THE EFFECTS OF ANTISERA UPON CLEAVAGE AND EARLY DEVELOPMENT OF FERTILIZED RANA PIPIENS EGGS

Thesis for the Degree of M. S.

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BARBARA JEANNE FREY

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THESIS

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ABSTRACT

THE EFFECTS OF ANTISERA UPON CLEAVAGE AND EARLY DEVELOPMENT OF FERTILIZED *RANA PIPIENS* EGGS

By Barbara Jeanne Frey

A series of experiments were conducted in which *Rana pipiens* eggs were treated externally and also injected with antisera against various *Rana pipiens* tissues.

Effects on cleavage and subsequent development by each serum were noted and compared with appropriate controls.

The results, though inconsistent, did show some cases of total inhibition of cleavage in eggs treated with anti-ovary serum.

It is postulated that this was due to an interaction of antibodies with complementary molecular configurations on the surface of the egg, which interfered with the normal function performed by these molecules in cleavage.

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By

Barbara Jeanne Frey

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I. INTRODUCTION

Numerous investigators have employed immunological techniques to study the macromolecular structure of gametes and the role of antigens in fertilization, cleavage, and development (Tyler, 1963, 1965). Knowledge of interacting, well-defined substances involved in such processes as the mechanism of the penetration of the egg surface by the sperm, initiation of the cortical reaction of the egg, the block to polyspermy, and the acrosomal reaction of the sperm is still largely obscure (Metz, 1961; Monroy, 1965).

The use of antigen-antibody reactions as a method of analysis of molecular structure and function is desirable in that antigen-antibody reactions are both sensitive and specific. It can be seen that if an antigen or its active site plays a role in fertilization or subsequent development, the antibodies produced against the antigen, when used at sublethal doses, would inhibit its normal function. Thus it would be possible to identify the antigen, localize it, and determine its developmental significance and nature of action (Edds, 1956; Tyler, 1955).

Lillie (1913, 1914) first observed that the sea water in which unfertilized sea urchin eggs had been standing ("egg water") agglutinated spermatozoa of the same

species and temporarily stimulated their motility. He named the agglutinating substance "fertilizin" and elaborated a theory, which stated that fertilizin was continuously secreted and diffused out into the jelly coat. This led to a number of investigations of fertilization in invertebrates (mainly sea urchins). Tyler (1959) modified Lillie's theory when he noted that fertilizin constituted essentially the whole jelly coat and surface proper of the egg, but that there was no evidence of continuous secretion of fertilizin from the unfertilized egg. He found fertilizin to be tissue and species-specific and when "anti-agglutinin" (sperm extract) and "agglutinin" (egg water) were mixed, there appeared a precipitation layer and neutralization of activity occurred.

Experiments by Motomura (1953) and Hagstrom (1956) indicated that a "cytofertilizin" existed in unfertilized, jellyless sea urchin eggs. This was later shown by the use of fluorescine-labelled antibodies against sea urchin fertilizin to be present in the plasma membrane rather than the vitelline membrane (Tyler, Seaton, and Signoret, 1961).

The material on the surface of the sperm complementary to fertilizin (Lillie's antifertilizin) has been investigated by many of the same techniques as have been used in the analysis of fertilizin (Tyler and O'Melveny, 1941). Presence of a substance in the egg proper identical to or closely related to antifertilizin (Tyler, 1940), led Tyler (1947) to the concept of a natural auto-antibody reaction. This concept states

that cells are composed of systems of complementary substances capable of interacting in a manner similar to antigens and antibodies, and that the formation of certain large molecular substances of cells proceeds by one of the interacting substances serving as a template for the other similar to one of the roles proposed for general antibody formation.

The results of these and other experiments seemed to indicate that, in the sea urchin, the interaction of these complementary substances on the plasma membranes of the gametes in a species-specific manner accounts for the specific adherence of the sperm to egg and incorporation of the sperm by a sort of pinocytotic process (Tyler, 1959).

To further study the roles of these interacting substances in fertilization and development in the sea urchin, antisera against various cell constituents were used. Treatment of eggs prior to fertilization (Perlmann, 1959) indicated the existence of four different types of macromolecules in the surface layers of the egg, three of which seemed to have some function in sperm attachment and egg activation.

Antisera against extracts of unfertilized eggs and of embryos at various stages of development were tested for effects on fertilization and on early development (Tyler, 1959, 1963; Tyler and Brookbank, 1956). Definite cytotoxic and inhibitory effects were noted on unfertilized and

fertilized eggs. In the case of the latter, cleavage was blocked by antisera against purified egg jelly (fertilizin) or whole egg homogenates, but not by antisera containing only antibodies against internal constituents of the egg.

Recently, papain-digested, univalent antibodies were tested for effects on fertilization and cleavage (Metz and Thompson, 1967). This non-agglutinating, non-precipitating, 3.5s form of gamma globulin failed to produce the morphological changes in eggs that result from treatment with multivalent, "7s" antibodies. These univalent antibodies against egg homogenate also failed to affect the fertilizability of dejellied or demembranated eggs. However, univalent anti-egg jelly gamma globulin did inhibit cleavage of fertilized eggs although to a lesser degree than the regular multivalent antibodies. Thus it was concluded in the sea urchin, Lytechinus variegatus, the fertilization-inhibiting action of multivalent antibodies depends upon the cross-linking of neighboring antigens, not on the blocking of specific antigenic sites by complementary antibodies. Whether this is true of the cleavage-inhibiting action remains to be ascertained.

Similar work has been done on anuran eggs suggesting that analogous substances and phenomena interact in fertilization and development in this group (Shaver, 1966).

Glaser (cited in Shaver and Barch, 1960) reported agglutination of sperm of Rana pipiens by "egg water" of

Rana pipiens. Bernstein (1952) and Shaver, Barch, and Shivers (1962) were unable to repeat this. However, Bernstein did find that the "egg water" of Rana clamitans irreversibly agglutinated Rana clamitans sperm. This was true of only jellied eggs. It was noted that the jelly of this species was highly soluble compared to that of Rana pipiens.

