EFFECT OF UNIVALENT ANTIBODY FRAGMENTS ON THE FERTILIZABILITY OF RANA PIPIENS EGGS

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY
HELEN KNAR ARAKELIAN

1971

ABSTRACT

EFFECT OF UNIVALENT ANTIBODY FRAGMENTS ON THE FERTILIZABILITY OF RANA PIPIENS EGGS

By

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Immunobiological techniques have been used to study the role of gamete surface components in sperm-egg interactions in amphibians. Rana pipiens eggs treated with multivalent antibodies to egg jelly show a marked decrease in fertilizability. Such inhibitory action has been interpreted by some workers as resulting from blockage of jelly receptor sites essential to interaction with sperm, and not from mechanical interference with sperm penetration, e.g., by precipitating the egg jelly, because an antiserum rendered non-precipitating (univalent) by papain digestion also inhibited fertilization in the frog. The present study presents data showing that univalent antibody fragments do not inhibit fertilization and suggests that such inhibition by multivalent antibody is in fact due to a precipitation barrier.

Univalent antibody fragments of rabbit anti-jelly serum were prepared from the purified gamma globulin fraction by papain digestion. Various absorbed and nonabsorbed sera

against frog tissues were employed as controls. It was demonstrated that univalent antibody fragments can inhibit the formation of antigen-antibody precipitates between multivalent antibody and egg jelly antigen in Ouchterlony agar diffusion plates. R. pipiens eggs treated with univalent antibody fragments did not show a decrease in fertilizability. This result suggests that the inhibition of fertilization by multivalent antibody can be explained solely on the basis of a mechanical barrier to sperm penetration and not to direct blocking of specific jelly receptor sites.

In order to ascertain whether, in fact, univalent antibody fragments were complexing with jelly antigens, the antiglobulin (Coombs') test was employed on intact eggs. Univalent antibody-pretreated eggs might be expected to lose fertilizability following subsequent treatment with multivalent sheep anti-rabbit gamma globulin serum. This expectation was not realized. Several reasons are discussed to explain this result.

However, the results of the Competition Test provided some indirect evidence that univalent fragments did bind to antigens in the intact jelly-coat. Eggs treated with univalent fragments of anti-ovary antibodies followed by treatment with the parent multivalent antibody did not show the marked inhibition of fertilization of eggs treated with multivalent antibody alone. This suggests that univalent

fragments neutralized jelly receptor sites and thereby prevented the subsequent attachment of multivalent antibody.

It remains to be conclusively demonstrated that there are specific receptor sites in the frog egg-jelly which can be blocked with antibody and which are involved in the fertilization reaction.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Zoology

1971

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and thanks to Dr. John R. Shaver, my major professor, for his interest, encouragement, and valued guidance during the course of this study. Special thanks are also due to Dr. Stephanie H. Barch for her helpful suggestions and unfailing assistance in the laboratory during this investigation.

I also acknowledge and thank most sincerely the members of my guidance committee, Drs. Evelyn Sanders and Hironobu Ozaki, for their helpful direction in the classroom and in the laboratory, and in the preparation of this manuscript.

Acknowledgement is made to the National Institutes of Health for a predoctoral traineeship in Developmental Biology (HD 00135-05).

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INTRODUCTION

Immunobiological techniques have been widely employed to study the mechanisms of the fertilization process. Sera containing antibodies against various constituents of the gametes have been used to investigate the role of gamete surface components in the activation, cleavage, and early development of the egg. Studies of this kind were prompted by the early proposal of Lillie (1913, 1919) that the specificity of the fertilization reaction was invoked by the interaction between egg and sperm receptors in a manner analogous to the reaction between antigen and antibody. The concept that highly specific interactions between macromolecules from sperm and egg are involved in fertilization gained considerable support from studies involving specifically the role of the jelly-coat of the eggs of amphibian and echinoderm species.

The general scheme of fertilization as proposed by Lillie (1913, 1919) in his fertilizin theory was based on the observation that sea water which had been in contact with sea urchin eggs agglutinated homologous sperm. He termed the substance from the eggs which caused agglutination, "fertilizin", which was subsequently confirmed to be identical to the jelly substance (Tyler, 1940). Presumably, the

fertilizin from the egg reacted with a receptor on the sperm surface, termed "antifertilizin", thereby promoting the initial specific adhesion of the gametes.

Various other aspects of fertilization were explained on the basis of this theory, with some subsequent modifications (for reviews, see Tyler, 1948, 1949, 1959). For example, Hagstrom (1956a, 1956b, 1959) demonstrated that the removal of the jelly of sea urchin eggs effected an increased fertilizability of the eggs, and suggested that the jelly substance actually presented an obstacle to sperm penetration. In addition, Tyler (1949) concluded that the specificity of the fertilizin-antifertilizin reaction could not, in itself, account for the specificity of fertilization. Nevertheless, the many studies on the effect of removal or addition of fertilizin and antifertilizin, and the effect of certain agents which block them (e.g., antisera) on the fertilizability of eggs or the fertilizing capacity of sperm, strongly suggest that specific receptor substances are involved (for reviews, see Metz, 1961, 1967; Monroy, 1965; Perlmann, 1957; Tyler, 1941, 1948, 1959). It is the interaction of these complementary substances which accounts, in part, for the specific adherence of sperm to egg, the initiation of the acrosomal reaction, and the incorporation of sperm.

Several studies have been made of the echinoderm egg and sperm surface antigens with special emphasis on their role in fertilization (for reviews see Perlmann, 1959;

Metz, 1967). The location and distribution of four surface and subsurface antigens in Arbacia sperm have been determined by Metz and Kohler (1960) and Flake and Metz (1962). Perlmann (1957b, 1957c, 1959) has identified four egg antigens using immunochemical techniques, three of which have some function in gamete attachment and egg activation. The role of these antigens in fertilization has been studied by determining the effect of blocking them with specific antisera. However, the information gained from such studies has been partially complicated by the many adverse effects of antisera on echinoderm eggs, e.g., parthenogenetic activation, wrinkling of the egg surface, precipitation of the egg jelly, and cortical damage (Perlmann, 1954, 1957a; Perlmann and Hagstrom, 1955, 1957). (For recent reviews, see Perlmann, 1959; Metz, 1967; Metz and Thompson, 1967).

Less extensive studies on amphibian eggs (and also mammalian eggs; for review, see Austin, 1961) have suggested that gamete surface components analogous to the fertilizin-antifertilizin system of echinoderms may interact in fertilization (for recent reviews, see Shaver, 1966; Metz, 1967). A current hypothesis as advanced by Shaver and co-workers (1966) and others concerning the role of antigenic components at the surface of amphibian eggs in fertilization involves a stepwise series of interactions between sperm and egg. The first of these would involve the complexing of sites on the sperm surface with jelly-coat molecules. The next series of interactions would involve combining sites on the sperm

and complementary sites on the egg surface proper. The involvement of the jelly-coat in the initial interaction has been supported by several lines of evidence, as described below.

It has been established that jelly-free body cavity eggs are not fertilizable, and that mechanically or chemically dejellied uterine eggs of amphibian species also cannot be fertilized (Bataillon, 1919; Good and Daniel, 1943; Tchou-Su and Wang, 1956; Shaver and Barch, 1960; Subtelny and Bradt, 1961; Barbieri and Raisman, 1969; Elinson, 1970b, 1971; Wolf and Hedrick, 1971), although they can be artificially activated (Bataillon, 1919; Subtelny and Bradt, 1961). Body cavity eggs can be fertilized, however, if artificially enrobed with jelly from uterine eggs (Tchou-Su and Wang, 1956, Subtelny and Bradt, 1961). The implication of these observations has been that the gelatinous envelopes around the eggs are necessary for the normal interaction of gametes; both physical and chemical mechanisms have been postulated for this interaction.

Kambara (1953) concluded that there was no chemical substance in the jelly important for fertilization in the toad, Bufo bufo asiaticus, since eggs deprived of their jelly-coats were not fertilizable after being covered with a homogenized jelly solution. Furthermore, this worker demonstrated that denuded eggs covered with agar or gelatin were fertilizable (15-50%). He concluded on this basis

that the jelly served simply as a mechanical foothold for sperm, and that the physical state of the material rather than any chemical substances in it was responsible for the indispensability of the jelly for fertilization. In this regard, Katagiri (1963) noted a thigmotactic response of sperm to enzyme-treated eggs with a small amount of jelly remaining, but sperm orientation was completely random when no jelly was present.

Further studies by Katagiri (1965, 1966a, 1966b) have not confirmed Kambara's observation that agar or gelatin can increase the fertilizability of denuded eggs. However, treatment of denuded toad (Katagiri, 1966a, 1967) and frog (Katagiri, 1966b) eggs with dialyzed jelly, polyvinyl-pyrrolidone (PVP), and egg albumin rendered the eggs fertilizable, a result also confirmed with dialyzed jelly on toad eggs by Barbieri and Raisman (1969). These results led Katagiri to conclude that these substances were perfect substitutes for the jelly envelopes in assuring close adherence of the sperm to the vitelline membrane and a high percentage of fertilization. In addition, it was demonstrated that the jelly material retained its effectiveness even after its molecular configuration was altered by treatment with KCN or pronase (Katagiri, 1966a), ultraviolet radiation (Barbieri and Raisman, 1969; Wolf and Hedrick, 1971) or mercaptoethanol (Wolf and Hedrick, 1971). Katagiri thus considered that the specific reactivity or penetrating

capacity of the sperm surface which allowed it to respond to surrounding stimuli was the important initial factor, and not the presence or absence of complementary molecular configurations in the jelly. But, as pointed out by Barbieri and Raisman (1969), the results with jelly substitutes do not invalidate the hypothesis that the jelly is essential for fertilization under "normal" physiological conditions.

Aside from any possible mechanical role of the jelly, many studies indicate that chemical substances in the egg jelly of some amphibian species are involved in fertilization, and suggest that specific receptor sites are present in the gelatinous capsules which interact with reciprocal sites on sperm.

Many workers have noted that the jelly layers of amphibian eggs swell considerably in water solutions (Rugh, 1951; Tchou-Su and Wang, 1956; Katagiri, 1961; Elinson, 1970a, 1971, Pereda, 1970), and that the eggs become increasingly refractory to fertilization as a result. Shivers (1961b) suggested that the loss of antigenic components from the jelly or egg immediately after immersion in water accounted for the decrease in fertilizability. Agar diffusion analysis of the "egg water" from jellied uterine eggs revealed that the diffused substances were antigenically related to the jelly material as well as to substances from the egg (Shaver et al., 1962). That the released substances in

"egg water" were responsible for the loss of fertilizability of hydrated eggs, and that these substances could be involved in interaction with sperm, was suggested by several experiments in which egg water had a "capacitating" effect on sperm.

For example, jelly-less body cavity eggs of Rana pipiens, normally not fertilizable, could be activated by sperm which had prior contact with jellied eggs (Shivers, as cited by Shaver, 1966; Shivers and James, 1970b). Elinson (1970b, 1971) fertilized KCN-dejellied oocytes of R. pipiens and Bufo americanus in the presence of egg water, and Roberts (1971) demonstrated that the block to fertilization of antibody-treated eggs could be bypassed with sperm treated with egg water ("capacitated"). In all cases, the dejellied or antibody-blocked eggs were not fertilizable by normal sperm suspensions.

Furthermore, recent work by Oliphant et al. (1970) indicated that one macromolecular component in the outermost jelly layer of Xenopus laevis eggs was capable of capacitating sperm. In addition, Barbieri and co-workers (1969) described a "diffusible factor" from the jelly of Bufo arenarum which was essential for fertilization. This factor was believed to be produced by the oviducts and retained within the jelly; as it readily diffused in water, it could activate sperm before the sperm actually penetrated the jelly (Barbieri and Raisman, 1969). Also, these latter

workers gave evidence for a non-diffusible factor which remained near the egg surface and which could be important in egg activation by sperm. The diffusible and non-diffusible substances might be similar to the dialyzable and non-dialyzable fractions of egg water which together ensured a high frequency of fertilization of dejellied oocytes of R. pipiens and Bufo americanus (Elison, 1970b, 1971).

These studies taken together suggest that some chemical substances from the jelly may play an important role in "capacitating" sperm, or activating them in some way, perhaps by initiating the acrosomal reaction. It should be noted, however, that no sperm agglutinins has been obtained from frog egg jelly, as has been the case for sea urchins and other animals: the claim by Bernstein (1952) that egg water of Rana clamitans agglutinates homologous sperm has not been confirmed by other workers. The studies cited above emphasize that the jelly has effects on sperm which have a bearing on their fertilizing capacity, and the failure to demonstrate complementary interacting substances between egg and sperm--as measured by sperm agglutination by egg water--may be due to other factors (Shaver, 1966).

