SPERM - EGG JELLY INTERACTIONS IN RANA PIPIENS

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ABSTRACT

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By

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Immunobiological techniques were used to examine the role of egg jelly in fertilization of Rana pipiens. Eggs of R. pipiens were treated prior to insemination with various antisera which block the normal function of the egg jelly and thus inhibit fertilization. Treated ("capacitated") sperm were then used for fertilization in an effort to by-pass the antisera inhibition. Treatment (capacitation) consisted of mixing sperm with egg water or various oviducal extracts for a period of time prior to insemination. A biochemical analysis of the basic components of the egg jelly was done to investigate possible chemical variations in different jelly layers. In addition a cytological study was done on the jelly producing organ, the oviduct, using light and transmission electron microscopy in order to investigate any structural differences at various oviducal levels which could be correlated to antigenic and histochemical differences that have been found by other investigators.

By capacitation of the sperm prior to insemination, the inhibition of fertilization due to the various treat-

ments with antisera of the eggs was by-passed. No major variations in the ability of the different "capacitating" solutions to affect a higher percentage of fertilization were noted. The factors present in the egg water which could be responsible for capacitation of sperm seems to be stable over a period of time and can be removed by centrifugation in the presence of excess sperm. A cytological study revealed some ultrastructural aspects of the female oviduct during egg jelly production but no evidence was found that could associate fine structural differences with histochemical and antigenic differences in the oviduct.

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By

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INTRODUCTION

Many animal eggs have some sort of surounding capsule. These capsules have a broad range of morphology from a mucus envelope to distinct layers of cells. In the case of the amphibian, the egg is surrounded by homogeneous thick, jelly-like capsules. Some of the earliest work done on the jelly envelopes of frogs appeared in 1883 (Giacosa,1883). The jelly was noted to swell enormously when contact was made with water after the eggs left the ovisac, and was assumed to have a protective function to save individual embroys from any sudden, harmful environmental change (eg. mechanical, chemical, or thermal shock). Later observations by E. Bataillon pointed toward another function of amphibian egg jelly besides that of protection. He noted that eggs taken from the body cavity (coelomic eggs) could not be fertilized. Such eggs do not have a jelly envelope since during ovulation, the eggs rupture the wall of the follicle and pass into the body cavity before being forced into the ostium of the oviduct by cilia (Noble, 1931 ; Rugh, 1935). As the egg progresses down the oviduct, it becomes surrounded by a mucoid or gelatinous material (the egg jelly) which is secreted by specialized cells of the oviduct. Additional evidence that the jelly envelopes of amphibians have a significant role in fertilization was

furnished by the observation that the eggs that have passed through the oviduct and then mechanically or chemically dejellied still could not be fertilized in any appreciable amount (Shaver, 1960; Kambara, 1953; Tchou-Su, 1956; Rugh, 1935).

One of the more important tools used by investigators to study the role of the egg jelly envelopes are immunobiological techniques. It has been demonstrated that the fertilizability of R. pipiens eggs, pretreated with antisera of rabbits immunized against the jelly coat of the eggs, was decreased (Shaver and Barch, 1960). Whether this inhibition is due to mechanical blockage of sperm via a precipitation lattice network, or to the blockage of specific sperm receptor sites in the jelly is not yet clear. Work with "univalent" antibodies (papain digested and thus non-precipitating) is inconclusive (Shivers and Metz, 1962; Arakelian, 1971). It is evident, however, that antibodies against the jelly-coat engage complementary sites in the jelly and prevent in some way the initial sperm-jelly interactions prerequisite for fertilization in amphibians.