In several species of Anura, coelomic eggs or eggs dejellied chemically or mechanically are generally unfertilizable. (Bataillon, 1919; Kambara, 1953; Tchou and Wang, 1956; Katagiri, 1965, 1966). Kambara (1953), working with Bufo vulgaris formosus, found that eggs devoid of jelly became fertilizable if covered with gelatin or agar. Thus, he postulated a thigmotactic role for the jelly in fertilization.

Katagiri (1966), however, investigating Bufo bufo formosus, could not repeat these results with agar, gelatin, or egg albumin, but found a high percentage of dejellied eggs were fertilized when they were inseminated in the presence of either dialyzed jelly material, pronase-digested jelly material, or polyvinylpyrrolidone (PVP).

In addition, Shivers (cited in Shaver, 1966) has demonstrated that jellyless body cavity eggs could be fertilized by spermatozoa which have had prior contact with jellied uterine eggs.

Other work by Katagiri (1965) seemed to indicate that in addition to the presence of jelly (layers J_1 and J_2

or layer J_4) the egg must be cytoplasmically mature to be fertilized. Therefore he suggested that the role of the oviduct may be to establish this cytoplasmic maturity.

In other investigations, Nace et al. (1960) found evidence of an antigen (A) in follicle cells, immature oocytes, oviducal epithelium, and oviducal eggs. Believed to be synthesized in the follicle cells and oviduct, this antigen was thought to be either a regulator of meiosis and mitosis, a sperm acceptor, or a factor which "matures" the eggs.

Glick and Shaver (1963) further demonstrated that Rana pipiens eggs from the middle and lower oviducal regions were more easily fertilized than those from upper segments.

Shaver (1966) treated spermatozoa of Rana pipiens with jelly coat materials of different origins and these spermatozoa were used to inseminate jellied uterine eggs. A significant decrease in fertilizing capacity of the sperm was produced by jelly material from oviducts of Rana pipiens, especially by extracts from the lower portion of the oviduct. It was noted that it is from this region that the outer layer of jelly would originate.

Shaver and Barch (1960) pretreated both spermatozoa and eggs of Rana pipiens with antisera against the jelly coat material. When the treated gametes were used in crosses with untreated gametes, a significant decrease in numbers of eggs fertilized resulted. Other experiments (Barch, personal

communication) in which antisera against other Rana pipiens tissues were used to treat eggs also have produced some inhibition. The percentages of inhibition of fertilization caused by these antibodies (against ovary, heart, and kidney of R. pipiens, R. clamitans, R. catesbiana, Axolotl, B. marinus, B. americanus, and against the egg jelly of the Arbacia punculata) roughly correspond to the number of antigenic components common to R. pipiens eggs and the tissues of the other species. Very recently, Shaver and Barch (1966) varied the time of insemination in relation to the time of treatment of eggs with various antisera. The results show that the antibodies probably reach the egg surface proper fairly rapidly. Therefore, sperm which have traversed the jelly layers may not be able to interact with egg surface sites due to prior blockage of these by the treatment with antibodies. Whether these results would support the existence of a "fertilizin-antifertilizin" system in Anuran gametes analogous to that of sea urchin eggs is uncertain.

Since the inhibiting action of the antisera could be due to a cross-linked lattice among the jelly molecules acting as a mechanical barrier to sperm, Shivers and Metz (1962) used papain-digested, univalent, non-precipitating anti-jelly serum. This also produced a significant inhibition of fertilization, but no precipitation of jelly layers occurred. Thus, the authors felt that a direct blocking by the antibodies of receptor sites in the jelly which were important in fertilization had occurred.

Different antigenic components have been shown to be present at different oviducal levels (lower and upper) by Barch and Shaver (1963). It was also noted that mature uterine eggs of Rana pipiens treated with antisera against the lower segment of the oviduct fertilized in significantly lower numbers than eggs treated with antibodies against the upper segment.

Corresponding to oviducal differences are the antigenic differences in the three jelly layer of mature uterine eggs, investigated extensively by Shivers. Shivers (1961) found that four species of Rana contained both species-specific and common components in their jelly layers. Eggs of Rana pipiens were pretreated with homologous and heterologous antibodies and fertilizability was decreased by antisera both against the species-specific components of Rana pipiens egg jelly and against the common components of heterologous egg jelly.

Using fluorescine-conjugated antibodies against homologous and heterologous egg jellies to stain the jellies of Rana pipiens eggs, Shivers (1962) found that the species-specific components were localized in the outer two jelly layers and the common component in the inner jelly layer. Investigating various genera and species of amphibians, Shivers (1965) also found that in no case where cross fertilization produced viable embryos were jelly antigens shared by the two species lacking.

All of these results have led Shaver (1966) to hypothesize that the egg jelly antigens are responsible for a series of reactions which involve the complexing of both species-specific and common combining sites on the sperm surface and jelly layers, followed by reactions of complementary sites on the egg and sperm surfaces.

Information about these hypothetical sites on the egg surface is sketchy and inconclusive and it is about these that this present investigation concerns itself.

Shaver, Barch, and Shivers (1962), using the double diffusion technique, found a tissue specificity of Rana pipiens egg jelly components similar to that described for the fertilizin of the jelly coat of sea urchin eggs. Some cross reactions did occur between jelly and ovarian material which seemed to indicate the presence in the jelly of material secreted by the egg or by oviducal cells.

Using antisera against mature ovaries and embryonic stages of Rana pipiens to treat coelomic eggs, an antigen on the surface of mature oocytes and fertilized eggs (antigen F) was found (Lavin, 1963; Nace and Lavin, 1963). Antisera against antigen F inhibited activation and cleavage. The role played by antigen F in activation and cleavage was believed to be consistent with the role suggested for mucosubstances in the egg.

Although Shivers (1965) showed by the use of fluorescine-conjugated antibodies, that egg jelly antigens

existed only in the jelly layers, Poirier (1964) found a significant inhibition of fertilization in coelomic eggs of Rana pipiens which had been treated with anti-egg jelly sera, transferred through the oviducts of ovarectomized females, and then inseminated.