In view of the demonstrated importance of the jelly-coat in fertilization, considerable interest in characterizing the chemical composition of the jelly has ensued. Echinoderm jellies have been studied extensively, especially

as sea urchin species have been the classical material in which to investigate the role of gamete surface components. The chemical composition of sea urchin fertilizin, which comprises the jelly-coat (Tyler, 1940), consists mainly of mucopolysaccharides with a rather high sulfate content (Tyler and Fox, 1940; Vasseur, 1948; Minganti and Vasseur, 1959) which gives a strongly acidic character to the jelly solution. Isaka et al. (1970), however, indicate that some species are practically devoid of sulfate. Echinoderm jellies lack hexosamines (Vasseur, 1948; Minganti, 1955). The high content of sialic acid residues (Isaka et al., 1970; Hunt, 1970) is thought to have some role in the sperm agglutinating activity of jelly solutions (Isaka et al., 1969, 1970). (For reviews of chemical composition, see Tyler, 1949; Monroy, 1965; Minganti and Vasseur, 1959; Metz, 1967.)

In addition, detailed histochemical analyses of the egg capsules and oviducal secretions (Humphries and Hughes, 1959; Freeman, 1968; Pereda, 1970; Umpierre, 1971; Kelly et al., 1970) of several amphibian species have been made, and it is apparent that the several egg jelly layers are not homogeneous with respect to chemical composition (Minganti, 1955; Salthe, 1963; Humphries, 1966; Shivers and James, 1970a; Steinke and Benson, 1970). The amphibian egg jellies are composed of mucopolysaccharides and protein, but unlike echinoderm egg jellies, possess hexosamines and were thought

to lack sulfate (Minganti, 1955). But recent evidence indicates that sulfate is present and incorporated into acid mucopolysaccharide of J1 (innermost layer) in the newt (Humphries, 1966, 1969) and in R. pipiens (Pereda, 1970; Umpierre, 1971). Also, sulfated mucopolysaccharides have been detected in several of the five cytochemically distinguishable layers of R. pipiens egg jelly (Kelly et al., 1970; Shivers and James, 1970a; Steinke and Benson, 1970).

As with echinoderms, amphibian egg jellies contain a large amount of sialic acid. Humphries et al. (1968), Humphries and Workman (1966), and Humphries (1966) have studied the distribution of sialic acid in the five jelly layers of several species of newts and present evidence that J2 and J4, and possibly J5 contain significant amounts. Lee (1967) reports also the presence of large amounts (13-20% of the dry weight of the jelly) of sialic acid in a hydrolyzed product of the jelly of R. pipiens eggs, and Bolgnani et al. detect high sialic acid contents in the egg jellies of R. latestei and Bufo vulgaris. Pereda (1970) confirms its presence in the three morphologically distinct jelly layers of R. pipiens, with the highest concentration in the middle layer, a result also confirmed in Xenopus laevis (Freeman, 1968). Several workers have suggested that sialic acid residues play an important role in penetration of sperm through amphibian jelly (Humphries, 1966; Humphries et al., 1968) and through the zona pellucida of mammalian

eggs (Soupart and Noyes, 1964; Soupart and Clewe, 1965). Thus, the distribution of these molecules may have some importance in gamete interaction.

Analyses of the composition of each of the 5-6 histochemically differentiated layers of the amphibian jelly-coat have not, however, resolved the role of specific molecules in fertilization. The possible function of sialic acids in sperm penetration has been noted. Steinke and Benson (1970) indicate that the arrangement of the different carbohydrate complexes in the several layers may be involved in progressively modifying the sperm to bring about complete sperm capacitation. These workers suggest, as do the analyses of Shivers (1965), that the outermost jelly layer (J5) in R. pipiens may be the site of specific receptors for sperm attachment, a conclusion supported by Humphries (1966) based on the presence of sialic acid in J5 in Triturus viridescens. Indeed, the relatively large amount of carbohydrate in the form of acidic and neutral mucopolysaccharides argues for its strong involvement, especially as both sulfate and sialic acids are associated with carbohydrate and have been implicated in reactions with sperm.

The possible role of protein components of jelly has also been considered. In the echinoderms, Vasseur (1948) has suggested that while sulfated carbohydrate may have agglutinating action on sperm, the protein moieties may be involved in the species specificity of fertilization.

But Isaka et al. (1970) have noted striking similarities in the relative amounts of various amino acids in several sea urchin species, suggesting that the polypeptide chain may form the backbone of the egg jellies; this argues against the role of protein in species specificity in fertilization, unless the distribution of such moieties was different. In amphibian species, Humphries (1966) and Shivers and James (1970a) demonstrated that the outer jelly layer was rich in protein. Since the outermost jelly layer is the first which confronts the sperm, it is natural to delegate to it primary importance in gamete interaction.

Gusseck and Hedrick (1971) have recently formulated an interesting molecular hypothesis for fertilization involving sulfhydryl and disulfide groups. These workers noted, as did Katagiri (1963), that disulfide linkages were involved in maintaining the structural integrity of the jelly-coat. Therefore, the reduction of or interchange with sulfhydryl groups of sperm could be a possible mechanism for sperm penetration through the jelly-coat layers; the sperm could possess free sulfhydryl groups on its external surface to promote interchange, or it could contain a "disulfide reductase" (Gusseck and Hedrick, 1971). According to these workers, modification of disulfide bonds in the protein moieties of the jelly could thereby act as a "gate" mechanism for sperm penetration. There is some evidence, however, that the integrity of disulfide bonds of jelly molecules is

not essential to the biological activity of the jelly in sperm capacitation (Katagiri, 1966a; Wolf and Hedrick, 1971).

Immunological studies in conjunction with the biochemical analyses have made possible the further understanding of the complex molecular species in the jelly and their possible role in fertilization. The nature of the antigenic components of amphibian jellies has been demonstrated by several workers (for reviews, see Shaver, 1966; Metz, 1967). Agar diffusion analyses have revealed that the jelly is tissue-specific (Shaver et al., 1962). Shivers (1965) found a minimum of six different antigens in the jelly of R. pipiens eggs, with considerable overlap in antigens from one jelly layer to the next adjacent layer (Shivers and James, 1970a). Furthermore, Barch and Shaver (1963) have demonstrated that different antigenic components (as well as some common components) are present in oviducal extracts from upper, middle, and lower segments of the oviduct; Pereda (1970) suggests that these antigenic properties of the different levels are linked to glycoproteins. Presumably, these antigens represent antigens (receptor sites) in the jelly, since anti-jelly serum produced the same precipitin band patterns in agar diffusion as did anti-oviduct serum.

The species-specificity of the fertilization reaction has been studied by experiments directed toward determining

the interspecific relationships of antigenic components of oviducal and jelly materials (for reviews, see Shaver, 1966; Metz, 1967). Agar diffusion analysis by Shivers (1962, 1965) of several amphibian jellies suggested that the species-specific antigens were localized in the outer two layers of the jelly, and that shared components were present in the inner layer. Recent evidence by Shaver et al. (1970) indicated that the regional distribution of antigens may be more complex and that the shared components may also be present in the outer layer.

Katagiri (1967), taking a somewhat different approach to the role of jelly components in interspecies fertilization, claimed that the antigenic components of egg jellies as determined by precipitation analysis did not reflect the existence of molecular configurations which performed an essential role in fertilization. He presented evidence that toad sperm acquired fertilizing capacity in response to a variety of heterologous jellies and even PVP, none of which formed immunologically detectable precipitin bands with antisera against the jelly of the homologous species.

With the above evidence that chemical and antigenic differences exist in the several layers of amphibian jelly-coats, several investigators have attempted to determine the relative importance of each of the layers in the fertilizability of eggs. One approach has been to remove eggs from various oviducal levels such that one or more jelly

layers surrounds the egg, and to test the fertilizability of such eggs. In this way, Kambara (1953), Glick and Shaver (1963), and Elinson (1970a, 1971) have shown that eggs with only the inner jelly layer (J1) seldom fertilized. As the inner layer was thought to contain shared antigenic components between species (Shivers, 1962; 1965), it may be that these components played little role in rendering the eggs fertilizable. Eggs with J1 and J2 showed increased fertilizability but did not approach those with all three morphologically distinct layers in this respect. Consequently, the increase in fertilizability of eggs taken from middle and lower oviducal segments could be correlated with the deposition around the egg of molecular configurations which promoted the adhesion and penetration of sperm.

A similar conclusion was reached by selectively reordering the jelly layers around the egg by surgical rearrangement of the oviduct (Elinson, 1970a, 1971). This worker found a high fertilizability of R. pipiens eggs with just J3 (but also with J1 and J3), indicating that the outer layer was sufficient for successful fertilization. This result was also noted in Bufo bufo eggs with only the outer jelly layer (Katagiri, 1965). These observations argue for the role of species-specific components in fertilization (Shivers, 1962, 1965).

Indeed, the evidence presented so far--based on a variety of approaches--has emphasized the importance of the

jelly-coat in mediating the reaction between egg and sperm. Studies on the mechanisms of fertilization at the molecular level have, in addition, been enhanced by the use of specific inhibitors such as antisera (Metz, 1961). Antisera to macromolecular surface components can be readily produced upon injection of cell extracts or homogenates into an appropriate foreign species. Since the reaction between antibodies and antigens is characterized by a high degree of specificity, the action of antisera on cells constitutes a valuable tool for the study of fertilization.

If antigenic sites in the jelly are necessary for the primary interaction with sperm, then presumably the blocking or neutralization of such sites with antibody would prevent fertilization. Accordingly, Rana pipiens eggs treated with rabbit anti-jelly sera showed a significant decrease in fertilizability (Shaver and Barch, 1960, Shivers, 1961a, 1961b). Furthermore, Shivers and Metz (1962) rendered the globulin fraction of anti-jelly sera non-precipitating (univalent) by papain digestion, and also found that R. pipiens eggs treated with this preparation showed decreased fertilizability. This result with univalent antibody fragments was thought to indicate an actual blocking of egg jelly sites that performed some essential interaction with sperm at fertilization. Furthermore, this finding supported the assumption that the multivalent antibody used by Shaver and Barch (1960) acted by blocking specific sites and not by

creating a mechanical barrier to sperm penetration by cross-linking neighboring jelly antigens, thereby producing a precipitation lattice in the jelly. However, studies of a similar nature in echinoderms, and amphibians (Shaver and Barch, unpublished) suggested that this interpretation was not entirely valid.

Multivalent antibodies against egg jelly and egg homogenates have been shown to cause visible precipitation of the jelly in frogs (Shaver and Barch, 1960; Katagiri, 1967), and in the sea urchin (Perlmann, 1956, 1957; Baxandall et al., 1964a, 1964b; Baxandall, 1966; Metz and Thompson, 1967; Metz et al., 1968), and also wrinkling of the egg surface in the sea urchin (Perlmann, 1955; Metz and Thompson, 1967). Furthermore, the antisera which produced these morphological effects in the sea urchin also caused decreased fertilizability of treated eggs (Metz and Thompson, 1967). Univalent antibody fragments obtained from such antisera, however, did not produce precipitation of the jelly of treated Lytechinus and Arbacia eggs (Graziano and Metz, 1967; Metz and Thompson, 1967). Moreover, sea urchin eggs treated with univalent fragments of anti-jelly antibody (Headrick and Metz, 1967) and with univalent fragments of anti-egg homogenate antibody (Graziano and Metz, 1967) did not show a decrease in fertilizability, in contrast to the results obtained with similar treatment of frog eggs (Shivers and Metz, 1962).

Thus, the results with echinoderm species suggested that essential jelly receptor sites were not blocked by antibody. Either the rabbit antibodies were not complementary to critical antigenic components involved in the reaction with sperm, or essential sites were not accessible to univalent fragments, or simple blocking of sites in the jelly or on the egg surface was not sufficient to inhibit fertilization (Graziano and Metz, 1967). This latter theory--that simple blocking of sites was not sufficient--was supported by the results of the antiglobulin (Coombs') test (Coombs et al., 1945): the addition of goat anti-rabbit gamma globulin serum to Arbacia eggs pretreated with univalent fragments of anti-jelly antibody caused a reduction in the fertilizability of these eggs (Headrick and Metz, 1967) with concomitant jelly precipitation. This result indicated that a physical barrier to fertilization was created by linking neighboring univalent fragments already bound to antigens in the jelly, and supported the theory that the inhibitory effect of multivalent antibodies could be explained solely on the basis of their structural effects on jelly material.

Subsequently, ultrastructural studies confirmed that a morphological basis existed for the inhibition of fertilization of echinoderm eggs treated with multivalent antisera or secondarily with multivalent antiglobulin sera (Metz et al., 1967, 1968). Electron micrographs of dejellied eggs treated

with multivalent anti-egg homogenate and anti-jelly antibodies showed a surface "gel" layer which was not present in univalent antibody fragment-treated eggs. Moreover, this "gel" layer was observed on univalent fragment-treated eggs after exposure to sheep antiglobulin serum. It was concluded that the "gel" layer was a true antigen-antibody precipitate formed by a cross-linking of antigenic sites by antibody. Since the antibody treatments which produced the surface precipitation were the same as those that inhibited fertilization, it was likely that a mechanical barrier to sperm-egg interaction was created. Protease treatment, in addition, partially removed the opaque surface layer and correlated well with the recovered fertilizability of antibody-treated eggs (Graziano and Metz, 1967; Metz et al., 1968).