To further investigate the role of antigenic components of the egg jelly, attention has been given to the various jelly layers found around amphibian eggs. Three to six layers have been noted in R. pipiens (Shaver, 1966; Nace et al.,1961; Shivers and James, 1970 A.). four in the toad Bufo bufo asiaticus (Kambara, 1953), and four in Hyla arborea japonica (Katagiri, 1963). The importance of different jelly layers has been demonstrated by noting

the fertilizability of eggs removed from various levels of the oviduct. Kambara used this technique with Bufo bufo asiaticus in which four distinct layers are visible in spawned eggs. Results showed that the first two layers deposited around the egg are necessary for fertilization to occur. The innermost alone is not sufficient, indicating that the second layer or an alliance of the two innermost layers (called C and D) is a necessary requirement. In a similiar manner, Glick and Shaver (1963) studied the fertilizability of eggs from various levels of the oviduct of R. pipiens. As in Kambara's experiments, an essential constituent(s) is put around the egg from the middle and lower levels of the oviduct. The lack of fertilizability of the upper oviducal eggs reflects the absence of these constituents from the other levels. This of course does not mean that the upper oviducal secretions are non-essential.

Using the agar-gel diffusion technique (Ouchterlony, 1949), Barch and Shaver (1963) have found antigenic components unique to the upper or lower levels of the oviduct. In addition there are antigenic components common to all levels. All of these antigens have also been shown to be present in the egg jelly.

Localization of these antigenic constituents in the egg jelly was achieved by using fluorescein-conjugated antibodies (Shivers, 1962). These antigens consist of two types: non-specific antigenic components which are shared between closely related species of Rana and species-specific antigenic components which are present in only one species of

Rana. The non-specific antigenic components appear to be confined to the inner layers of the egg jelly whereas the species-specific antigenic components appear to be located in the middle and outer layers.

The significance of these egg jelly antigens in fertilization was tested by noting the effects of antibodies directed against these antigens (Shivers, 1961). It seems that both the species-specific and the non-specific antigens are necessary for fertilization. In addition, The presence of an antigen produced by the egg itself has been detected (Nace and Lavin, 1963) and shown to have a significant role in fertilization.

Examination of the function of the egg jelly in fertilization has been accomplished by treatment of frog sperm prior to imsemination with secretions of the female reproductive tract, as has been done with sperm of other animal groups (Austin, 1951; Austin and Bishop, 1958; Chang, 1951; Kirton and Hafs, 1965). It has been shown that sperm of mammals and of several invertebrate species require a period of contact or residency within the female reproductive tract before being able to fertilize. During such exposure the sperm becomes "capacitated" and thereby becomes able to initiate fertilization. It is thought that capacitation is a process which in some way produces the acrosome reaction which is important in sperm-egg membrane fusion (Dan, 1956; Colwin and Colwin, 1964). Several studies with invertebrate and mammalian spermatozoa indicate that a release of sperm materials, believed to be lytic enzymes (lysins), from the

acrosome is accomplished when contact has been made with some activator substance(s) of the female reproductive tract (Monroy, 1965; Stambough and Buckley, 1969).

Recent studies (Shivers and James, 1970 B.; Gusseck and Hedrick, 1971; Wolf and Hedrick, 1971) indicate that the sperm of R. pipiens and Xenopus leavis can also be "capacitated" by exposure to jelly coat materials. By such exposure the sperm was able to fertilize mechanically and chemically dejellied Xenopus laevis eggs and the body cavity (coelomic) eggs of R. pipiens. Thus the role of the egg jelly has been experimentally demonstrated through pre-treatment (capacitation) of the sperm with jelly coat materials. Electron micrographic studies of R.pipiens spermatozoa (Poirier, 1970) indicate that acrosomal structures do exist and are, in general, similiar to those of invertebrate and mammalian sperm. However, the classical acrosome reaction has yet to be observed in Rana species with electron microscopy. Although the enzymatic nature of the acrosomal reaction has been demonstrated in mammals (Hartree and Allison, 1970; Stambough and Buckley, 1969; Monroy, 1956), the number and nature of acrosomal substances have not been explicitly defined in amphibian spermatozoa.