Cerny (1963) also found a lower number of cleavages in Rana pipiens eggs which had been fertilized, dejellied with potassium cyanide, and then treated with anti-jelly serum.

Thus is seemed that by treating dejellied Rana pipiens eggs, at different times after fertilization, with antisera against various Rana pipiens tissues, further knowledge of the antigenic components of the frog egg would be gained. The results of exposing eggs to antisera both in culture and by injection will be presented.

II. MATERIALS AND METHODS

A. Production and Fractionation of Antisera

Rana pipiens egg-jelly, heart, and ovary antigens were prepared in the following manner: Jelly was manually removed from mature uterine eggs, lyophilized, and homogenized (5 mg./ml.) in one-tenth full strength Holtfreter's solution. Heart and ovary were each homogenized (one gram in ten ml.) in one-tenth full strength Holtfreter's solution. Each homogenate was then separately added to equal parts of complete Freund's adjuvant and emulsified. Next, 1.5 ml. of these emulsions were injected half on each side into the subscapular region of rabbits. An equal dose, with incomplete Freund's adjuvant, was injected one week later. In about 3-4 weeks, if antibodies could be detected, bleedings were begun and continued every other week. Antigen injections were also continued every two weeks. In all cases, serum was collected from the rabbits, before antigen injections, to be used as control serum.

The control serum and the antisera which had been demonstrated, by the Ouchterlony double-diffusion technique (Ouchterlony, 1949; Shaver, 1961), to have antibodies to homologous antigens were each pooled and then fractionated

as follows: First the serum was filtered and an equal amount of ammonium sulfate was added to cause a precipitation. This was placed in the refrigerator overnight. Then it was centrifuged at 2 degrees C. for twenty minutes at a speed of 10,000 rpm. The supernatant (albumin fraction) was poured off and the residue (globulin fraction) was rinsed with 50% ammonium sulfate to get rid of the trapped albumins and centrifuged as before. Again, the supernatant was discarded. The residue was dissolved in one-tenth full strength Holtfreter's solution (one-half the amount of the original volume of serum filtered). This dissolved globulin was then dialyzed against amphibian saline (0.65% NaCl) for 48 hours, removed, and frozen for later use.

B. General Procedure for Jelly Removal and External Treatment of Fertilized *Rana pipiens* Eggs

Ovulation in female *Rana pipiens* was induced by pituitary gland injection (Rugh, 1934). The number of pituitaries injected depended on the season. Twenty-four to forty-eight hours after injection, eggs from 1-4 frogs were stripped onto glass slides and fertilized with sperm suspension obtained by the maceration of two testes of *Rana pipiens* per 20 ml. of one-tenth full strength Holtfreter's solution. After 6-8 minutes, excess sperm suspension was decanted and each slide of eggs was placed in a finger bowl half-full of aerated water. There the jelly was hydrated for fifteen minutes. One slide of fertilized eggs was left in the

aerated water with the jelly intact as a control on the cleavage percentages of that particular batch of eggs. The rest of the eggs were put into 100 ml. of 0.1N cysteine HCl (M. W. 175. 64) which had been buffered to a pH of about 7.5 with 1N sodium hydroxide. The eggs were vigorously swirled in this solution for about twenty minutes. The cysteine HCl solution was poured off the eggs and 50 ml. of fresh solution was added and swirling was continued. Another 50 ml. was added and by this time most of the eggs were visibly dejellied. This could be seen by the way they lay in the beaker touching each other with no jelly coats separating them. At this point they were washed six times with one-sixth or one-tenth full strength Holtfreter's solution and placed into the various treatment solutions for different lengths of time (15, 20, 30, and 45 minutes). These treatments began 50-55 minutes, 2 hours, and 4 hours after fertilization. The following treatment solutions were used at various times during the course of this work: full strength Holtfreter's solution; one-sixth full strength Holtfreter's solution; one-tenth full strength Holtfreter's solution; 7.7% Ringers; aerated water; control sera and antisera diluted 1:1, 1:2, 2:1, and 3:1 with one-sixth or one-tenth full strength Holtfreter's solution. In the cases where the eggs were not put into the treatment solutions for 2 or 4 hours after fertilization, the dejellied eggs were kept in one-tenth full strength Holtfreter's solution until

included: one-tenth full strength Holtfreter's solution; control serum; heart, ovary, and egg jelly antisera. All the sera were diluted 1:2, 1:4, and 1:8 with one-tenth full strength Holtfreter's solution. A fertilized, non-injected, control slide of eggs was included in each experiment. All eggs were cultured in finger bowls of aerated water at room temperature. Cleavage, gastrula, and tailbud counts were made.

III. RESULTS

The results of the experiments involving the external treatment of the dejellied, fertilized frog eggs are presented in Tables I-1X. Each trial in experiments 1-7 in Tables I-VII involved eggs from just one frog (except Exp. 4-H). Cleavage and gastrula counts were made about 4-6 hours and 24-34 hours, respectively, after fertilization. These correspond approximately to Shumway (1940) stages 5 and 11. Although counts were not recorded past gastrula, embryos were allowed to develop and were observed for as long as ten days. More died as the days progressed, but some did appear normal at the end of this period.

There are some figures in the data indicating that the number of eggs which successfully reached gastrulation was larger than the number which was recorded as having cleaved. This does not represent a "recovery" but rather a few eggs which were slower to divide at first but had "caught up" by the gastrula stages.

Eggs which were not counted as developing showed a variety of abnormalities. Often there was cytoplasmic mottling or streaking, especially along the cleavage furrows. In other cases the eggs had burst due either to a cytotoxic effect or from being extremely fragile without the jelly

coat. Many eggs were misshapen and/or contained irregular folds. Some had patches of smooth undivided cytoplasm amidst areas of normally divided calls. Occasionally there were eggs which had partially pushed out of the vitelline membrane to form a dumb-bell shaped embryo. Many showed diffuse yolk plugs and/or arrested yolk plug closure.