Clearly, therefore, the interpretation of the effects of both univalent antibody fragments and multivalent antibody on echinoderm eggs was in conflict with that initially formulated for the amphibian system. It thus seemed desirable to repeat the original experiment employing univalent antibody fragments on R. pipiens eggs.

The present study was done with the purpose of investigating further the role of jelly receptor sites of the amphibian jelly-coat in fertilization. The objective was to determine the effect of nonprecipitating, univalent antibody fragments of anti-jelly serum on the fertilizability of

Rana pipiens eggs. Such an experiment would reveal whether there are critical jelly receptor sites which can be blocked by antibody and which are necessary for sperm-egg interaction in fertilization. In addition, such a study could clarify the nature of the previously demonstrated inhibitory effect of multivalent antibody on the fertilizability of amphibian eggs.

MATERIALS AND METHODS

Preparation of Antigens

Adult Rana pipiens were obtained from commercial dealers in Wisconsin and Vermont. Tissue antigens prepared as described below were used for immunization of rabbits, for absorption of antisera, and in Ouchterlony agar diffusion tests.

Jelly antigen was prepared from oviducts as follows. Whole oviducts were removed from ovulating females and eggs were stripped from the oviducts. The macerated oviducal tissue was immersed in a large amount of distilled water and mechanically agitated for several hours to insure that the jelly secretion diffused into the medium. The mixture was filtered through glass wool to separate the jelly from any remaining oviducal tissue. The filtrate was lyophilized in a Virtis freeze-mobile and the lyophilate was stored in a dessicator for future use. Jelly antigen used in these experiments was reconstituted by dissolving 5 mg. of the lyophilate per 1 ml. of full strength Holtfreter's solution, and the thick mixture was mechanically agitated for about 30 minutes.

Ovary and kidney antigens were prepared as follows. The tissues were removed from the adult frog and weighed;

1 gm. of tissue per 10 ml. of full strength Holtfreter's solution was homogenized in a glass homogenizer. The homogenate was centrifuged at low speed to remove debris, and the supernatant was used as antigen.

Sperm antigen was prepared by macerating whole testes obtained from pithed frogs in one-tenth percent full strength Holtfreter's solution, 1 testis per 7-8 ml.

Preparation of Antisera

Antisera were prepared in Giant German Checker rabbits. Blood for control serum was withdrawn from the marginal ear veins of the animals prior to immunization. In no case was a cross-reaction observed between rabbit control serum and any frog tissues tested.

Rabbits were immunized employing Freund's adjuvant (Difco Labs., Detroit, Mich.). Each rabbit was injected via the subscapular route with 2 ml. of emulsion prepared by mixing equal volumes of complete Freund's adjuvant mixture and antigen. One week later, a second injection was given, this time using equal volumes of incomplete adjuvant mixture and antigen; four weeks later, this injection was repeated. One week after this third injection, blood was taken from the marginal ear vein and tested, and bleedings were continued every other week thereafter. Booster injections with incomplete adjuvant were given every six weeks during the entire period that serum was obtained from the rabbits.

Sera obtained from successive bleedings were tested separately for the presence of antibodies on agar diffusion plates. At least two rabbits were immunized against the same antigen, and their sera were pooled after testing had indicated antibody activity. Small amounts of Merthiolate (Lilly) were added as a preservative.

Sheep anti-rabbit gamma globulin serum was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Control nonimmune sheep serum was kindly supplied by Dr. E. Sanders. Protein concentration was determined at 280 mμ with a Coleman UV spectrophotometer. As the antisera were of the same approximate concentrations, they were diluted in buffered physiological rabbit saline (1:5) to a final concentration of 40 mg. protein per ml.

Preparation of Ouchterlony Agar Diffusion Plates

Ouchterlony plates were prepared according to the method of Ouchterlony (1949, 1958) as modified by Shaver (1961). A 1% solution of "Ionagar" (Colab Laboratories Inc., Chicago Heights, Ill.) in rabbit saline was heated and applied to glass Petri dishes while still hot. After the agar was completely cooled, various arrangements of wells were cut and sealed with a drop of hot agar. Plates were stored at 4°C until used.

Tests on sera were done in the following manner. Each antiserum well was first preloaded, allowed to diffuse, and

refilled, at which time the antigen was added. Usually 5-6 applications of antibody and 3-4 applications of antigen were necessary for maximal precipitation reaction. When the plate was fully developed, usually after 10 days at room temperature (18-20°C), a final drawing was made of all lines, and the plate photographed for a permanent record. The details for the inhibition of precipitation test in agar diffusion vary from the procedure above, and will be described in the Results.

In some cases, lines of precipitation were better visualized by staining the developed plates with Amidoschwarz. The stain was washed over the plates for about 30 seconds, and excess stain was removed by repeated rinses with a methanol-acetic acid-water mixture (45-10-45).

Absorption of Antisera

Four types of rabbit sera were used: sera against jelly, ovary, kidney, and control nonimmune serum. Whole antisera from the original pooled batches were absorbed. Absorption of each of the sera was deemed desirable to make them as tissue-specific as possible, and to eliminate any antibodies which might react with sperm and ovarian antigens. In most cases, the nonabsorbed antisera reacted with all the heterologous antigens tested (except for the control serum). The lines of identity between different antigens with each antiserum indicated that it might be possible to absorb each with

one heterologous antigen and thereby remove most heterologous components, leaving some but not all of the tissue-specific components. Preliminary absorptions and testing indicated this was feasible.

Absorption of antisera was accomplished by mixing equal volumes of antiserum with antigen in glass tubes. The mixtures were shaken to ensure even mixing and were refrigerated for a minimum of 24 hours. The absorption mixtures were centrifuged at 10,000 x g for 20 minutes at 4°C to remove antigen-antibody precipitates. The supernatant antisera, now one-half the concentration of the untreated whole antisera, were brought to original volume by concentration with a 20% solution (Goldman, 1968) of polyvinylpyrrollidone (PVP-360; Sigma Chemical Co., St. Louis, Mo.), as modified from a method by Kohn (1959).

All reconcentrated, absorbed sera were then tested on Ouchterlony plates in the usual way. Unfortunately, it was necessary to discard the kidney-absorbed anti-kidney serum, for repeated manipulations resulted in extensive bacterial contamination.

As a control for the absorbed antisera, antigen preparations were treated in a manner similar to the antisera. Equal volumes of each of the antigen preparations (jelly, ovary, kidney, sperm) and full strength Holtfreter's solution were mixed, shaken, refrigerated for 24 hours, and reconcentrated. Since little material remained after one

precipitation with $(NH_4)_2SO_4$, the antigen control solutions were not treated any farther.

Preparation of Univalent Antibody Fragments

Univalent antibody fragments (3.5S) were prepared by the papain-digestion procedure of Porter (1958, 1959). The globulin fraction of whole sera (absorbed and nonabsorbed) was obtained as follows. Each serum was first precipitated in 33⅓% $(NH_4)_2SO_4$ at 4°C overnight; the suspension was centrifuged in an IEC International Centrifuge at 10,000 x g for 20 minutes at 4°C, the precipitate washed in fresh $(NH_4)_2SO_4$ solution, and recentrifuged. The resulting precipitate was dissolved in distilled water at 4°C and dialyzed in the cold against a .65% saline solution for three days with six changes of the dialysate. All subsequent centrifugations and dialyses were carried out under the same conditions except where noted. Further purification was carried out by precipitation with 18% Na_2SO_4 for 6 hours at room temperature (Kekwick, 1940). This precipitate was centrifuged at room temperature, washed, dissolved in distilled water, and dialyzed as above. Protein determinations at 280 mμ were made of the globulin solutions.

The digestion mixture of Porter (1958, 1959) contained the following solution buffered at pH 7.0: 0.1 M KH_2PO_4, 0.002 M EDTA, 0.01 M cysteine. Papain (2x crystallized, Worthington Biochemical Co., Freehold, N. J.) was used at a

concentration of 1 mg. papain/100 mg. globulin. The complete digestion mixture--buffer, papain, globulin--was incubated in a water bath at 37°C for 20 hours. As indicated by Porter (1959), complete digestion occurs at 16 hours or less. In every digestion, control samples of undigested globulin from the parent stock were prepared by subjecting them to the complete procedure except that the buffer was substituted for the papain. To stop digestion, 0.2 M iodoacetamide was added to each sample and the digestion mixtures were dialyzed as above to remove cysteine and EDTA.

Digested and undigested globulins were tested on Ouchterlony plates, based on the inhibition of precipitation method described by Shivers and Metz (1962) and detailed here in the Results. No further dilution of the globulin preparations was made.

Experimental Procedure for the Treatment of Eggs

Mature eggs were obtained from adult R. pipiens females by artificially inducing ovulation (Smith et al., 1968) by intraperitoneal injection of a pituitary gland (obtained from adult frog and frozen until use) in a small volume of one-tenth percent full strength Holtfreter's solution, plus injection of 2.5-5 mg. progesterone in corn oil (Wright and Flathers, 1961) in the dorsal lymph sac. Generally, 36-48 hours were required to obtain ovulated uterine eggs.

The first 10-20 eggs stripped from a female were always discarded since these generally have reduced fertilizability (Rugh and Exner, 1940). Thirty to fifty eggs were extruded onto glass microscope slides supported by Syracuse dishes. The eggs were then covered completely by various antibody preparations. After 5 minutes, the solutions were washed off the eggs by thorough pipetting with one-tenth percent full strength Holtfreter's solution. The eggs were then inseminated with a normal sperm suspension (one testis macerated in 10 ml. of one-tenth percent full strength Holtfreter's solution), and after 10 minutes exposure to sperm, excess sperm was decanted and the eggs placed in finger bowls containing aerated tap water. Egg cultures were maintained at room temperature (18-20^{o}C). Three to five hours later, the number of cleaving eggs and the total number of eggs were counted and recorded. Generally the eggs were at 8-32 cell stage. Cleavage is considered an accurate end-point of these experiments rather than rotation and the emission of the second polar body (Shaver and Barch, 1966).

Statistical Analyses

Samples of eggs from one female frog were always treated with all the preparations in any series of treatments. Eggs from a number of females were used to replicate the treatments. Furthermore, only those females whose eggs showed high normal fertilizability (80-100%) upon preliminary testing

were used. The percentage cleavages were transformed into arc sin equivalents and significant differences were established by an analysis of variance for each experiment. Significant differences between means at the 5% level were calculated by the Q test and in one case with the Scheffé method (Snedecor and Cochran, 1967).

There is great variability between eggs from different females. Therefore, by using the eggs of one female for all of a given series of treatments, the variability due to animals was accounted for in the type of statistical analysis employed here.

RESULTS

Analysis of Sera

Agar Diffusion Analysis of Absorbed and Nonabsorbed Antisera

The purpose of absorbing the various antisera was to remove antibodies to certain cross-reacting antigens of jelly, ovary, kidney, or sperm extracts, and thus to make each antiserum as tissue-specific as possible. Absorption with homologous antigen was the control for these absorptions. In no case was a reaction seen with absorbed or nonabsorbed control nonimmune sera.

The results of Ouchterlony agar diffusion analyses can be summarized as follows, based on drawings of representative interactions (Plate I). All drawings were made from negatives obtained from photographing the fully-developed plates, as it is difficult to visualize distinct lines of precipitation in photographs.

1). Anti-jelly serum: In Figure 1, at least six precipitin bands are present in the reaction between anti-jelly serum (AJ) and jelly antigen (J). In addition, at least one component of AJ is identical with both ovary antigen (O) and sperm antigen (Sp), as indicated by the continuous curved band. As shown in Figure 2, absorption of AJ with ovary

(AJ+O) removes all antibodies from the serum which also react with O and Sp. Moreover, as this plate was stained with Amidoschwarz, the jelly-specific bands are more pronounced. In Figure 3, it is apparent that absorption with homologous antigen (J) did not completely remove all antibodies; however, no visible reaction was observed until the plate was stained.

2). Anti-ovary serum: Figure 4 shows at least five precipitin bands between the anti-ovary serum (AO) and ovary antigen (O). At least one component of AO is identical with both J and Sp. Figure 5 represents the reaction between the sperm-absorbed anti-ovary serum (AO+Sp) and these antigens. Even after repeated absorption, it was not possible to remove the antibodies in the serum which reacted with J. The single precipitin band was only visualized after staining. No reaction with Sp was demonstrated, indicating that antibodies to this antigen were essentially removed. The homologous absorption with ovary antigen (AO+O) removed all anti-ovary antibodies (Figure 6), but the single band with jelly persisted (visualized by staining only). Therefore, with both AJ and AO, it was very difficult to remove antibodies to some jelly components.