The tissue responsible for the production of frog egg-jelly material has also been investigated as to possible clues to early sperm-egg interactions. The oviducts are not uniform in their distribution of glands and mucus cells (LeBrun, 1891; Lee, 1965). The gross structure of the oviduct can be divided into three parts: a short straight

upper portion around the ostium, a long curled, twisted middle portion, and a lower curved protion near the ovisac and cloaca. Histological studies have revealed three cell types which constitute the oviduct (Lee, 1965): jelly gland (tubular) cells, mucus cells, and ciliated epithelial cells which line the lumen. In addition connective tissue anchors the oviduct to the body wall. The structural differences in the various portions of the oviduct seem to be reflected by the distinct layers of jelly materials seen in the jelly envelopes (Kambara, 1953; Metz and Monroy, 1967; Shivers and James, 1970 A.) In R. pipiens six distinct regions have recently been reported by Shivers and James (1970 A.) using differential histochemical staining techniques on the cells of the oviduct. It was also noted that the jelly was deposited in six layers instead of the three reported by other workers. Based on an average oviducal length of 39 cm., the regions measured 1, 5, 6, 3, 20, and 3 cm. respectively, in a cranial - caudal direction. Histochemically, all regions except the fifth (20 cm.) showed large amounts of acid mucopolysaccharides with sulfated mucopolysaccharides found in regions 1,3,4, and 6. Neutral mucopolysaccharides were found in regions 2 and 5. These different histochemical properties possibly reflect the distribution of oviducal antigens previously noted (Barch and Shaver, 1963). Recent evidence (Pereda, 1971) on the nature and distribution of sialomucins and proteins also showed differences in the oviduct which could be correlated to the jelly layers.

In analyzing the chemical composition of amphibian egg jelly, the most common method used other than histochemical techniques has been acid hydrolysis (Flood et al., 1948). Such hydrolysates yeild chiefly monosaccharides and associated proteins by splitting of glycosidic bonds.

Amphibian egg jelly consists of two types of compounds: carbohydrates and proteins. The major carbohydrate contents of the egg jelly are glucosamine, galactosamine, fucose, and galactose (Folkes et al., 1950; Minganti, 1955; Hiyama, 1945; Schultz and Becher, 1935; Bray and James, 1949; Schultz and Ditthorn, 1900). Forty to fifty percent of the frog egg jelly consists of these reducing sugars. The protein moiety consists about an equal porportion of the egg jelly. A complete spectrum of the twenty amino acids is present (Metz and Monroy, 1967) but the exact stoichiometric relationships between the proteins and the carbohydrates as mucopolysaccharides is not clear. Disulfide bonds have been shown to be important in maintaining the structural integrity of the jelly (Gusseck and Hedrick, 1971). However, variance in the chemical composition of the egg jelly from species to species is apparent (Monroy, 1965) indicating again that species-specific chemical differences may be involved in mechanisms of fertilization.

This report further investigates the role of the egg jelly in fertilization of R. pipiens. Immunobiological techniques were employed to examine initial sperm reactions, sometimes termed "capacitation" of sperm. The normal role of the egg jelly is blocked by the use of inhibitory antisera.

Sperm was then treated with various capacitating solutions in order to overcome this block. The capacitating solutions consisted of egg water and dilute extracts from various levels of the oviduct. Various levels of the oviduct were used in preparing capacitating solutions in order to note if their different antigenic composition could have different effects on enabling the sperm to fertilize antisera-blocked eggs. The work of Shivers and James (1970 A.) was used as a guideline in obtaining extracts from various oviducal levels. A general biochemical analysis of oviducal materials was done in order to correlate antigenic differences with the basic chemical composition. A cytological study was also performed which perhaps reflects structural differences in the oviduct which could be linked to histological and antigenic differences.

MATERIALS AND METHODS

Capacitation of Sperm

In all experiments R. pipiens purchased from commercial dealers in Wisconsin and Vermont were used. Eggs were obtained by pituitary and hormone induced ovulation (Wright and Flathers, 1961). Five mg. of progesterone in corn oil was injected into the dorsal lymph sac plus one adult frog pituitary injected into the body cavity for each female. Sperm was obtained by dissection of the testis and maceration in 1:10 Holtfreter's solution. A "normal" sperm suspension contained one testis per 10 ml. of solution. The eggs of females used in all the experiments and those eggs used to obtain the egg water were pretested by inseminating small samples with a normal sperm suspension and noting the success of fertilization. This was done to insure that fertilizable eggs were used.