Comparing the percentages, there is a disturbing variation among frogs. This could be due to differences in egg sensitivity or differences among the antisera used. However, the differences among antisera were thought to have been minimized by pooling them. The only antisera that seemed to have effects significantly different from the others were those against ovary. The most striking results appear in the five trials (Experiment 2-A and B; Experiment 4-A, B, and C) where there was no development at all after treatment with anti-ovary antisera. Every egg was smooth without a single cleavage furrow. Since development was satisfactory to excellent after the other treatments, the eggs can be considered normal in these trials. These five trials were done with the same dilutions of sera and with similar times of treatment (20 and 30 minutes).

These results could not be repeated although the same methods were used. There were, however, other trials in which noticeably lower percentages of eggs treated with anti-ovary serum reached the gastrula stage. This is shown by the results in Experiment 3-A and B. Although the

percentages reaching gastrulation were low after treatment with anti-ovary serum in Experiment 4-F and Experiment 6-B, there were also low percentages for eggs treated with anti-heart and anti-jelly sera in both these experiments. The differences were usually greater in the gastrula stages.

Sometimes a much lower percentage of eggs reached gastrulation in the jelly controls, or after treatment with 1/10 full strength Holtfreter's solution or with the control sera. This appeared only occasionally and did not seem to be due to anything other than differences among eggs from different females.

Tables VIII and IX represent experiments in which the eggs were treated with antisera after development had proceeded for 2 or 4 hours. The eggs which were treated at the end of four hours were counted for cleavage after four hours of development, then treated, and counted again an hour after treatment. Of course those which were treated after two hours could not be counted before treatment because it was too early to note cleavage. In each of these trials a regular set of treatments were run in which the eggs were treated immediately as in all the other work. Thus, this served as an overall control for the eggs which were treated after 2 or 4 hours of development. No noticeable differences occurred consistently between the immediate or delayed treatments or between the different types of antisera in each experiment. There were some incidences of lower

percentages of development in anti-ovary serum. However, these were accompanied by an equally poor development in anti-heart and anti-jelly sera (Exp. 8-B; Exp. 9-C). Apparently the time at which the eggs were treated was not a decisive factor here.

Eggs from more than one frog were used in each of the trials just mentioned (Experiments 8 and 9). It was hoped that this would minimize differences in egg sensitivities. The eggs used in this phase of the work were cultured in aerated water after treatment rather than Holtfreter's solution, as were those recorded in Tables I-VII. This procedure seemed to be more favorable for survival.

Often it was difficult to keep the fractionated sera fully dissolved in the Holtfreter's solution. During the course of the time treatments, a precipitate (probably of denatured protein) would form on the bottom of the dish and around the eggs. This usually only happened with the control and anti-heart sera. Perhaps this meant that active anti-bodies were not effectively reaching the egg surface, but since the results did not show consistent differences in the development of the eggs in these antisera, the significance of the precipitate on the experiments is uncertain. Unfractionated serum did not precipitate in this fashion, so one experiment was done with unfractionated sera. However, it was not repeated since the unfractionated sera seemed very toxic to the eggs and many more eggs stopped developing earlier.

Tables X-XIV represent the results obtained from injecting antisera into fertilized, jellied Rana pipiens eggs 45 minutes and one hour after fertilization. There was no doubt that the eggs were indeed punctured, since a hole and/or exovate was noticeable. These punctures did not seem to be the reason that development did not proceed normally because many healed as they developed normally.

Abnormalities were not as varied here as in the external treatments. Most of them showed cytoplasmic streaking, diffuse yolk plugs in gastrulation, or irregularly shaped embryos in the tailbud stage (often due to edema).

Comparison of the percentages here shows even less consistency than the previously mentioned results. There was a general increase in death rate from the four-cell stage to the tailbud stage. This was even true of the non-injected controls and almost all of the eggs injected with 1/10 Holtfreter's solution and control serum. Thus, rather than a specific effect by one or more antisera, there appeared to be a general collapse due to the condition of the eggs. The injections were all done in April and May, so perhaps the eggs were not as good as they were earlier in the year.

It can only be definitely stated that the percentage of eggs which reached cleavage was good in almost every experiment and that of embryos which reached the tailbud stage was low. Thus, the only place where differences could be

noticed was in the percentage that reached the gastrula stage. However, the number of embryos reaching the gastrula stage was low regardless of the type of serum injected, although injection with anti-ovary serum produced the smallest number of gastrulae.

A dilution of 1:1 was not used since the 1:4 dilution seemed sufficiently strong to give an effect and the more concentrated antisera were extremely hard to draw into the pipette. There was also a problem with the fractionated sera precipitating and clogging the needle, so unfractionated sera were tried but proved to be more toxic, as they had in the external treatments. As before, many of these eggs did develop into more advanced normal embryos although no exact counts were made beyond the tailbud stage.

TABLE I
Experiment 1

Sera diluted 1:2 with 1/6 f.s. Holt.-30 minutes

	Solution	Total	Cleaved	Percent	Gastrula	Percent
A.	J. Cont.*	41	32	78.0	32	78.0
	1/6 Holt.	57	55	96.5	55	96.5
	Control	57	55	96.5	55	96.5
	Heart	52	49	94.2	50	96.2
	Ovary	55	54	98.2	55	100.0
	Jelly	53	51	96.2	53	100.0
B.	J. Cont.	64	62	96.9	62	96.9
	1/6 Holt.	73	70	95.9	71	97.3
	Control	84	82	97.6	82	97.6
	Heart	64	64	100.0	64	100.0
	Ovary	81	78	96.3	78	96.3
	Jelly	72	68	94.4	68	94.4

TABLE II
Experiment 2

Sera diluted 1:1 with 1/6 f.s. Holt.-20 minutes

	Solution	Total	Cleaved	Percent	Gastrula	Percent
A.	J. Cont.	70	70	100.0	70	100.0
	1/6 Holt.	88	86	97.7	86	97.7
	Control	41	41	100.0	38	92.7
	Heart	119	116	97.5	117	98.3
	Ovary	70	0	0	0	0
	Jelly	58	56	96.6	56	96.6
B.	J. Cont.	85	82	96.5	82	96.5
	1/6 Holt.	94	85	90.4	85	90.4
	Control	79	71	89.9	72	91.1
	Heart	64	63	98.4	63	98.4
	Ovary	85	0	0	0	0
	Jelly	96	94	97.9	90	93.8

*Eggs with jelly, cultured in aerated water after fertilization--no treatment.