3). Anti-kidney serum: In Figure 7, several precipitin bands are seen in the reaction between anti-kidney serum (AK) and kidney antigen (K). Several of these components are identical to those in the reaction between AK and J and O,

Drawings of agar diffusion plates showing immunoprecipitation bands obtained when testing nonabsorbed and absorbed anti-jelly, anti-ovary, and anti-kidney sera with various tissue antigens.

Figure 1
AJ--anti-jelly serum
Sp--sperm antigen
J--jelly antigen
O--ovary antigen

Figure 2
AJ+O--anti-jelly serum absorbed with ovary
Sp--sperm antigen
J--jelly antigen
O--ovary antigen
(Plate stained with Amidoschwarz)

Figure 3
AJ+J--anti-jelly serum absorbed with jelly
Sp--sperm antigen
J--jelly antigen
O--ovary antigen
(Plate stained with Amidoschwarz)

Figure 4
AO--anti-ovary serum
Sp--sperm antigen
O--ovary antigen
J--jelly antigen

Figure 5
AO+Sp--anti-ovary serum absorbed with sperm
Sp--sperm antigen
O--ovary antigen
J--jelly antigen
(Plate stained with Amidoschwarz)

Figure 6
AO+O--anti-ovary serum absorbed with ovary
Sp--sperm antigen
O--ovary antigen
J--jelly antigen
(Plate stained with Amidoschwarz)

Figure 7
AK--anti-kidney serum
O--ovary antigen
K--kidney antigen
J--jelly antigen
Sp--sperm antigen

Figure 8
AK+O--anti-kidney serum absorbed with ovary
O--ovary antigen
K--kidney antigen
J--jelly antigen
Sp--sperm antigen
(Plate stained with Amidoschwarz)

as indicated by the continuous lines between the antigen wells. No reaction was seen with Sp. Results of absorption of AK with ovary (AK+O) are seen in Figure 8 (stained plate). At least three kidney-specific antigens remained after absorption. Moreover, it appears that some antibodies against J and O are present, even after a repeated absorption, as the precipitin bands curve suspiciously between these wells and the antiserum. But most, if not all, of the several cross-reacting components to the heterologous antigens are removed.

Agar Diffusion Analysis of Undigested Globulin Preparations and Sheep Serum

Separate agar diffusion analyses of each of the three antisera types were carried out to demonstrate that common tissue-specific antibodies were retained between the non-absorbed and heterologously-absorbed globulin preparations. In all cases, the undigested (multivalent) globulin preparations were used, after having been incubated for the same period of time as the preparations digested with papain.

The results are presented in Plate II. In each agar plate, only the homologous antigen was used. All plates were stained with Amidoschwarz in this series. The figures in Plate II can be summarized as follows:

1). Anti-jelly globulin preparations (Figure 9): A comparison of the precipitin bands between the jelly antigen (J) and the two wells with undigested, nonabsorbed anti-jelly globulin (UJ) and the undigested, ovary-absorbed

anti-jelly globulin (UJ+O) indicates reactions of identity, except in one case where a line appeared opposite UJ that did not appear with UJ+O. This means that all the antibodies against jelly which remained in UJ+O were also present in UJ but that absorption with heterologous antigen also removed some antibodies against jelly in UJ+O.

Also, UJ+J gives one faint band with J, and this band shows a reaction of identity with both UJ and UJ+O. Therefore, as confirmed previously, absorption was not complete as desired (see Plate I, Figure 3), although the antibody titer was reduced considerably.

2). Anti-ovary globulin preparations (Figure 10): All precipitin bands present between the ovary antigen and the two wells with undigested, nonabsorbed anti-ovary globulin (UO) and the undigested, sperm-absorbed anti-ovary globulin (UO+Sp) represent reactions of identity. In this case, absorption apparently did not remove any tissue-specific antibodies from the serum. Homologous absorption of the anti-ovary globulin (UO+O) did not completely remove all antibodies to ovary, as indicated by the single precipitin band opposite UO+O well (which was not seen in Plate I, Figure 6). Therefore, antibodies to at least one common ovary component were retained.

3). Anti-kidney globulin preparations (Figure 11): Multiple precipitin bands are visible in the reaction between undigested, nonabsorbed anti-kidney globulin (UK)

and kidney antigen (K). Many of these bands are lost in the reaction between ovary-absorbed anti-kidney globulin (UK+O) and the antigen, indicating that the titer of this serum was considerably reduced. However, the four precipitin bands opposite UK+O show reactions of identity with those opposite UK, indicating that the kidney-specific antibodies retained in UK+O are also present in UK.

In reactions not shown, none of the undigested, absorbed or nonabsorbed control globulins showed any reaction in agar diffusion with jelly antigen.

Also, an analogous series of plates was prepared as above with the respective digested globulin preparations. In no case was there a positive reaction, confirming the lack of precipitable multivalent antibodies in these preparations.

4). Sheep anti-rabbit gamma globulin serum (Figure 12): The reactions between this immune sheep serum (IS) and two digested (DJ, DC) and undigested (UJ, UC) globulin preparations are shown in an agar diffusion plate in Figure 12. Numerous precipitin bands are seen between the IS well and UJ and UC. These bands, which extend toward the DJ and DC wells, indicate that the undigested preparations contain antigenic components not shared by DJ and UC. Such an analysis, however, confirms that it is feasible for the anti-globulin serum antibodies to complex with univalent antibody fragments as well as with whole globulin (Amiraian and Leikhim, 1961). As expected, no reaction was seen between

Drawings of agar diffusion plates showing immunoprecipitation bands obtained when testing undigested globulin preparations of anti-jelly, anti-ovary, and anti-kidney sera with homologous antigens, and when testing sheep anti-rabbit gamma globulin serum with papain-digested and undigested globulins. Plates stained with Amidoschwarz.

Figure 9 UJ+O--undigested anti-jelly globulin absorbed with ovary
UJ--undigested anti-jelly globulin
UJ+J--undigested anti-jelly globulin absorbed with jelly
J--jelly antigen

Figure 10 UO+Sp--undigested anti-ovary globulin absorbed with sperm
UO--undigested anti-ovary globulin
UO+O--undigested anti-ovary globulin absorbed with ovary
O--ovary antigen

Figure 11 UK+O--undigested anti-kidney globulin absorbed with ovary
UK--undigested anti-kidney globulin
K--kidney antigen

Figure 12 IS--sheep anti-rabbit gamma globulin serum
DC--papain-digested control globulin
UC--undigested control globulin
DJ--papain-digested anti-jelly globulin
UJ--undigested anti-jelly globulin

PLATE II

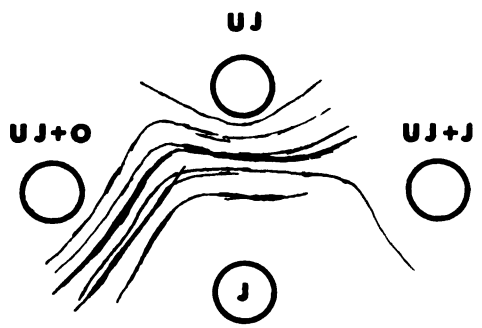


Figure 9

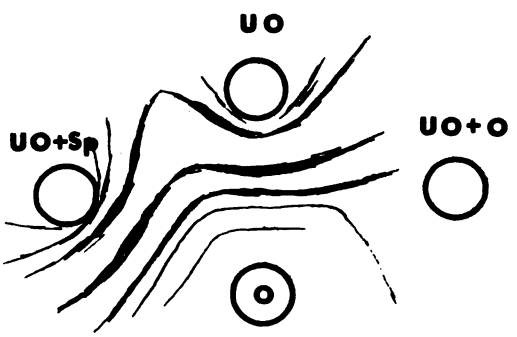


Figure 10

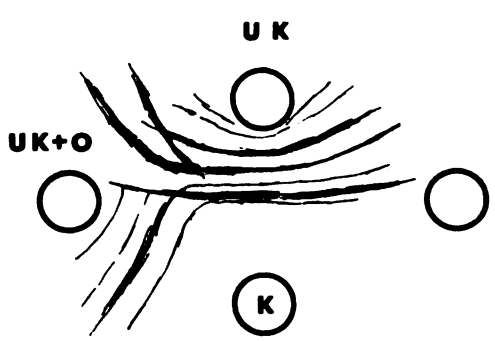


Figure 11

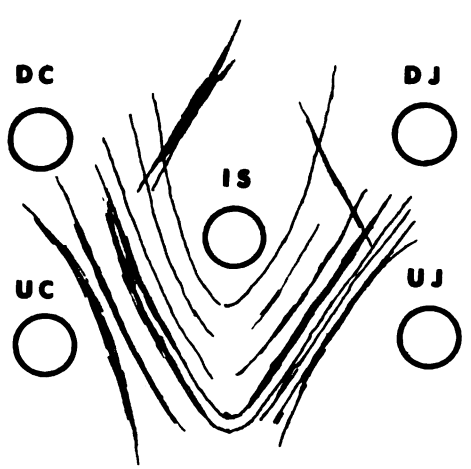


Figure 12

the same globulin preparations and control sheep serum. In addition, neither immune nor control sheep serum demonstrated the presence of antibodies to jelly or ovary antigens in agar diffusion.

Agar Diffusion Analysis of Univalent Antibody Fragments in the Inhibition of Precipitation Test

The activity of non-precipitating, univalent antibody fragments was effectively demonstrated in agar diffusion as described below. Inhibition of precipitation of parent undigested antibody with homologous antigen by univalent antibody fragments was shown using the special design of the agar plate (Shivers and Metz, 1962) as seen in Plate III. Two series of applications of antibody were made as follows:

1). Wells 1 and 2 (above antigen trough) were first filled with a digested immune globulin preparation, e.g., univalent anti-jelly fragments. At the same time, wells 3 and 4 (below antigen trough) received digested control globulin. Five applications of each of the above were made within a 72 hour period, after which time the antibodies presumably had completely diffused into the agar. No antigen was added in the center trough during this time.

2). Wells 1 and 3 were then filled with an undigested immune globulin preparation, e.g., multivalent anti-jelly globulin. Simultaneously, wells 2 and 4 received undigested control globulin. Before drying, a second application of these preparations was made. The center trough was filled with antigen at this time.

The four serum types used in egg treatments, e.g., anti-jelly, anti-ovary, anti-kidney, and control serum, were examined separately with their homologous antigens only (jelly antigen in the case of control serum) in this way. Homologously and heterologously absorbed preparations were analyzed in addition to the nonabsorbed preparations. The results from Plate III can be summarized as follows:

1). As seen in Figures 13-18, no precipitin bands appear between wells 2 and 4 and the antigens. In all cases, therefore, the lack of reaction between well 2 and the antigen indicates that the digested immune globulins (DJ, DJ+O, DO, DO+Sp, DK, DK+O) do not contain a sufficient amount of multivalent antibody to cause visible precipitation. As expected, the lack of reaction opposite well 4 confirms the absence of antibodies in the control serum preparations.

2). As predicted (Well 3, Figures 13-18), specific precipitin bands are present in the reaction between the antigen and the undigested immune globulins (UJ, UJ+O, UO, UO+Sp, UK, UK+O) in the presence of undigested control globulin. With both absorbed and nonabsorbed globulins, e.g., AJ and AJ+O (Figures 13 and 14), multiple bands formed opposite well 3, a good indication that some tissue specific antibodies to jelly were retained in the absorbed preparation. With the AK+O, however, one kidney-specific band did not appear opposite well 3; this is not surprising in view of the number of components lost in absorption of this serum (Plate I, Figure 8).

3). Furthermore, faint bands appear opposite well 1 (Figures 13, 14, 15 and 17). These bands were always late-appearing and were rather diffuse. Clearly, therefore, pretreatment of well 1 with digested immune globulin caused considerable inhibition of antigen-antibody precipitation of the multivalent immune globulin also in this well. Presumably, then, the univalent, non-precipitating antibody fragments reacted with the diffusing antigen and thereby protected the reactive sites of these antigens from combination with multivalent, precipitating antibody. The failure to get complete inhibition of precipitation (in well 1) was also noted by Shivers and Metz (1962), and is probably due to insufficient titer of univalent fragments, such that all reactive sites on the antigen are not precomplexed with them before addition of multivalent antibody.

In similar agar diffusion tests not shown here, none of the control globulin preparations showed any precipitation whatsoever with jelly antigen. None of the homologously-absorbed globulins (AJ+J, AO+O) showed visible precipitation in this test, either, suggesting complete absorption, but more probably a result of reduced titer of these preparations (Plate I, Figures 3 and 6).

PLATE III

Drawings of agar diffusion plates showing immunoprecipitation bands obtained when testing papain-digested and undigested globulin preparations of nonabsorbed and absorbed sera (inhibition of precipitation test).