Antisera used in all experiments were obtained by previously employed methods (Barch and Shaver, 1963; Shaver et al. 1970). Approximately 5 mg. of antigen was mixed with 1 ml. of 1:10 Holtfreter's solution and suspended in 1 ml. of complete Freund's adjuvant. This mixture was then injected into the subscapular muscles of rabbits. After one week a similiar dose with incomplete adjuvant was administered.

Every 4 - 6 weeks thereafter booster shots of the antigen - incomplete adjuvant mixture was given. The rabbits were bled from a marginal ear vein. Blood for control sera was taken prior to immunization. All sera were tested for antibody content on Ouchterlony double diffusion plates (Ouchterlony, 1949; Shaver, 1961) and dialized against 0.65% NaCl prior to use.

In the first experiment sperm was treated (capacitated) by exposure to egg water for various time intervals. Egg water was obtained by stripping all the eggs of a female into 75 - 80 cc of 1:10 Holtfreter's solution, allowing them to sit at room temperature in the Holtfreter's solution for 1 to 1½ hrs. and drawing off the fluid with a Pasteur pipette. A protein determination, using an Hitachi Model 101 UV-Vis spectrophotometer, was run on all egg water used.

Eggs were exuded onto glass microscope slides and covered with antijelly serum, control serum, or 1:10 Holtfreter's solution for 2 minutes. The eggs were washed by thorough pipetting with 1:10 Holtfreter' solution. Batches of eggs from each of the three treated groups were then inseminated with the different sperm-egg water suspensions listed below or with normal sperm suspension. In this first experiment the eggs from 16 different frogs were used.

Capacitated sperm were obtained by diluting 1 ml. of a concentrated sperm suspension (1 testis per 1 ml. of 1: 10 Holtfreter's solution) with 9 ml. of egg water. This mixture was used immediately or allowed to react for 5, 10,

or 15 minutes before being applied to treated eggs. All sperm suspensions (capacitated and normal) were allowed to react for 10 minutes with eggs, which were then placed in finger bowls filled with aerated water.

After several hours the number of cleaving eggs (2 - 8 cell stage) and the total number of eggs were counted and recorded. The percent of cleavage was calculated and transformed into arc sin equivalents for a statistical test (analysis of variance; Snedecor, 1956). Significant differences were calculated by the Q test (see appendix).

In the second experiment capacitated sperm were prepared as described previously except the sperm were exposed to egg water for one time period (10 minutes) only. Eggs on glass microscope slides were treated as before with 10 different types of antisera from immunized rabbits, non-immune control sera, and 1: 10 Holtfreter's solution. The antisera were directed against R. pipiens egg jelly, upper oviduct material, middle oviduct material, lower oviduct material, spleen, kidney, ovary, sperm, and R. clamitans egg jelly. Batches of eggs from each of the above treated groups were then inseminated with the capacitated sperm or with the normal sperm suspension. The eggs from 23 different females were used. After several hours the eggs were counted and a statistical analysis of variance was done as in experiment # 1.

Sperm capacitation in the third experiment was accomplished by mixing sperm for 10 minutes with materials exuded from various regions of the oviduct of R. pipiens

and with egg water. Oviducts fron non-ovulating adult females were dissected and divided into segments corresponding to the regions described by Shivers and James (1970 A.) or into thirds (Figure 1). A whole oviduct was also used to obtain a capacitating solution. All oviducal segments were individually homogenized in 1:10 Holtfreter's solution and stirred for several hours. The extracts were filtered through glass wool and stored in the cold. A protein determination was made on each extract. The extracts were kept in a highly diluted form so as to prevent excess gel formation. Eggs on glass microscope slides were treated as previously mentioned with either antisera against the egg jelly or non-immune control sera. Batches of eggs from these two treated groups were then inseminated with the different solutions of capacitated sperm or with normal sperm suspension. The eggs of 19 different frogs were used. After several hours the eggs were counted and an analysis of variance was done as in experiment #1 (see appendix).