TABLE III
Experiment 3

Sera diluted 1:1 with 1/6 f.s. Holt.-30 minutes

	Solution	Total	Cleaved	Percent	Gastrula	Percent
A.	J. Cont.	62	59	95.2	59	95.2
	1/6 Holt.	92	90	97.8	90	97.8
	Control	104	102	98.1	102	98.1
	Heart	95	94	98.9	94	98.9
	Ovary	73	69	94.5	42	57.5
	Jelly	77	72	93.5	65	84.4
B.	J. Cont.	41	32	78.0	32	78.0
	1/6 Holt.	57	55	96.5	55	96.5
	Control	51	49	96.1	37	72.5
	Heart	43	42	97.7	39	90.7
	Ovary	39	38	97.4	22	56.4
	Jelly	52	51	98.1	38	73.1
C.	J. Cont.	64	62	96.9	62	96.9
	1/6 Holt.	73	70	95.9	71	97.3
	Control	93	92	98.9	91	97.8
	Heart	57	54	94.7	53	93.0
	Ovary	81	78	96.3	78	96.3
	Jelly	84	81	96.4	79	94.0

TABLE IV
Experiment 4

Sera diluted 1:1 with 1/10 f.s. Holt.-30 minutes

	Solution	Total	Cleaved	Percent	Gastrula	Percent
A.	J. Cont.	56	55	98.2	56	100.0
	1/10 Holt.	43	43	100.0	43	100.0
	Control	54	48	88.9	52	96.3
	Heart	55	54	98.2	54	98.2
	Ovary	65	0	0	0	0
	Jelly	56	52	92.9	43	76.8
B.	J. Cont.	59	48	81.4	48	81.4
	1/10 Holt.	36	27	75.0	28	77.8
	Control	41	31	75.6	33	80.5
	Heart	34	26	76.5	27	79.4
	Ovary	45	0	0	0	0
	Jelly	50	37	74.0	30	60.0
C.	J. Cont.	90	88	97.8	84	93.3
	1/10 Holt.	55	50	90.9	49	89.1
	Control	62	51	82.3	51	82.3
	Heart	91	83	91.2	83	91.2
	Ovary	72	0	0	0	0
	Jelly	85	69	81.2	69	81.2
D.	J. Cont.	88	84	95.5	85	96.6
	1/10 Holt.	74	72	97.3	79	94.6
	Control	90	87	96.7	87	96.7
	Heart	76	72	94.7	72	94.7
	Ovary	63	57	90.5	57	90.5
	Jelly	72	69	95.8	70	97.2

TABLE IV, Experiment 4--Continued

Solution	Total	Cleaved	Percent	Gastrula	Percent
E. J. Cont.	164	146	89.0	146	89.0
1/10 Holt.	29	25	86.2	23	79.3
Control	45	36	80.0	40	88.9
Heart	46	41	89.1	43	93.5
Ovary	45	42	93.3	42	93.3
Jelly	25	25	100.0	25	100.0
F. J. Cont.	144	112	77.8	112	78.5
Aerat. H_2O	53	46	86.8	45	84.9
1/10 Holt.	49	42	85.7	42	85.7
Control	25	22	88.0	21	84.0
Heart	38	24	63.2	23	60.5
Ovary	71	51	71.8	48	67.6
Jelly	43	27	62.8	24	55.8
G. J. Cont.	39	37	94.9	33	84.6
Aerat. H_2O	71	63	88.7	56	78.9
1/10 Holt.	59	59	100.0	59	100.0
Control	57	50	87.7	48	84.2
Heart	55	53	96.4	49	89.1
Ovary	70	66	94.3	63	90.0
Jelly	85	83	97.6	78	91.8
**H. J. Cont.	98	86	87.8	83	84.7
1/10 Holt.	90	79	87.8	78	86.7
Control	76	72	94.7	72	94.7
Heart	82	73	89.0	73	89.0
Ovary	90	82	91.1	72	80.0
Jelly	50	48	96.0	33	66.0

**Eggs used were from four different frogs.

TABLE V
Experiment 5

Sera diluted 2:1 with 1/10 f.s. Holt.-15 minutes

	Solution	Total	Cleaved	Percent	Gastrula	Percent
A.	J. Cont.	164	146	89.0	146	89.0
	1/10 Holt.	19	17	89.5	16	84.2
	Control	22	21	95.5	20	90.9
	Heart	27	25	92.6	25	92.6
	Ovary	40	37	92.5	39	97.5
	Jelly	31	28	90.3	28	90.3

TABLE VI
Experiment 6

Sera diluted 2:1 with 1/10 f.s. Holt.-30 minutes

	Solution	Total	Cleaved	Percent	Gastrula	Percent
A.	J. Cont.	164	146	89.0	146	89.0
	1/10 Holt.	19	17	89.5	16	84.2
	Control	27	24	88.9	25	92.6
	Heart	18	18	100.0	18	100.0
	Ovary	24	18	75.0	19	79.2
	Jelly	34	29	85.3	29	85.3
B.	J. Cont.	144	112	77.8	113	78.5
	Aerat. H_2O	53	46	86.8	45	84.9
	1/10 Holt.	49	42	85.7	42	85.7
	Control	62	46	74.2	46	74.2
	Heart	69	42	60.9	24	34.8
	Ovary	66	34	51.5	23	34.8
	Jelly	38	21	55.3	12	31.6

TABLE VII
Experiment 7

Sera diluted 1:1 with 1/10 f.s. Holt.-45 minutes

	Solution	Total	Cleaved	Percent	Gastrula	Percent
A.	J. Cont.	164	146	89.0	146	89.0
	1/10 Holt.	27	21	77.8	21	77.8
	Control	47	44	93.6	43	91.4
	Heart	31	28	90.3	28	90.3
	Ovary	36	29	80.6	29	80.6
	Jelly	38	36	94.7	37	97.4