Figure 13 Well 1--papain-digested anti-jelly globulin followed by undigested anti-jelly globulin
Well 2--papain-digested anti-jelly globulin followed by undigested control globulin
Well 3--papain-digested control globulin followed by undigested anti-jelly globulin
Well 4--papain-digested control globulin followed by undigested control globulin

Figure 14 Well 1--papain-digested ovary-absorbed anti-jelly globulin followed by undigested ovary-absorbed anti-jelly globulin
Well 2--papain-digested ovary-absorbed anti-jelly globulin followed by undigested control globulin
Well 3--papain-digested control globulin followed by undigested ovary-absorbed anti-jelly globulin
Well 4--papain-digested control globulin followed by undigested control globulin

Figure 15 Well 1--papain-digested anti-ovary globulin followed by undigested anti-ovary globulin
Well 2--papain-digested anti-ovary globulin followed by undigested control globulin
Well 3--papain-digested control globulin followed by undigested anti-ovary globulin
Well 4--papain-digested control globulin followed by undigested control globulin

Figure 16 Well 1--papain-digested sperm-absorbed anti-ovary globulin followed by undigested sperm-absorbed anti-ovary globulin
Well 2--papain-digested sperm-absorbed anti-ovary globulin followed by undigested control globulin
Well 3--papain-digested control globulin followed by undigested sperm-absorbed anti-ovary globulin
Well 4--papain-digested control globulin followed by undigested control globulin

Figure 17 Well 1--papain-digested anti-kidney globulin followed by undigested anti-kidney globulin
Well 2--papain-digested anti-kidney globulin followed by undigested control globulin
Well 3--papain-digested control globulin followed by undigested anti-kidney globulin
Well 4--papain-digested control globulin followed by undigested control globulin

Figure 18 Well 1--papain-digested ovary-absorbed anti-kidney globulin followed by undigested ovary-absorbed anti-kidney globulin
Well 2--papain-digested ovary-absorbed anti-kidney globulin followed by undigested control globulin
Well 3--papain-digested control globulin followed by undigested ovary absorbed anti-kidney globulin
Well 4--papain-digested control globulin followed by undigested control globulin

PLATE III

Anti-jelly

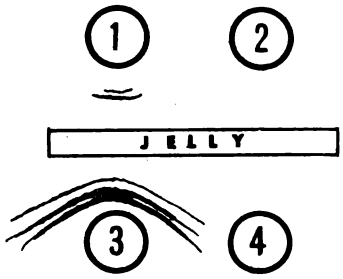


Figure 13

Anti-jelly abs. ova.

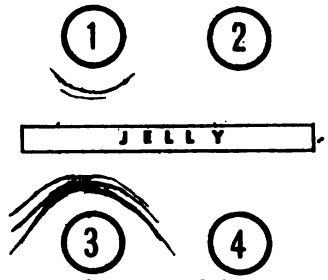


Figure 14

Anti-ovary

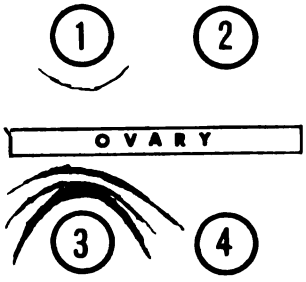


Figure 15

Anti-ovary abs. sp.

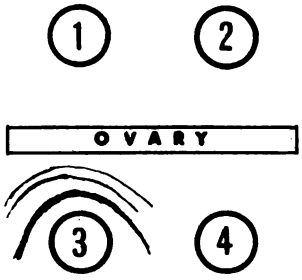


Figure 16

Anti-kidney

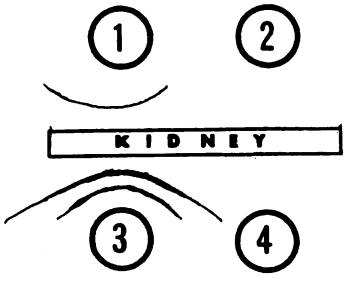


Figure 17

Anti-kidney abs. ova.

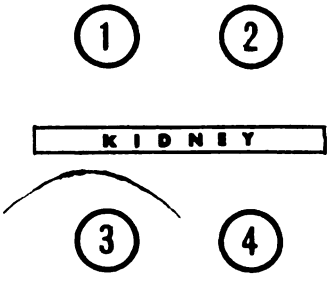


Figure 18

Effect of Antibodies on the Fertilizability of Rana Pipiens Eggs

Effect of Univalent Antibody Fragments and Multivalent Antibody on Fertilizability

The results obtained from inseminating eggs that had been previously treated with papain-digested (univalent) and undigested (multivalent) antibodies are presented in Text Figure 1. All globulin preparations were used full strength as prepared. The eggs of 15 females were used and the results expressed as the mean of the arc sin equivalents of cleavage percentages. (For individual arc sin equivalents, see Appendix, Tables I and III). A three-way analysis of variance (Appendix, Tables II and IV) and the Q test established significant differences between means at the 5% level (Snedecor and Cochran, 1967).

Because of the loss of kidney-absorbed anti-kidney serum, it was necessary to analyze egg treatments with anti-kidney globulins separately in a one-way analysis of variance. To be able to compare the means of the eggs treated with anti-kidney globulins with the means of the other treatments, the following statistical manipulations were performed. Since the two experiments were run at the same time with the eggs of the same females, the experiments were drawn from the same population, and thus the variances were the same and the means followed a normal distribution. The mean square error terms of the two experiments were pooled and the average mean

square was determined. The means of the different treatments were then compared using the Scheffé method (Snedecor and Cochran, 1967), which is a more conservative analysis than the Q test.

The results from Text Figure 1 can be summarized as follows:

1). The fertilizability of any group of eggs after treatment with any of the digested globulin preparations, nonabsorbed (DJ, DO, DK) or heterologously absorbed (DJ+O, DO+Sp, DK+O) is not significantly different from that of any other group or from that of the controls (DC, DC+Sp, DC+O). All eggs treated with these preparations show high fertilizability, based on cleavage percent values. In this regard, for example, there is no significant difference between the digested globulin fractions of DJ and DO on cleavage of treated eggs. Moreover, there is no difference between these two treatments and their corresponding absorbed preparations (DJ+O, DO+Sp). Thus, there is no effect to be ascribed to nontissue-specific antibodies which could conceivably react with sperm in the nonabsorbed preparations. From these observations, it appears that the presence of non-precipitating antibody to egg jelly is not sufficient to prevent the interaction of jelly-coat substances and sperm.

2). Eggs treated with digested, homologously-absorbed globulins (DJ+J, DO+O) show high fertilizability, and are not significantly different from the corresponding

heterologously-absorbed preparations (DJ+O, DO+Sp) in this regard. Also there is no significant difference from any of the control globulins. Therefore, all digested preparations act as do the controls in producing no significant effect on fertilizability of the eggs.

3). All undigested (multivalent) globulin preparations (UJ, UJ+O, UO, UO+Sp, UK, UK+O) show marked inhibitory effects on the fertilizability of treated eggs which are significantly absent with the controls (UC, UC+Sp, UC+O). None of these treatments with multivalent antibody, however, is significantly different from each other. Therefore, neither the type of globulin nor heterologous absorption has any relevance in preventing the inhibitory effect on fertilization. It seems that these preparations contain substances which can significantly inhibit the fertilization reaction or some post-fertilization event leading to cleavage.

4). In comparing the arc sin values, although they are not different in statistical significance, there is apparently more inhibition by UO and UO+Sp than by UJ and UJ+O. This suggests that the block to fertilization may be at the surface of the egg rather than in receptor sites in the jelly.

5). The homologously-absorbed, undigested globulins (UJ+J, UO+O) act as do controls in that they show the non-inhibition to be expected if all antibodies have been removed. In fact, some antibodies are still present in these

Legend for symbols used in Text Figure 1: various treatments of Rana pipiens eggs prior to insemination.

DC--papain-digested control globulin

UC--undigested control globulin

DC+O--papain-digested control globulin absorbed with ovary

UC+O--undigested control globulin absorbed with ovary

DC+Sp--papain-digested control globulin absorbed with sperm

UC+Sp--undigested control globulin absorbed with sperm

DJ--papain-digested anti-jelly globulin

UJ--undigested anti-jelly globulin

DJ+O--papain-digested anti-jelly globulin absorbed with ovary

UJ+O--undigested anti-jelly globulin absorbed with ovary

DJ+J--papain-digested anti-jelly globulin absorbed with jelly

UJ+J--undigested anti-jelly globulin absorbed with jelly

DO--papain-digested anti-ovary globulin

UO--undigested anti-ovary globulin

DO+Sp--papain-digested anti-ovary globulin absorbed with sperm

UO+Sp--undigested anti-ovary globulin absorbed with sperm

DO+O--papain-digested anti-ovary globulin absorbed with ovary

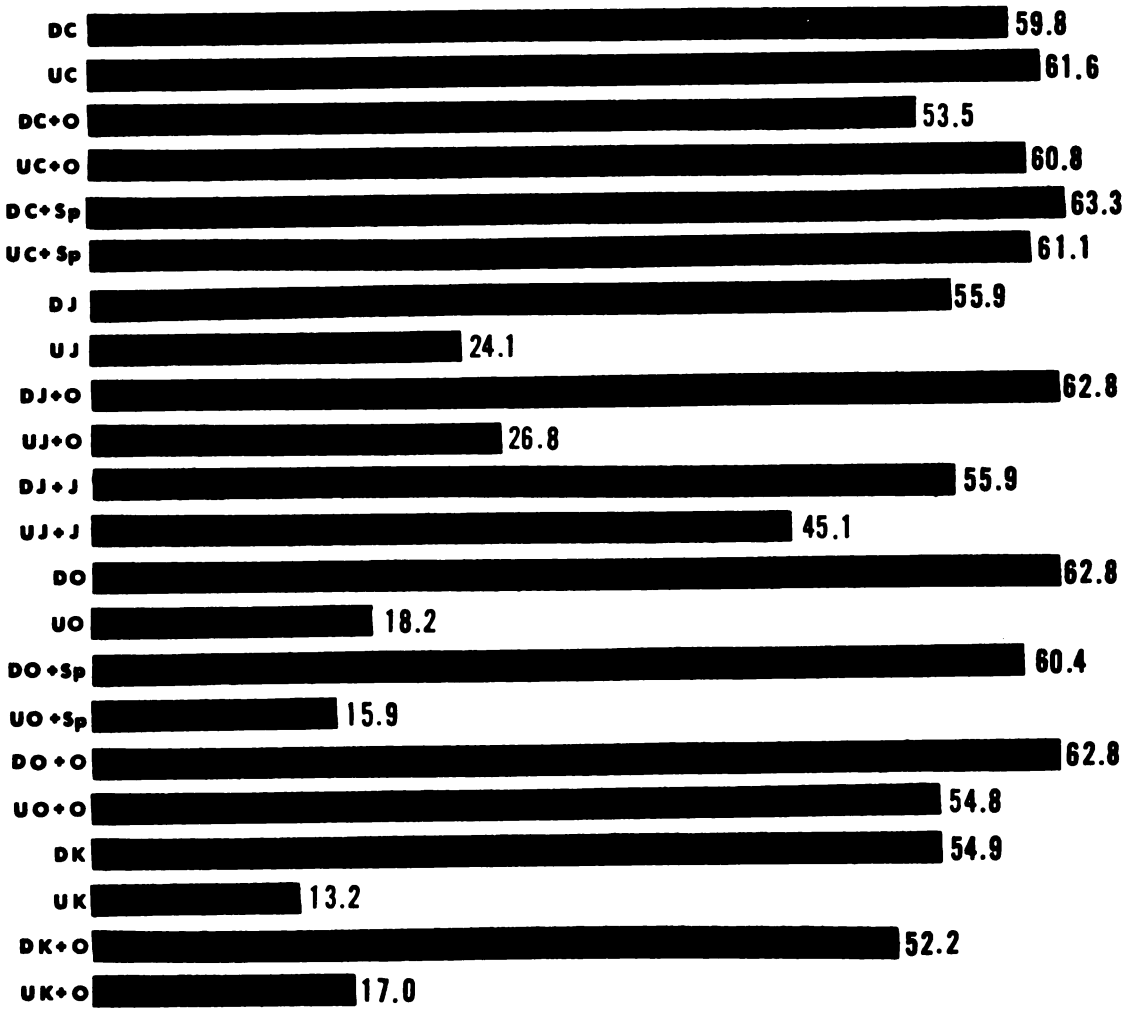
UO+O--undigested anti-ovary globulin absorbed with ovary

DK--papain-digested anti-kidney globulin

UK--undigested anti-kidney globulin

DK+O--papain-digested anti-kidney globulin absorbed with ovary

UK+O--undigested anti-kidney globulin absorbed with ovary



TEXT FIGURE 1. Fertilization of Rana pipiens eggs, in arc sin equivalents of percentages after 5 min. treatment with papain-digested and undigested globulin preparations of anti-jelly, anti-ovary, anti-kidney, and control sera. Each serum was absorbed prior to digestion procedures with homologous and heterologous antigens; nonabsorbed preparations were also used.

two preparations (Plate I, Figures 3 and 6). While UJ+J is significantly different in its effect on fertilization from UJ and UJ+O, suggesting that inhibitory antibodies are absent in UJ+J, fertilizability with UJ+J does not quite approach the undigested control (UC, Text Figure 1).