In the fourth experiment, four different solutions were tested as to their capacitating ability. The four solutions were a) normal egg water, b) egg water stored 24 hrs. in the cold and allowed to reach room temperature prior to use, c) egg water spun at 11,500 x g in an International High Speed Refrigated Centrifuge Model HR-1 for 15 minutes, d) the supernatant of a dense sperm - egg water suspension. To obtain the supernatant, 4 testis were

Figure 1. Oviducts dissected and cut up into regions 1 through 6 according to Shivers and James (1970 A.) and into thirds. These segments were used to obtain extracts for capacitation experiment # 3

macerated in 10 ml. of fresh egg water and allowed to react for 10 minutes. This suspension was then spun at 11,500 x g for 15 minutes and the supernatant saved. A protein determination was done on all four solutions. Sperm were mixed with these solutions 10 minutes prior to insemination. Eggs on glass microscope slides were treated as mentioned above either with antisera against the egg jelly or with non-immune control sera. Batches of eggs from these two treated groups were then inseminated with the different sperm - capacitating solution mixtures or with normal sperm suspension. The eggs of 15 different frogs were used. After several hours the success of fertilization was noted and an analysis of variance was done as in experiment # 1 (see appendix).

Chemical Analysis

Biochemical tests were run on lyophylized extracts from the whole oviduct of R. pipiens. The extracts were obtained by homogenizing whole oviducts from adult females in 1:10 Holtfreter's solution and stirring for several hours. The extracts were then filtered through glass wool and lyophylized (freeze-dried). In all of the chemical methods listed below, 2 mg. of lyophylized extract was hydrolyzed in 1 N H_2SO_4 for 1½ hrs. at 105 C. The chemical composition was determined by the following methods: reducing sugars by the Nelson test (Nelson, 1944), inorganic phosphate by the modified Fiske - Subbarow method (Fiske and Subbarow, 1925), protein by the Lowry method (Lowry et al., 1951), pentose sugar by the Mejbaum method (Schneider,1957) , and deoxypentose by the

Dische method (Dische, 1955). The colorimetric determinations were done on a Spectronic 20.

An amino acid analysis was performed on lyophylized extracts prepared in the manner described above from the whole oviduct of R. pipiens, upper ⅓ oviduct, middle third oviduct, lower third oviduct, the outer jelly layers of R. pipiens eggs, and the whole oviduct extracts from R. clamitans, R. catesbieana, and Bufo fowleri. In the case of each of the above materials, 6 mg. were hydrolyzed in 2 ml. of 6 N HCl for 24 hrs. at 105 C in sealed glass tubes. The HCl was evaporated and replaced with distilled water so that an approximate protein concentration of 1 mg. per ml. was present. A 100 ul sample was analyzed by an amino acid analyzer using Technicon Chromobeads C. The separated amino acids were detected with a Gilford Micro-Sample Spectrophotometer set at 540 mu. Use of the amino acid analyzer was kindly provided by Dr. Derek Lamport, AEC Plant Research Lab, M.S.U.

Microscopy

For electron microscopy whole oviducts from adult female R. pipiens were fixed at room temperature with 6.25 % glutaraldehyde in Sorensen's phosphate buffer (.2M) at pH 7.2 for 30 minutes. They were then divided into six segments corresponding to the regions described by Shivers and James (1970 A.) and washed thoroughly with Sorensen's phosphate buffer (.2 M). The tissues were post-fixed in 1 % osmium tetroxide in Sorensen's phosphate buffer (.2 M)

for 45 minutes and dehydrated through an alcohol series of 25%, 50%, 75%, 95% ETOH for 10 minutes each and 100% ETOH twice for 15 minutes each. The tissues were transferred into two changes of propylene oxide of 30 minutes each and then placed into a 1:1 mixture of propylene oxide and Epon 812 mixture (Luft, 1961). This 1:1 mixture was changed four times over a 48 hr period. The tissues were flat embedded in Epon 812 and left in a dessicator for 48 hrs. to allow for complete penetration. The Epon was polymerized in a 60 oven for 48 hrs. Thin sections, with an interference color of silver to gold, approximately 75 to 100 nm thick,were cut with glass knives on a Porter - Blum MT-2 ultramicrotome. The sections were collected on 150 or 200 mesh uncoated copper ethene grids. The sections were stained for 15 minutes by floating them on a concentrated aqueous uranyl-acetate solution and then for 5 minutes on an aqueous lead citrate solution (Reynolds, 1963).

