat on fertilization in sea urchins. Exptl. Cell Research, 10:740-743.

Hagstrom, B. E. 1956 Studies on the fertilization of jelly-free sea urchin eggs. Exptl. Cell Research, 10: 24-28.

Hagstrom, B. E. 1956 Further studies on cross-fertilization in sea urchins. Exptl. Cell Research, 11:506-510.

Hathaway, R. R. and Metz, C. B. 1961 Interactions between Arbacia sperm and S^{35}--labelled fertilizin. Biol. Bull., 120:360-369.

Holtfreter, J. 1943 Properties and functions of the surface coat in amphibian embryos. J. Exptl. Zool., 93:251-323.

Holtfreter, J. 1947a Experiments on the formed inclusions of the amphibian egg. III J. Exp. Zool., 103:81.

Holtfreter, J. 1947b Changes of structure and the kinetics of differentiating embryonic cells. J. Morph., 90:57.

Holtfreter, J. 1948 Significance of the cell membrane in embryonic processes. Ann. N. Y. Acad. Sci., 49:709-760.

Horesji, J. and Smetana, R. 1956 The isolation of gamma globulin from blood serum by rivanol. Acta Scand. Med., 155:63-70.

Kambara, S. 1953 Role of jelly envelope of toad eggs in fertilization. Annotat. Zool. Japon., 26:78-84.

Lewis, W. H. 1949 Gel layers of cells and eggs and their role in early development. Lecture Series, Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Maine. pp. 59-77.

Lillie, F. R. 1913 Studies of fertilization. V. The behavior of the spermatozoa of Nereis and Arbacia with special reference to egg extractives. J. Exp. Zool., 14:515-574.

Lillie, F. R. 1921 Studies of fertilization. IX. On the question of superposition of fertilization on parthenogenisis in Strongylocentrotus purpuratus, Biol. Bull., 40:23-31.

Metz, C. B. 1957 Specific egg and sperm substances and activation of the egg. In The Beginnings of Embryonic Development. Am. Assoc. Adv. Sci., Washington, D. C., pp. 23-70.

Minganti, A. 1955 Chemical investigations on amphibian egg jellies. Exptl. Cell Research, Suppl. 3:248-251.

Motomura, I. 1952 Cortical granules in the eggs of the frog. Annot. Zool. Japon., 25:238-241.

Moore, J. A. 1940 Adaptative differences in the egg membrane of frogs. Am. Naturalist, 74:89-93.

Moore, J. A. 1955 Abnormal combinations of nuclear and cytoplasmic systems in frogs and toad. Advances in genetics, 7:139-182.

Perlmann, P. 1959 Immunochemical analysis of the surface of the sea urchin egg - an approach to the study of fertilization. Experientia, 15:41.

Rothschild, Lord 1956 Fertilization, Methusen, London.

Rugh, R. 1934 Induced ovulation and artificial fertilization in the frog. Biol. Bull. 77:233-257.

Rugh, R. 1935 Ovulation in the frog. II Follicular rupture to fertilization. Jour. Exp. Zool., 71:163.

Rugh, R. 1948 Experimental Embryology: A Manual of Techniques and Procedures. Burgess Publishing Company, Minneapolis, Minn., p. 356.

Rugh, R. 1951 The Frog. Blakiston, Philadelphia.

Runnstrom, J., Hagstrom, B., and Perlmann, P. 1959 The Cell. Vol. 1, p. 327. Academic Press, New York.

Shaver, J. R., and Barch, S. H. 1960 Experiments on the jelly coat of Rana eggs. Acta Embryol. et Morphol. Exptl., 3:180-189.

Spiegel, M. 1954a The role of specific surface antigens in cell adhesion. Part I. The reaggregation of sponge cells. Biol. Bull., 107:130-148.

Spiegel, M 1954b The role of specific surface antigens in cell adhesion. Part II. Studies on embryonic amphibian cells. Biol. Bull., 107:149-155.

Spiegel, M. 1955 The reaggregation of dissociated sponge cells. Ann. N. Y. Acad. Sci., 60:1056-1076.

Snedecor, G. W. 1956 Statistical Methods Fifth Edition, The Iowa State College Press. pp. 237-328.

Subtelny, S. and Bradt. 1961 Transplantations of blastula nuclei into activated eggs from body cavity and from the uterus of Rana pipiens. Part II. Development of the recipient body cavity eggs. Develop. Biol., 2:96-114.

Tyler, A. 1948 Fertilization and immunity. Physiol. Revs., 28:180-219.

Tyler, A. 1949 Properties of fertilizin and related substances of eggs and sperm of marine animals. Am. Naturalist, 83:195-219.

Tyler, A. 1955a Gametogenesis, fertilization and parthenogenisis. In Analysis of Development, B. H. Willier, P. A Weiss, and V. Hamburger, editors. W. B. Saunders Company, Philadelphia, Pa., pp. 170-212.

Tyler, A. 1955b Ontogeny of immunological properties. In Analysis of Development, B. H. Willier, P. A. Weiss, and V. Hamburger, editors. W. B. Saunders Company, Philadelphia, Pa., pp. 556-573.

Tyler, A. 1957 Immunological studies of early development. In The Beginnings of Embryonic Development. Am. Assoc. Adv. Sci., Washington, D. C., pp. 341-382.

Tyler, A. 1959 Some immunological experiments on fertilization and early development in sea urchins. Exptl. Cell Research, Suppl. 7:183-199.

Tyler, A. and Fox, S. A. 1940 Evidence for the protein nature of the sperm agglutinins of the keyhole limpet and the sea urchin. Biol. Bull., 79:153-165.

Vasseur, E. 1948 Chemical studies on the jelly coat of the sea urchin egg. Act. Chem. Scand., 2:900-913.

Woerdeman, M. W. 1955 Immunobiological approach to some problems of induction and differentiation. In Biological Specificity and Growth, E. G. Butler, editor. Princeton University Press, Princeton, N. J., pp. 33-53.

[redacted] 126

 14