6). Data not presented here indicates that eggs from 16 females treated with the antigen control preparations, as described in Materials and Methods, fertilize normally. None of the four treatments is significantly different from each other or from control eggs. Consequently, no effect may be ascribed to any uncomplexed antigens perhaps remaining in the absorbed sera.

The dramatic difference between the effects on eggs of digested and undigested preparations of a given serum suggests that there is a mechanical barrier effect with multivalent antibody. The differences in effects seem to depend on whether the antibody has one active site or more than one. Apparently, simple blocking of jelly receptor sites by univalent antibody fragments is not sufficient to prevent gamete interaction. That such blocking occurs is inferred from the demonstrated activity of univalent fragments in agar diffusion (Plate III), though this latter test does not prove that this activity is manifest with the intact jelly-coat.

Effect of Univalent Antibody Fragments on the Fertilizability of Eggs Subsequently Treated with Sheep Antiglobulin Serum (Coombs' Test)

To further understand the non-inhibitory effects of univalent antibody fragments, it was deemed desirable to demonstrate their actual binding to antigens of the jelly-coat. Theoretically, it should be possible to mimic the effect of multivalent antibody, e.g., precipitation lattice formation and inhibition of fertilization, by first treating eggs with univalent fragments and subsequently adding sheep anti-rabbit gamma globulin serum. The antiglobulin serum should complex with and link adjacent univalent fragments, thereby creating a lattice in the jelly.

Each treatment with the various digested (DC, DJ, DO) and undigested (UC, UJ, UO) preparations was followed by treatment with each of the three following treatments: 1) one-tenth percent full strength Holtfreter's solution (H); 2) sheep anti-rabbit gamma globulin serum (IS, 1:5 dilution); 3) control sheep serum (CS, 1:5 dilution). Both series of treatments were for 5 minutes with a wash in between.

The results of the antiglobulin (Coombs') test in arc sin equivalents of percentages are shown in Text Figure 2. (For individual arc sin equivalents, see Appendix, Table V.) A statistical analysis of variance of the data (Appendix, Table VI) indicated that the three main factors were significant. There was no interaction between the second treatments (H, IS, CS) and the antiserum types or digestions.

The second treatments were significant at the 1% level, however, so a Q test was performed on pooled data, separating only the second treatments.

The following points can be made from the data (Text Figure 2):

1). There is no significant difference in fertilizability between eggs treated with IS or CS following initial treatment with any of the digested globulins (DJ, DO). All eggs treated in this way show high fertilizability. Therefore, it seems that immune sheep serum does not, in fact, create the postulated precipitation lattice, or at least such an effect is not monitored in terms of a decrease in the fertilizability of univalent antibody fragment-treated eggs.

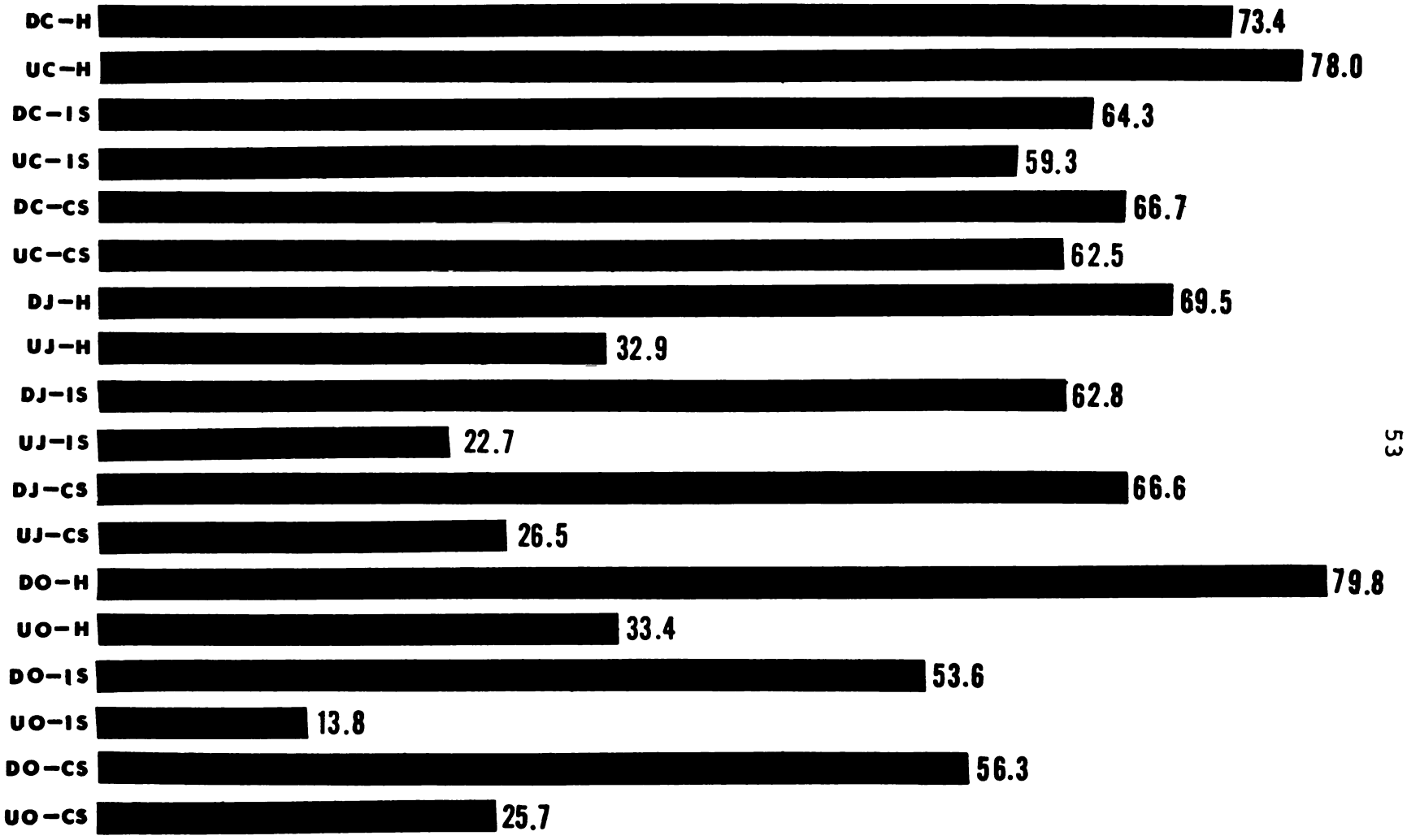
2). Moreover, there is no significant difference in the fertilizability of eggs treated with any of the undigested globulins (UJ, UO) followed by either IS or CS. The inhibition is of the same relative degree as previously demonstrated (Text Figure 1). However, in comparing the arc sin values for UO-IS and UO-CS (Text Figure 2), it seems that the IS treatment considerably lowers the fertilizability of eggs, suggesting that IS enhances the normal inhibitory effect of multivalent antibody. Presumably this is a result of a double lattice formation, the first created by linking antigens in the jelly by undigested antibody, and the second created by linking undigested antibody with IS antibodies. Furthermore, this effect with UO suggests that the mechanical

Legend for symbols used in Text Figure 2: various treatments of Rana pipiens eggs prior to insemination.

DC-H--treatment with papain-digested control globulin followed by one-tenth percent full strength Holtfreter's solution
UC-H--treatment with undigested control globulin followed by one-tenth percent full strength Holtfreter's solution
DC-IS--treatment with papain-digested control globulin followed by sheep anti-rabbit gamma globulin serum
UC-IS--treatment with undigested control globulin followed by sheep anti-rabbit gamma globulin serum
DC-CS--treatment with papain-digested control globulin followed by control sheep serum
UC-CS--treatment with undigested control globulin followed by control sheep serum

DJ-H--treatment with papain-digested anti-jelly globulin followed by one-tenth percent full strength Holtfreter's solution
UJ-H--treatment with undigested anti-jelly globulin followed by one-tenth percent full strength Holtfreter's solution
DJ-IS--treatment with papain-digested anti-jelly globulin followed by sheep anti-rabbit gamma globulin serum
UJ-IS--treatment with undigested anti-jelly globulin followed by sheep anti-rabbit gamma globulin serum
DJ-CS--treatment with papain-digested anti-jelly globulin followed by control sheep serum
UJ-CS--treatment with undigested anti-jelly globulin followed by control sheep serum

DO-H--treatment with papain-digested anti-ovary globulin followed by one-tenth percent full strength Holtfreter's solution
UO-H--treatment with undigested anti-ovary globulin followed by one-tenth percent full strength Holtfreter's solution
DO-IS--treatment with papain-digested anti-ovary globulin followed by sheep anti-rabbit gamma globulin serum
UO-IS--treatment with undigested anti-ovary globulin followed by sheep anti-rabbit gamma globulin serum
DO-CS--treatment with papain-digested anti-ovary globulin followed by control sheep serum
UO-CS--treatment with undigested anti-ovary globulin followed by control sheep serum



TEXT FIGURE 2. Fertilization of Rana pipiens eggs, in arc sin equivalents of percentages after 5 min. treatment with papain-digested and undigested globulin preparations followed by 5 min. treatment with sheep anti-rabbit gamma globulin serum (antiglobulin or Coombs' test), control sheep serum, or Holtfreter's solution.

barrier may be near the egg surface where ovary components may be diffused in the inner jelly layer.

3). A slight effect of sheep serum is apparent, for those eggs whose second treatment was the Holtfreter's solution (H) show a significant difference (higher fertilizability) from those whose second treatment was either sheep serum (IS or CS). However, the magnitude of differences in fertilizability between eggs first treated with digested or undigested immune preparations was the same, regardless of whether the subsequent treatment was H, IS, or CS.

In summary, the data indicates that it is not possible to decrease the fertilizability of eggs treated with univalent antibody fragments followed by treatment with sheep antiglobulin serum. This suggests that univalent antibody fragments are not binding, or are binding in insufficient numbers to allow for the creation of a mechanical barrier to sperm penetration in the antiglobulin test.

Effect of Univalent Antibody Fragments on the Fertilizability of Eggs Subsequently Treated with Parent Multivalent Antibody (Competition Test)

The competition test was devised to demonstrate the binding of univalent antibody fragments after the expectations of the antiglobulin test were not realized. In principle, the competition test is identical to the Ouchterlony inhibition of precipitation test (Plate III) previously described except that intact jellied eggs are

used instead of antigen. For example, if univalent antibody fragments are bound to jelly receptor sites, the effect of subsequent addition of parent multivalent antibody should be reduced; there should be considerably less inhibition of fertilization than in eggs treated with multivalent antibody only. Presumably univalent antibody fragments, first applied, would neutralize jelly receptor sites and prevent the subsequent attachment of multivalent antibody.

The results of this experiment are presented in Text Figure 3. (For individual arc sin equivalents see Appendix, Table VII.) The same six females were employed as in the antiglobulin test, and the arc sin values for treatments with control globulin preparations (DC, UC), and digested (DJ, DO) and undigested (UJ, UO) preparations--all followed by treatment with Holtfreter's solution--are also the same. Both first and second treatments were for five minutes, with a wash in between. Statistical analysis of variance revealed that differences due to various sequences of treatments were significant at the 1% level (Appendix, Table VIII).