TABLE VIII
Experiment 8

Sera Diluted 1:1 with 1/10 f.s. Holt.-30 minutes

**A. Immediate treatment

Solution	Total	Cleaved	Percent	Gastrula	Percent
J. Cont.	101	94	93.1	94	94.1
1/10 Holt.	52	50	96.2	50	96.2
Control	79	72	91.1	73	92.4
Heart	86	80	93.0	80	93.0
Ovary	68	66	97.1	66	97.1
Jelly	74	69	93.2	64	86.5

Delayed treatment after 4 hours

Solution	Tot.	4 Hrs. after Fert. (Before Treat.)	%	6 Hrs. after Fert. (1 Hr. after Treat.)	%	Gast.	%
1/10 Holt.	57	54	94.7	54	94.7	54	94.7
Control	65	64	98.5	64	94.7	64	98.5
Heart	73	70	95.9	70	95.9	70	95.9
Ovary	70	64	91.4	63	90.0	63	90.0
Jelly	73	68	93.2	68	93.2	68	93.2

**B. Immediate Treatment

Solution	Total	Cleaved	Percent	Gastrula	Percent
J. Cont.	89	87	97.8	87	97.8
1/10 Holt.	45	45	100.0	45	100.0
Control	39	28	71.8	32	83.1
Heart	44	25	56.8	29	65.9
Ovary	64	35	54.7	33	51.6
Jelly	51	46	90.2	47	92.2

Delayed treatment after 4 hours

Solution	Tot.	4 Hrs. after Fert. (Before Treat.)	%	6 Hrs. after Fert. (1 Hrs. after Treat.)	%	Gast.	%
1/10 Holt.	48	37	77.1	37	77.1	36	75.0
Control	46	40	87.0	40	87.0	15	32.6
Heart	47	38	80.9	38	80.9	4	8.51
Ovary	36	30	83.3	30	83.3	5	13.9
Jelly	47	37	78.7	37	78.7	22	46.8

**Eggs used were from three different frogs.

TABLE IX
Experiment 9

Sera diluted 1:1 with 1/10 f.s. Holt.-30 minutes

Solution	Total	Cleaved	Percent	Gastrula	Percent
*A. Immediate treatment					
J. Cont.	92	62	67.4	62	67.4
1/10 Holt.	40	24	60.0	27	67.5
Control	46	26	56.5	26	67.5
Heart	55	33	60.0	34	61.8
Ovary	47	29	61.7	28	59.6
Jelly	56	38	67.9	43	76.8
Delayed treatment after 2 hours					
1/10 Holt.	56	43	76.8	43	76.8
Control	54	37	68.5	40	74.1
Heart	56	40	71.4	40	71.4
Ovary	52	26	50.0	11	21.2
Jelly	60	42	70.0	37	88.1
**B. Immediate treatment					
J. Cont.	56	51	91.1	52	92.9
1/10 Holt.	36	27	75.0	25	69.4
Control	41	33	80.5	33	80.5
Heart	34	17	50.0	20	58.8
Ovary	45	32	71.1	30	66.7
Jelly	58	45	77.6	45	77.6
Delayed treatment after 2 hours					
1/10 Holt.	38	17	44.7	7	18.4
Control	48	24	50.0	32	66.7
Heart	44	34	77.3	27	61.4
Ovary	44	32	72.7	29	65.9
Jelly	37	23	62.2	21	57.8

*Eggs used were from three different frogs.
**Eggs used were from two different frogs.

TABLE IX, Experiment 9, Continued

Solution	Total	Cleaved	Percent	Gastrula	Percent
*C. Immediate treatment					
J. Cont.	112	100	89.3	100	89.3
1/10 Holt.	61	57	93.4	56	91.8
Control	70	65	92.9	65	92.9
Heart	52	39	75.0	39	75.0
Ovary	93	70	75.3	72	77.4
Jelly	57	35	61.4	32	56.1
Delayed treatment after 2 hours					
1/10 Holt.	48	41	85.4	40	83.3
Control	46	43	93.5	43	93.5
Heart	61	48	78.7	41	67.2
Ovary	77	53	68.8	32	41.6
Jelly	47	28	59.6	22	46.8

*Eggs used were from three different frogs.
**Eggs used were from two different frogs.

TABLE X
Experiment 10

Sera diluted 1:2 with 1/10 f.s. Holt.-Inj. 1 hour after fert.

	Solution	Total	Cleaved	Percent	Gast.	Percent	Tailbud	Percent
A.	No Inj.	56	38	67.9	25	54.6	19	33.9
	1/10 Holt.	41	18	43.9	18	43.9	12	29.3
	Control	24	13	54.16	7	29.2	0	0
	Heart	27	14	51.9	0	0	0	0
	Ovary	38	27	71.0	23	60.5	18	47.4
	Jelly	44	31	70.5	19	43.2	9	20.5
B.	No Inj.	49	48	98.0	41	83.7	38	77.6
	1/10 Holt.	32	29	90.6	29	90.6	28	87.5
	Control	30	15	50.0	8	26.7	5	16.7
	Heart	26	11	42.3	1	3.8	3	11.5
	Ovary	25	7	28.0	3	12.0	2	8.0
	Jelly	26	10	38.5	4	15.4	6	23.1

TABLE XI
Experiment 11

Sera diluted 1:8 with 1/10 f.s. Holt.-Inj. 1 hour after fert.