The sections were observed on a Philips 100 B transmission electron microscope operating at 60 KV. Pictures were taken with 35 mm fine grain release positive film.

For light microscopy, whole oviducts were fixed in 10% neutral formalin for 1 hour. The oviducts were divided into segments corresponding to the six regions described by Shivers and James (1970 A.) and dehydrated through a series of alcohol changes as described above and embedded in parafin. Sections were cut on an A.O. Spencer No 820 Rotary Microtome at a thickness of 5 u meters. The sections were stained in Mayer's hematoxylin and eosin (Luna, 1968)

and observed with an A.O. Spencer light microscope. Pictures were taken with Kodak Pan-X 35 mm negative film.

RESULTS

Capacitation of Sperm

The first sperm capacitation experiment indicated that preinsemination treatment of sperm with egg water enhanced the percentage of cleavage of antijelly sera - treated eggs. The fertilization values from this experiment are shown in figure 2. Except for the eggs inseminated with sperm exposed to egg water for 15 minutes (J-15') the enhancement in cleavage does not reach the levels shown by the controls. The increase, however, is significantly higher than the antijelly sera - treated eggs inseminated with normal sperm. The use of control sera and Holtfreter - treated eggs inseminated with the treated sperm and the untreated sperm demonstrated two points. First the antijelly sera treatment was inhibitory to fertilization and second that the sperm exposure to egg water was not deleterious to normal fertilization. The capacitated sperm had the same fertilizing ability as normal sperm when inseminating Holtfreter or control sera - treated eggs. For example, the cleavage of Holtfreter - treated eggs was the same whether the sperm was exposed to egg water for 15 minutes (H-15') or untreated (H-U). These values were not significantly different from those of control sera - treated eggs inseminated with normal sperm (C-U) or sperm exposed to egg water for 15 minutes (C-15').