The following points can be made from the data (Text Figure 3:

1). The arc sin value of eggs treated with DJ followed by treatment with UJ (DJ-UJ) was not significantly different from that of eggs treated with UJ followed by Holtfreter's solution (UJ-H). Considerable inhibition was evident in both cases. Eggs treated with DJ followed by UC (DJ-UC) or

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

DC-H--treatment with papain-digested control globulin followed by one-tenth percent full strength Holtfreter's solution

UC-H--treatment with undigested control globulin followed by one-tenth percent full strength Holtfreter's solution

DJ-H--treatment with papain-digested anti-jelly globulin followed by one-tenth percent full strength Holtfreter's solution

UJ-H--treatment with undigested anti-jelly globulin followed by one-tenth percent full strength Holtfreter's solution

DJ-UJ--treatment with papain-digested anti-jelly globulin followed by undigested anti-jelly globulin

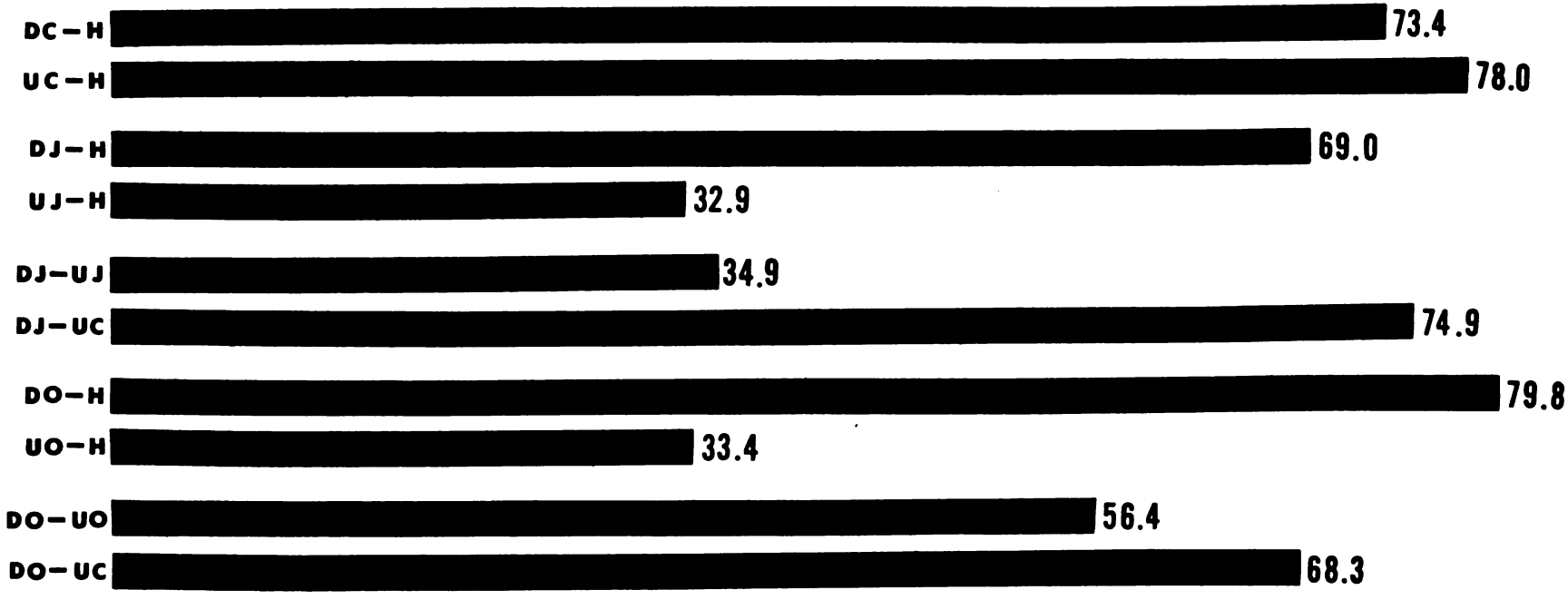
DJ-UC--treatment with papain-digested anti-jelly globulin followed by undigested control globulin

DO-H--treatment with papain-digested anti-ovary globulin followed by one-tenth percent full strength Holtfreter's solution

UO-H--treatment with undigested anti-ovary globulin followed by one-tenth percent full strength Holtfreter's solution

DO-UO--treatment with papain-digested anti-ovary globulin followed by undigested anti-ovary globulin

DO-UC--treatment with papain-digested anti-ovary globulin followed by undigested control globulin



TEXT FIGURE 3. Fertilization of Rana pipiens eggs, in arc sin equivalents of percentages after 5 min. treatment with papain-digested anti-jelly and anti-ovary globulins followed by 5 min. treatment with undigested parent multivalent antibody (Competition Test).

H(DJ-H) showed significantly higher fertilizability than those treated with DJ-UJ. Therefore, as the inhibition of fertilization with DJ-UJ and UJ-H was of the same magnitude, it apparently was not possible to preload a sufficient number, if any, of the jelly sites with DJ to prevent the attachment and inhibitory effect of UJ.

2). Significant competition was, however, apparent with anti-ovary globulins. Eggs pretreated with DO followed by treatment with UO (DO-UO) did not show the marked inhibition of fertilization as those treated with UO followed by Holtfreter's solution (UO-H), and the difference was statistically significant. Moreover, the result with DO-UO was not statistically different from the controls, DO-UC and DO-H. This indicates that prior treatment with DO reduced the inhibitory effect of UO to control levels of fertilizability. In this case, then, DO antibody fragments do bind to the jelly or egg surface, at least in sufficient numbers to prevent the multivalent antibody (UO) block to fertilization.

3). Support for the contrasting conclusions in the two points above was the observation that the treatment DO-UO was significantly different from treatment DJ-UJ. Why DO shows binding ability in this system and DJ does not is not presently obvious.

DISCUSSION

The results presented in this paper concern the effects of univalent antibody fragments and multivalent antibody on the fertilizability of Rana pipiens eggs with respect to the role of macromolecular components of the jelly-coat in initial gamete interaction. The cleavage-inhibiting effect of multivalent antibodies (Shivers, 1961a; Shaver, 1966; Shaver and Barch, 1960) was confirmed. However, non-precipitating, papain digested anti-jelly antibodies did not inhibit fertilization, in contrast to the results obtained by Shivers and Metz (1962). This latter observation requires reconsideration of the previous interpretations of the modes of action of antibodies on the jelly-coats of amphibian eggs, and suggests that they may be similar to that observed in echinoderms.

The analyses of the three different immune antisera in agar diffusion plates (Plate I) demonstrated that antibodies to a number of frog tissue antigens were present in each sera. Of particular interest to this study was the presence of antibodies to ovary antigens in the anti-jelly serum; ovary components found in the jelly material probably come from the egg surface or from the oviduct before the jelly is deposited (Shaver et al., 1962). Absorption of the sera

with heterologous tissue antigens partially removed antibodies to certain tissue antigens, but all antibodies to sperm were effectively removed (Plate I). Ideally, complete absorption was desired; however, the lack of significant effects of both the absorbed and nonabsorbed digested preparations on fertilization indicated that incomplete absorption was not critical to the interpretation of the results. Absorption of the sera with homologous tissue antigens was used as a control. However, agar diffusion analyses showed that complete absorption was not achieved, and the slight but not significant inhibition of fertilization shown with UJ+J treatment could be interpreted on this basis.

The action of papain on the antibody molecule has been known since the detailed work of Porter (1958, 1959). This worker demonstrated that degradation with papain yields two fragments (Fab), each with a molecular weight of 50,000-55,000, with one active site, and of almost identical chemical composition and antigenic properties (Mandy et al., 1961). A third fragment (Fc), of somewhat higher molecular weight (80,000), differed from the first two in its amino acid content, its lack of an active site, and its readily crystallizable property. Moreover, several investigators (Karush, 1959; Nisonoff et al., 1960) showed that there was no impairment of binding ability of the papain-digested, univalent antibody fragments when compared to the parent multivalent antibody. Also, univalent fragments were found to retain the antibody specificity of the parent molecule, and their

total yield accounted fully for the ligand-binding activity of the intact molecule (Porter, 1959; Nisonoff et al., 1960).

An additional property of univalent fragments (Porter, 1958, 1959)--that they are no longer capable of specific precipitation but can inhibit or compete with the precipitation reaction of the undigested antibody with homologous antigen--was demonstrated in the present study by Ouchterlony agar diffusion tests. Thus, papain digestion of a purified globulin fraction of various antisera against frog tissues did not affect the ability of the resultant univalent antibody fragments to complex with homologous antigen, and thereby prevented precipitation of parent multivalent antibody (Plate III). Similar results were obtained by Shivers and Metz (1962), who employed anti-jelly preparations.

A possible explanation for the failure of complete inhibition of precipitation by univalent fragments, as shown by some faint precipitin bands opposite wells 1 (Plate III), despite the postulated retention of binding ability of univalent fragments, may be that papain digestion lowered the "affinity" of the antibody. This would result in a greater dissociability of the antigen-univalent fragment complex as compared with the complex formed with multivalent antibody. As a consequence, multivalent antibody should compete more successfully with and "displace" the univalent material from the antigen. In addition, lowered affinity of the

papain-digested preparations may also explain why it was necessary to preload the wells several times with univalent antibody fragments in order to demonstrate inhibition of precipitation of the parent antibody with antigen. In this regard, Amiraian and Leikhim (1961) found that it was only possible to block sheep erythrocyte agglutination with multivalent antibody by pretreating cells with univalent antibody fragments at a high univalent to multivalent molar ratio, 20:1. This may also explain the failure to demonstrate binding of DJ in the competition test, where the univalent to multivalent ratio added to eggs was only 1:1.

Having demonstrated that univalent antibody fragments retained their binding ability in agar diffusion, their lack of significant effect on the fertilizability of pretreated eggs was of considerable interest. No significant inhibition of cleavage was demonstrated with any of the univalent fragments employed. But significant inhibition was always apparent with multivalent antibody. These two observations together support the assumption that fertilization inhibition by multivalent antibody can be explained solely on the basis of a mechanical barrier effect which results from cross-linking neighboring antigens in the jelly, creating a precipitation lattice which could secondarily mask essential antigenic sites involved in interaction with sperm. Jelly receptor sites which are presumably neutralized by univalent fragments may not, then, play any direct role in gamete

interaction in amphibian or echinoderm species (Graziano and Metz, 1967; Headrick and Metz, 1967; Metz and Thompson, 1967).

The inhibitory effect on fertilization of multivalent antibodies to various frog tissues as well as against jelly material was previously noted by Shivers (1961), Shaver (1966), and Shaver and Barch (1960). The latter workers suggested that antibodies against frog tissue antigens may reach the egg surface proper with relative rapidity such that sperm which have successfully traversed the jelly layers would be unable to interact with egg-surface sites owing to their prior blockage by antibodies. This suggests that the level of inhibition may be different for anti-jelly antibodies than for those against other tissues.

The discrepancy between these results with univalent antibody fragments and those of Shivers and Metz (1962) in Rana pipiens may be due to several factors. First, these latter workers averaged the results from only four experiments from a total of 160 eggs. Second, no statistical analysis of the data was made. This is to be compared to the fifteen experiments with fifteen females for an approximate total of 450 eggs, and the statistical analysis employed here. Other factors may also be involved which are less easy to control, and which are not clear from the information given by Shivers and Metz. For example, it is well-known that antibody preparations may differ in the amount of

antibody to the antigen with which the rabbit has been immunized (Carpenter, 1965). Variations in the titers of antibodies from those used by Shivers and Metz may be significant.

The binding ability of univalent fragments was exhibited in agar diffusion. The failure to exhibit such binding in the intact jelly-coat in the antiglobulin (Coombs') test suggests the following interpretations of the results of the antiglobulin test. First, univalent anti-jelly antibody fragments may not be complexing with antigens in the jelly; no physical barrier to sperm penetration is thus created by the antiglobulin serum. Alternatively, univalent fragments may complex with antigens in the jelly, but a decrease in fertilizability upon application of the antiglobulin serum might not be demonstrated for the following two reasons:
1) It may be that only a relatively small number of antigenic sites in the jelly were blocked by univalent fragments so that any cross-linking of these fragments by antiglobulin antibodies is insufficient to create a continuous, complete physical barrier. This may also explain the lack of fertilization inhibition by univalent fragments alone, for it implies that there may be sites in the jelly which are involved in sperm-egg interaction but which are not sufficiently blocked, to prevent sperm attachment and penetration;
s) Secondly, it is possible that the titer of antibody in the sheep antiglobulin serum was not high enough to effect

complete linkage between univalent fragments in the jelly; if lattice formation is incomplete, it is likely that sperm penetration would not be prevented. This implies that there may be antigenic sites in the jelly complexed to unlinked univalent fragments, but that neutralization of these sites alone is not sufficient to affect fertilizability.

The competition test provided some indirect evidence that binding of univalent fragments actually occurred. Eggs treated with digested antibodies to ovary (DO) followed by undigested antibodies to ovary (UO) did not show the inhibition of fertilization of UO alone, suggesting that univalent fragments complexed and prevented the attachment of multivalent antibody. As this was not demonstrated with the anti-jelly preparation, it may be that univalent fragments of these antibodies did not bind, as it would be expected they would, or were bound in insufficient numbers, leaving sites open for complexing by multivalent antibody. Differences in antibody titer between these two preparations may account for the discrepancy in the results of this test.

Pertinent to this study is the observation that univalent fragments of antibodies against sperm reduce the fertilizing capacity of sea urchin sperm (Flake and Metz, 1962; Metz et al., 1964). Similarly, Mowbray et al (1970), working with selected crustacean species, determined that both bivalent and univalent fragments of sperm antibodies reduced the capacity of treated sperm to attach to eggs.

Furthermore, these latter workers were able to show that binding of univalent fragments to sperm did occur (Coombs' test), suggesting that specific sperm surface antigens were involved in sperm-egg attachment. It appears that essential sperm fertilization antigens were blocked by univalent antibody in these studies.

Fertilizability of eggs was the standard criterion used in the present study. However, it was hoped that morphological effects on the jelly or on the egg surface could be detected with different antibody treatments and correlated to the fertilizability of the eggs, as had been successful with the sea urchin (for review, see Metz and Thompson, 1967). This expectation was not realized in R. pipiens. It was especially apparent that a morphological criterion would have been extremely useful in the antiglobulin test to demonstrate some binding of univalent antibody fragments. Considerable attempts to find reproducible differences in the intact jelly-coat upon treatment of eggs with multivalent antibody, or with univalent fragments followed by antiglobulin were not successful.