	Solution	Total	Cleaved	Percent	Gast.	Percent	Tailbud	Percent
A.	No Inj.	53	47	88.7	47	88.7	45	84.9
	1/10 Holt.	45	45	100.0	39	86.7	26	57.8
	Control	38	34	89.5	23	60.5	9	23.7
	Heart	32	30	93.8	28	87.5	23	71.9
	Ovary	45	39	86.7	34	75.6	20	44.4
	Jelly	48	45	93.8	40	83.3	29	60.4
B.	No Inj.	47	43	91.5	42	89.4	42	89.4
	1/10 Holt.	61	56	91.8	17	27.9	3	4.9
	Control	46	35	76.1	13	28.3	5	10.9
	Heart	34	29	95.3	14	41.2	2	5.9
	Ovary	35	32	91.4	1	2.9	1	2.9
	Jelly	45	41	91.1	22	48.9	11	24.4

TABLE XII
Experiment 12

Sera diluted 1:4 with 1/10 f.s. Holt.-Inj. 1 hour after fert.

	Solution	Total	Cleaved	Percent	Gast.	Percent	Tailbud	Percent
A.	No Inj.	44	36	81.8	34	77.3	22	50.0
	1/10 Holt.	36	19	52.8	14	38.9	3	8.3
	Control	27	13	48.1	11	40.7	4	14.8
	Heart	53	36	69.2	9	17.3	2	3.9
	Ovary	23	12	52.2	3	13.0	0	0
	Jelly	46	31	67.4	19	41.3	5	10.9
B.	No Inj.	68	63	92.6	57	85.3	48	70.6
	1/10 Holt.	40	34	85.0	8	20.0	2	5.0
	Control	42	31	73.8	6	14.3	1	2.4
	Heart	44	40	90.9	13	29.5	8	18.2
	Ovary	38	33	86.8	14	36.8	0	0
	Jelly	38	36	94.7	11	28.9	1	2.6
C.	No Inj.	52	39	75.0	36	69.2	26	50.0
	1/10 Holt.	39	29	74.4	4	10.3	0	0
	Control	33	23	69.7	5	15.2	1	3.0
	Heart	55	46	83.6	6	10.9	0	0
	Ovary	40	33	82.5	0	0	0	0
	Jelly	30	27	90.0	8	26.7	1	33.3
D.	No Inj.	64	38	59.4	25	39.1	5	7.8
	1/10 Holt.	46	37	80.4	18	39.1	2	4.34
	Control	37	26	70.3	11	29.7	3	8.1
	Heart	49	40	81.6	13	26.5	0	0
	Ovary	34	29	85.3	6	17.6	0	0
	Jelly	33	15	45.5	7	21.2	0	0
E.	No Inj.	45	44	97.8	43	95.6	41	91.1
	1/10 Holt.	31	25	80.6	21	67.7	16	51.6
	Control	38	33	86.8	27	71.1	14	36.8
	Heart	45	45	100.0	31	68.9	16	35.6
	Jelly	34	31	91.2	21	61.8	12	35.3

TABLE XIII
Experiment 13

Sera diluted 1:8 with 1/10 f.s. Holt.Inj. 45 min. after fert.

	Solution	Total	Cleaved	Percent	Gast.	Percent	Tailbud	Percent
A.	No Inj.	35	26	74.3	8	22.9	7	20.0
	1/10 Holt.	44	27	61.4	1	2.3	0	0
	Control	22	17	77.3	1	4.5	0	0
	Heart	32	22	68.8	4	12.5	1	3.1
	Ovary	38	29	76.3	0	0	0	0
	Jelly	18	10	55.6	0	0	0	0

TABLE XIV
Experiment 14

Sera diluted 1:4 with 1/10 f.s. Holt.-Inj. 45 min. after fert.

	Solution	Total	Cleaved	Percent	Gast.	Percent	Tailbud	Percent
A.	No Inj.	30	26	86.7	16	53.3	4	13.3
	1/10 Holt.	25	16	64.0	7	28.0	0	0
	Control	30	25	83.3	7	23.3	2	6.7
	Heart	29	18	62.1	5	17.2	2	6.9
	Ovary	26	20	76.9	3	11.5	0	0
	Jelly	19	14	73.7	8	42.1	0	0
B.	No Inj.	59	49	83.1	46	78.0	42	71.2
	1/10 Holt.	33	28	84.8	12	36.4	1	3.0
	Control	35	27	77.1	10	28.6	0	0
	Heart	29	23	79.3	10	34.5	5	17.2
	Ovary	35	30	85.7	1	2.9	0	0
	Jelly	46	37	80.4	18	39.1	5	10.9
C.	No Inj.	55	50	90.9	54	98.2	49	89.1
	1/10 Holt.	38	33	86.8	20	52.6	14	36.8
	Control	35	32	91.4	26	74.3	24	68.6
	Heart	38	36	94.7	29	76.3	25	65.8
	Ovary	30	23	76.7	5	16.7	4	13.3
	Jelly	29	26	89.7	17	58.6	14	48.3

IV. DISCUSSION

A. External Treatments

The inconsistent and varied effects of the different antisera and even of the controls are indeed difficult to interpret as a significant blockage by specific antisera. Instead they must be explained in terms of differences in sensitivity of eggs from different female frogs and differences among antisera. The eggs do vary in their reactions to any kind of manipulations at different times of the year. The health of the frog, the size of the eggs, and the amount and condition of the jelly can all have significant influences on the response of the eggs to treatments. The antisera produced by one rabbit can also vary in titer of antibodies in relation to the general condition of the animal. Considering the number of animals used and the number of bleedings performed, it is reasonable to suppose that the titer of antibodies would differ, even though they were pooled.

In addition to these inherent variations in the materials used, the process of dejellying the eggs could have had a general deleterious effect on the outcome of the experiments. Eggs with their jelly coats removed are obviously more sensitive to any manipulations. Although

great care was used in the dejellying process, poor development of the dejellied eggs, treated with both control serum and Holtfreter's solution, in a number of trials, is proof that some protection is afforded by the jelly in manipulations of this sort. Eggs without jelly coats are also more sensitive to slight variations in osmolarity. Thus, it can be seen that any cytotoxicity of antisera in general is more likely to have an effect on dejellied eggs.