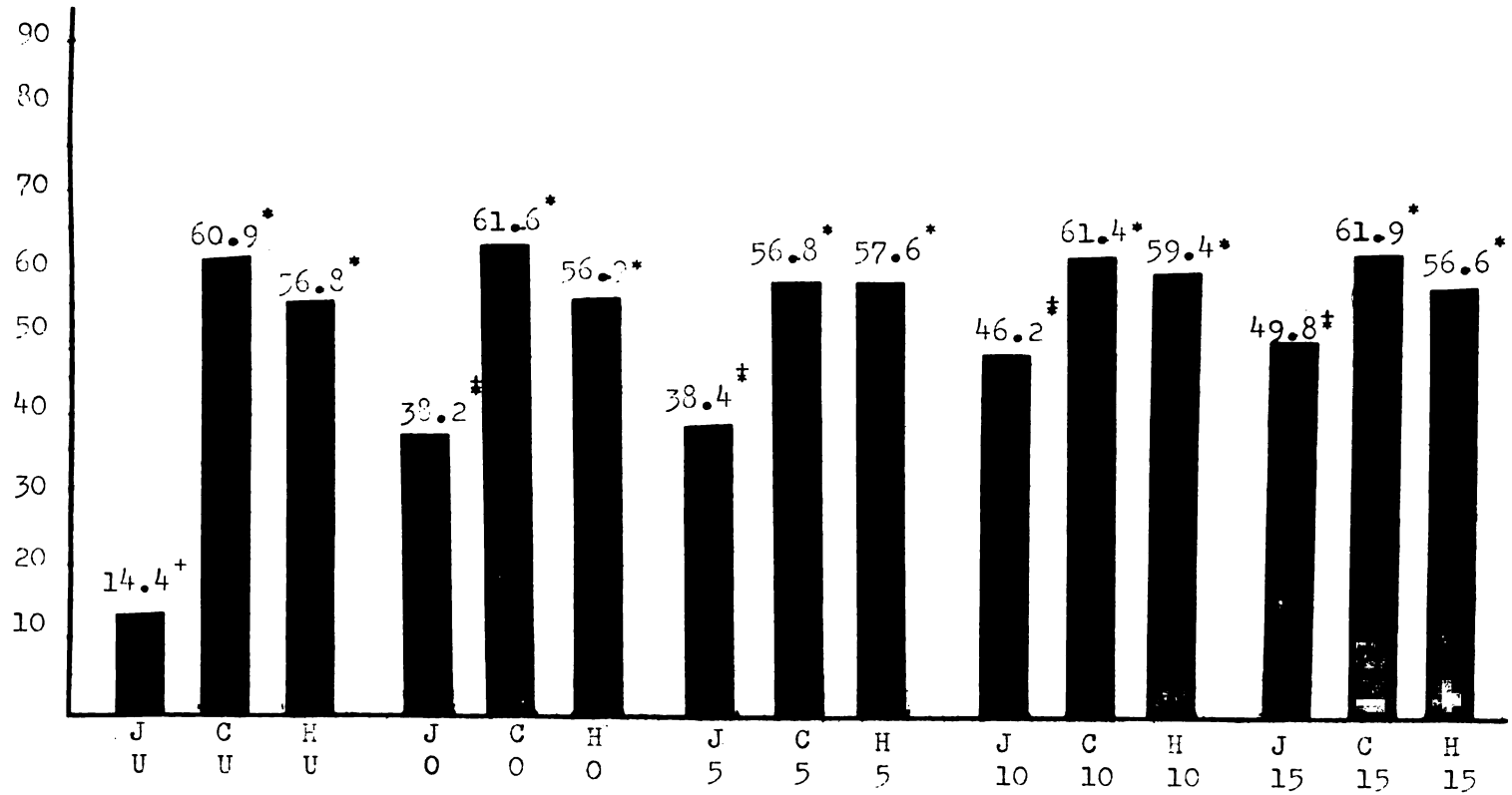

Figure 2. Capacitation experiment #1 : Fertilization values in arc sin equivalents of percentages of cleaving R. pipiens eggs after proinsemination treatment with antisera against the jelly coat (J), non-immune control sera (C), or Holtfreter's solution (H). Sperm capacitation was accomplished by exposure of sperm to egg water for 0 minutes (0), 5 minutes (5), 10 minutes (10), or 15 minutes (15). U = normal sperm suspension. * = values significantly higher than J-U. + = values significantly different than C-U.

When other types of antisera were used as in experiment # 2 (figure 3), it can be noted that in all cases, except that of antispleen sera (Sp-U and Sp-10'), treatment of sperm with egg water succeeded in allowing more eggs to be fertilized. As seen before in experiment # 1 the exposure to the egg water was not successful in increasing the fertilization of the eggs to the level seen with the control sera or Holtfreter - treated eggs (C-U, H-U, C-10', H-10'). In all cases treatment of sperm with egg water increased the success of fertilization of eggs treated with inhibitory antisera to approximately the same degree. The enhancement seen also corresponds to that observed in experiment # 1, keeping in mind that the sperm were exposed only for 10 minutes.

Antisperm sera were the most inhibitory of all sera used. Treatment with egg water significantly increased the cleavage of the antisperm sera - treated eggs (S-10') but the percent of cleavage was still far below the control treatments. Antijelly, antilower oviduct, and antimiddle oviduct sera inhibited to approximately the same degree. Anticlamitans jelly sera treatment produced similiar values. Antiovary, antikidney, and antiupper oviduct sera were also inhibitory to about the same degree, which was less than the inhibition mentioned above. Antispleen serum was the only antiserum used (except control serum) which showed no significant inhibition.

In experiment # 3 different parts of the oviduct were used for making capacitating solutions, as well as egg water.