If specific molecular configurations of the egg jelly-coat are involved in amphibian fertilization, future approaches must be directed to determining the specific antigens involved and their precise location in the jelly. It remains to be conclusively demonstrated that there are specific receptor sites in the frog egg jelly which can be blocked with antibody and which are involved in the

fertilization reaction. It may very well be that the molecular species, or portions of them which are antigenic in the rabbits used to produce the antibodies, are not the same which are involved in interaction with sperm. Whereas the reactions between antigens and antibodies are confined to rather restricted areas on the surface of molecules, the specificity of sperm attachment is likely to be more complex. It may be that the essential sites in the jelly are not accessible to direct interaction with antibody. If this is the case, then the use of antibodies to elucidate the function of macromolecular components in the jelly may not be the best approach. If univalent fragments do not attach, or if they do but do not thereby prevent fertilization, then it may not be possible to use this technique to identify or localize the postulated jelly receptor sites in the amphibian jelly-coat. Further studies must be directed to determining the specific effects of the jelly substances on sperm adhesion, sperm penetration, and induction of the acrosomal reaction.

SUMMARY

1). Antisera were prepared by immunizing rabbits with egg jelly and several tissues of the frog, Rana pipiens. The sera were absorbed with various tissues to make the preparations tissue-specific. Samples of the gamma globulin fraction of both absorbed and nonabsorbed antisera were degraded to the non-precipitating, univalent form by papain digestion.

2). Univalent antibody fragments inhibited the precipitation of multivalent antibody with homologous antigen in Ouchterlony agar diffusion plates, indicating that the antibody activity of the univalent fragments was retained.

3). Univalent antibody fragment-pretreated eggs did not show the marked inhibition in fertilizability of eggs pretreated with multivalent antibody. Neither the type of antisera nor heterologous absorption was significant in producing these effects.

4). Univalent antibody fragment-pretreated eggs followed by treatment with sheep anti-rabbit gamma globulin serum (antiglobulin or Coombs' test) did not show the expected inhibition of fertilization. While this suggested that univalent fragments did not complex with jelly antigens in the intact jelly-coat, other explanations were offered.

5). Eggs pretreated with univalent anti-ovary fragments followed by treatment with parent multivalent antibody (Competition Test) did not show the marked inhibition of fertilization of multivalent antibody alone. This provided indirect evidence that univalent fragments were binding to antigens of the jelly-coat, although this was not demonstrated with anti-jelly preparations.

6). It was concluded that the presumptive direct blocking of receptor sites in the jelly-coat was not sufficient to prevent fertilization. This observation confirmed that the inhibitory effect of multivalent antibodies on fertilization was due to the structural effects on the egg jelly which produced a mechanical barrier to fertilization, and not to direct blocking of jelly substances involved in gamete interaction.

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APPENDIX

Table Ia.--Individual arc sin equivalents of cleavage percentage values of Rana pipiens eggs treated with various absorbed and nonabsorbed digested and undigested globulins, excluding anti-kidney globulins.

Animals \ Treatments	DJ	UJ	DO	UO	DC	UC	DC+Sp	UC+Sp
1	46.1	18.4	41.6	00.0	48.2	55.7	49.1	36.3
2	38.0	34.7	65.0	10.5	52.9	55.1	48.2	47.9
3	61.6	28.1	56.3	00.0	65.0	64.5	90.0	55.5
4	66.6	17.9	61.3	23.3	33.7	38.1	55.6	67.5
5	18.4	00.0	38.4	23.1	40.2	55.9	39.2	42.4
6	52.7	25.4	55.5	00.0	58.5	61.1	60.0	54.0
7	20.7	12.9	68.1	31.1	57.9	63.4	70.2	71.6
8	63.8	10.5	59.5	00.0	51.7	68.6	53.1	74.6
9	74.1	33.2	61.9	23.4	79.9	75.2	90.0	79.5
10	74.6	30.0	69.3	19.1	70.9	61.7	73.3	79.1
11	62.6	27.0	66.9	46.4	68.9	67.5	73.3	90.0
12	69.3	42.6	7±.3	22.2	75.6	75.0	79.5	71.6
13	75.0	33.2	79.5	24.1	68.2	62.4	37.1	42.8
14	38.2	00.0	68.6	10.5	56.3	55.7	67.8	39.9
15	77.1	47.1	79.1	39.8	69.3	67.5	63.4	63.9
$\bar{X}$	55.9	24.1	62.8	18.2	59.8	61.6	63.3	61.1

Table Ib.--cont.

Animals \ Treatments	DJ+J	UJ+J	DJ+O	UJ+O	DO+Sp	UO+Sp	DO+O	UO+O	DC+O	UC+O
1	47.6	26.0	40.2	12.9	38.7	17.3	45.0	32.3	35.2	28.5
2	51.4	33.2	50.8	18.4	52.9	11.8	90.0	75.0	60.6	78.2
3	45.0	57.7	59.2	16.7	42.6	00.0	67.8	63.4	70.5	67.3
4	33.7	18.4	64.5	24.1	67.4	18.4	35.2	57.7	48.5	53.3
5	18.4	18.4	48.2	00.0	22.8	00.0	34.0	32.8	22.6	35.2
6	55.5	21.4	71.6	24.1	39.0	00.0	56.8	48.9	55.7	55.3
7	47.9	52.9	53.3	33.2	71.1	11.5	66.9	69.9	16.7	42.9
8	68.1	29.3	66.4	23.3	63.4	00.0	73.3	64.0	46.7	61.9
9	46.4	75.0	78.5	48.9	72.2	28.1	73.5	55.5	78.8	78.5
10	77.3	63.4	78.9	34.9	68.6	24.1	68.4	61.1	68.6	67.8
11	60.5	64.8	65.9	55.3	77.8	33.2	61.6	69.7	73.3	75.0
12	64.4	57.3	66.9	49.8	68.7	47.6	78.0	72.2	75.2	79.5
13	69.3	56.3	54.0	12.0	73.9	26.1	45.7	32.3	24.1	57.2
14	63.4	38.4	54.1	15.5	56.6	00.0	65.9	36.5	57.7	59.0
15	90.0	64.4	90.0	33.2	90.0	20.1	79.1	50.8	68.6	71.6
$\bar{X}$	55.9	45.1	62.8	26.8	60.4	15.9	62.8	54.8	53.5	60.8

Table II.--Three-way analysis of variance of effect of exposing eggs of Rana pipiens to various absorbed and nonabsorbed, digested and undigested globulins, excluding anti-kidney globulins.

Source of Variation	Degree of Freedom	Sum of Squares	Mean Square	F
Animal	14	32582.6	2327.3	17.7**
Sera(S)	2	12720.0	636.0	4.8**
Absorptions(A)	2	6358.6	3179.3	24.2**
Digestions(D)	1	23782.1	23782.1	180.9**
Interaction(SA)	4	3368.0	842.0	6.4**
Interaction(SD)	2	15368.4	7684.2	58.4**
Interaction(AD)	2	4789.3	2394.7	18.2**
Interaction(SAD)	4	4942.8	1235.7	9.4**
Error	238	31293.7	131.5	

**Significant to the 1% probability level.

Table III.--Individual arc sin equivalents of cleavage percentage values of Rana pipiens eggs treated with digested and undigested anti-kidney and ovary-absorbed anti-kidney globulins.

Animals \ Treatments	DK	UK	DK+O	UK+O
1	37.1	00.0	16.4	11.5
2	46.0	26.1	54.8	16.4
3	58.9	00.0	62.4	00.0
4	35.2	00.0	49.8	12.3
5	19.5	00.0	18.9	00.0
6	58.8	00.0	50.2	00.0
7	33.5	00.0	66.7	17.9
8	49.7	00.0	58.8	15.0
9	67.9	18.9	72.7	36.7
10	64.6	20.7	51.7	30.0
11	69.3	28.1	62.4	28.7
12	77.1	50.1	74.6	30.0
13	64.4	9.8	51.2	20.7
14	51.4	17.3	49.1	15.9
15	90.0	26.6	43.5	19.1
$\bar{X}$	54.9	13.2	52.2	17.0

Table IV.--One-way analysis of variance of effect of exposing eggs of Rana pipiens to digested and undigested anti-kidney and ovary-absorbed anti-kidney globulins.

Source of Variation	Degree of Freedom	Sum of Squares	Mean Square	F
Treatment	3	22375.8	7458.6	65.0**
Animal	14	8998.7	642.8	5.6**
Error	42	4816.5	114.7	
Total	59	36191.0	613.4	

**Significant to the 1% probability level.

Table V.--Individual arc sin equivalents of cleavage percentage values of Rana pipiens eggs treated with digested and undigested globulins followed by sheep anti-globulin serum (Coombs' test).

Animals \ Treatments		DJ-H	DJ-IS	DJ-CS	UJ-H	UJ-IS	UJ-CS	DO-H	DO-IS	DO-CS
1		50.2	70.5	59.2	32.3	37.8	26.1	65.9	30.4	70.2
2		68.9	79.4	78.0	30.6	28.1	11.5	90.0	55.5	37.5
3		74.6	58.2	65.9	27.6	35.2	43.6	76.7	71.1	75.0
4		72.0	50.8	69.9	21.4	9.1	32.7	80.1	40.5	54.8
5		73.3	58.8	61.5	44.1	17.0	15.9	75.9	67.8	32.5
6		77.9	59.3	65.3	41.5	9.1	29.4	90.0	56.3	67.8
	$\bar{X}$	69.5	62.8	66.6	32.9	22.7	26.5	79.8	53.6	56.3

		UO-H	UO-IS	UO-CS	DC-H	DC-IS	DC-CS	UC-H	UC-IS	UC-CS
1		35.2	00.0	17.2	64.5	60.8	69.7	71.6	52.5	41.6
2		30.9	00.0	19.7	67.2	68.2	56.2	65.9	41.5	74.2
3		45.0	36.4	28.7	71.6	75.0	66.9	78.0	74.5	62.9
4		19.5	11.5	30.0	68.6	47.6	63.9	72.4	50.4	60.0
5		43.1	17.6	28.6	90.0	54.8	53.4	90.0	73.9	61.1
6		26.6	17.2	30.0	78.5	79.4	90.0	90.0	63.0	75.2
	$\bar{X}$	33.4	13.8	25.7	73.4	64.3	66.7	78.0	59.3	62.5

Table VI.--Three-way analysis of variance of effect of exposing eggs of Rana pipiens to digested and undigested globulins followed by sheep antiglobulin serum (Coombs' test).

Source of Variation	Degree of Freedom	Sum of Squares	Mean Square	F
Animal	5	2394.2	478.8	3.7**
Antisera(A)	2	11848.9	5924.4	45.8**
Digestions(D)	1	18910.4	18910.4	146.2**
Second Treatments(T)	2	4287.5	2145.8	16.6**
Interaction(AD)	2	8391.3	4195.7	32.3**
Interaction(AT)	4	714.1	178.5	1.4
Interaction(DT)	2	52.1	26.0	0.2
Interaction(ADT)	4	519.5	129.9	1.0
Error	75	9702.7	129.4	

**Significant to the 1% probability level.

Table VII.--Individual arc sin equivalents of cleavage percentage values of Rana pipiens eggs treated with digested anti-jelly and anti-ovary globulins followed by parent multivalent antibody (Competition Test).

Animals \ Treatments	DJ-H	UJ-H	DJ-UJ	DJ-UC	DO-H	UO-H	DO-UO	DO-UC	DC-H	UC-H
1	50.2	32.3	26.6	77.8	65.9	35.2	56.2	51.7	64.5	71.6
2	65.9	30.6	28.1	52.7	90.0	30.9	59.6	65.9	67.2	65.9
3	74.6	27.6	27.6	69.8	76.7	45.0	59.2	78.0	71.6	78.0
4	72.0	21.4	24.1	69.3	80.1	19.5	40.7	56.4	68.6	72.4
5	73.3	44.1	47.2	90.0	75.9	43.1	64.4	67.8	90.0	90.0
6	77.9	41.5	55.6	90.0	90.0	26.6	58.5	90.0	78.5	90.0
$\bar{X}$	69.0	32.9	34.9	74.9	79.8	33.4	56.4	68.3	73.4	78.0

Table VIII.--One-way analysis of variance of effect of exposing eggs of Rana pipiens to digested anti-jelly and anti-ovary globulins followed by parent multivalent antibody (Competition Test).

Source of Variation	Degree of Freedom	Sum of Squares	Mean Square	F
Treatment	9	20115.3	2235.0	33.6**
Animal	5	2933.4	586.7	8.8**
Error	45	2989.7	66.4	
Total	59	26038.3	441.3	

**Significant to the 1% probability level.