The results obtained in five trials with anti-ovary sera were very interesting and are similar to some other preliminary work done in this laboratory. Barch (personal communication) has injected antisera (diluted 1:1 with one-tenth full strength Holtfreter's solution) against Rana pipiens egg jelly, ovary, heart, and control serum into the body cavity of female Rana pipiens. This was done every other day for four weeks, if possible (most frogs died earlier). The effects on the eggs produced by these females were noted. The frogs injected with anti-ovary serum ovulated fewer eggs when artificially stimulated with pituitary glands and when they did ovulate, the percentages of eggs which cleaved were lower. Although this work is incomplete, the general trend seemed to be consistent with the effects of anti-ovary sera on fertilized dejellied eggs. The fact that the inhibition of cleavage in the latter could not be repeated is probably due to differences in sensitivities of the eggs as discussed previously.

The reasons that antisera against frog ovary can inhibit cleavage in this way can only be tentatively interpreted on the basis of the antigenic components of the egg surface and the ovarian tissues. Agar diffusion studies (Shaver, Barch, and Shivers, 1962) have shown that no tissue of Rana pipiens except the oviduct, where the jelly is secreted, has been found to have components identical to those in egg jelly. This is not true of ovary tissue, however, where many antigens common to other frog tissues, including sperm extracts, have been identified. It has also been shown by Barch (personal communication) that there is an ovary (egg)-specific, rather than a species-specific, antigen apparently active in fertilization. This supports the presence of an "E" antigen on the surface of the egg as hypothesized by Shaver (1966).

Since ovary is mostly egg material, antisera against frog ovary could be expected to contain antibodies which are complementary to antigens in or on the surface of the frog egg.

A mucosubstance in Rana pipiens, presumably having a significant role in activation and cleavage, was identified and named "Antigen F" by Lavin (1963). Fluorescine-tagged antisera localized this antigen on the surface (cortex) of developing oocytes and fertilized eggs. Anti-F antisera inhibited both activation and cleavage of fertilized eggs.

It is doubtful that antibodies could effectively pass through the vitelline or fertilization membrane

(Flickinger and Nace, 1952; Tyler, 1963). Kemp (1956, 1967) has demonstrated with electron microscopy that, in growing oocytes of Rana pipiens, the folded cortex extends as microvilli into the substance of the developing vitelline membrane intertwining with the microvilli of follicular cells. However, in the mature oocyte, microvilli are withdrawn and the surface becomes smooth. The cortex is separated from the vitelline membrane by a narrow perivitelline space containing small vesicles and flocculent material. After fertilization, the perivitelline space increases as the cortical granules erupt and discharge their contents into this space. The short pseudopodial protrusions which had existed in the space soon retract. Thus, the vitelline membrane represents a considerable barrier to any antibody which might react with a cortical antigen. It is possible, however, that these antigenic substances, if present on the tips of the microvilli, could have been deposited on the vitelline membrane before retraction. This would allow interaction of antibodies complementary to these so-called surface antigens.

Assuming that there are antigens that could be reached by antibodies in the anti-ovary sera, there is still a question as to the nature of the antigen-antibody reaction and how it could inhibit cleavage. It seems reasonable that the interaction of antibodies with surface antigens of the fertilized egg could alter its permeability or other

properties so as to upset the metabolic and structural organization for cleavage. The inhibition here appeared to be a primary effect and not secondarily due to cytolysis, since cytolysis in uncleaved eggs occurred only after a period of time. Perhaps the antigens and antibodies form a cross-linked lattice with deeper layers or with neighboring unrelated sites since the antibodies used were multivalent. However, no precipitation layer appeared on the egg surfaces and no clumping of eggs occurred.

Tyler and Brookbank (1956) observed that treatment with antiserum increases the tension at the surface of sea urchin eggs. If this is also true of Rana pipiens, the increase in tension, perhaps caused by a lattice of antigens and antibodies, could have an external effect on the formation of the cleavage furrows or could lead to later developmental abnormalities.

B. Injection of Eggs

The damage to the eggs due to the injection of antisera can be attributed to the time of year and the time after fertilization at which they were performed. The eggs in April and May are much more sensitive to any kind of treatments as the poor controls show. In many cases, the general injury caused by a puncture was apparently too much of a shock to the eggs.

Perhaps the time at which the eggs were injected in relation to insemination was too critical. Various developmental

events could have been irreversibly disrupted such as: spindle formation and chromosome movements, furrowing and pinching off of polar bodies, or general protoplasmic streaming. Any drastic derangements could cause immediate cytolysis or later death during the critical periods of gastrulation and hatching.

Additional work in these areas with some modifications of techniques might lead to more conclusive results than were obtained in this study. External treatment of the egg as soon as possible after fertilization, if the time of jelly removal can be shortened, might be more effective. Injections at a later time after fertilization might also lead to better development. If inhibition similar to that produced here by anti-ovary sera can be repeated, observation of sections of the eggs would give a more complete picture of the nature and extent of the blockage. In evaluating the role of egg and jelly-coat antigens in early development, it must be remembered that when an antibody combines with a macromolecule, it does not necessarily follow that it combines with the region of the molecule responsible for the particular developmental activity in question.

V. SUMMARY

There is considerable literature concerned with the role of antigens in development as investigated by immunological methods. On the basis of the previous work done on sea urchin and frog eggs, it was of interest to the author to observe, what effect, if any, antisera against egg jelly and various tissues of the frog had on the cleavage and subsequent development of frog eggs.

Fertilized, dejellied Rana pipiens eggs were treated externally at different times after fertilization, with different concentrations of antisera against various Rana pipiens tissues.

The same antisera were also injected into jellied Rana pipiens eggs at 45 minutes and 1 hour after insemination.

Cleavage, gastrulation, and tailbud (only of the injected eggs) counts were made and percentages of successful development in the various antisera and controls were noted.

The differences were too varied to be considered significant, except for five trials in which external treatment of eggs with anti-ovary sera totally inhibited cleavage in all the eggs involved.

The possible reasons for the inconsistency of results were discussed.

Due to the inability of the author to reproduce the effect with anti-ovary sera, definite conclusions cannot be drawn. However, the possibility of an egg surface antigen, active in cleavage, which could have reacted with antibodies in the anti-ovary sera is discussed in relation to the nature of the blockage involved.

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