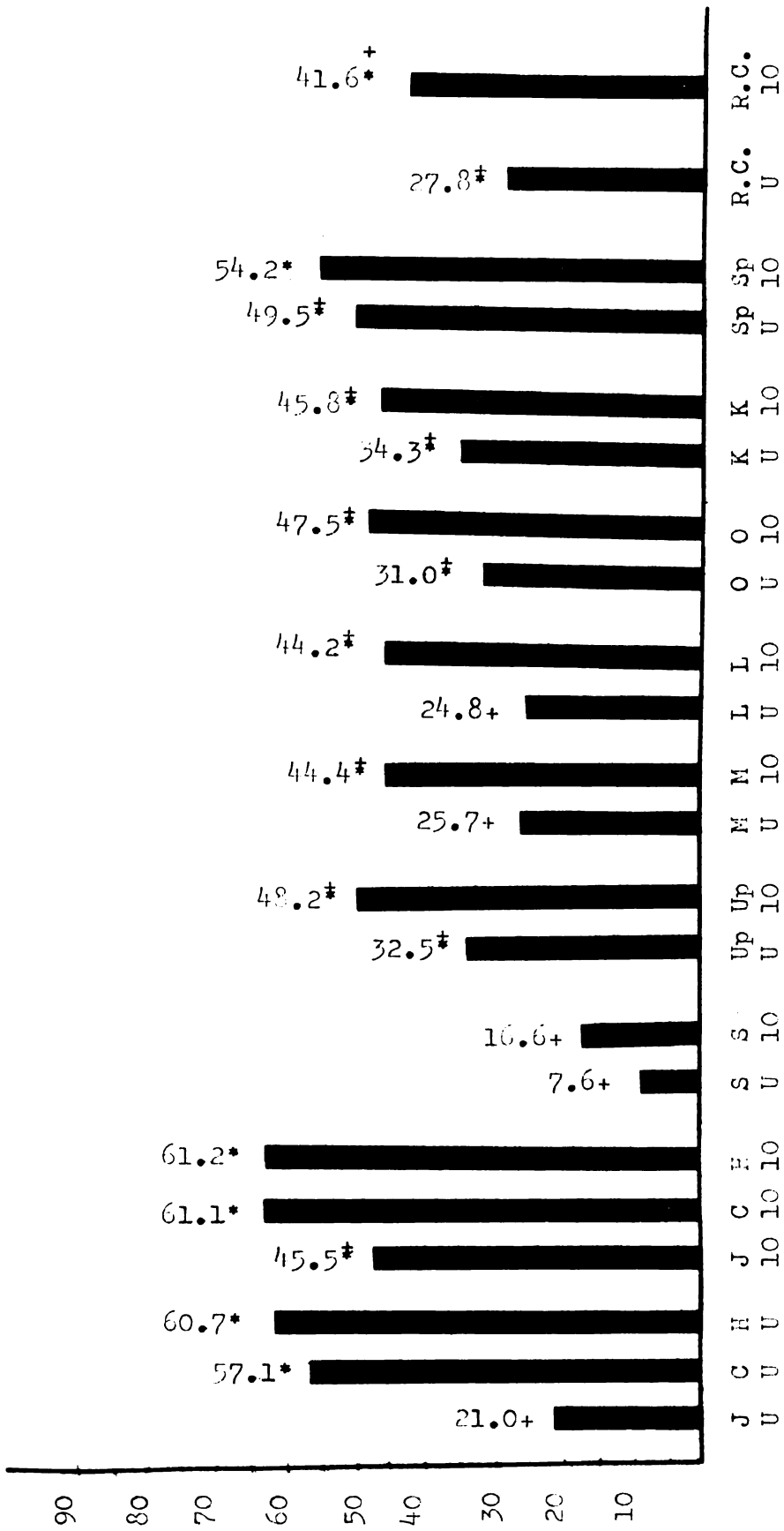

Figure 3. Sperm capacitation experiment # 2: Fertilization values in arc sin equivalents of percentages of cleaving R. pipiens eggs after presemination treatment with antisera against the jelly coat (J), sperm (S), upper oviduct (Up), middle oviduct (M), lower oviduct (L), ovary (O), kidney (K), spleen (Sp), R. clamitans jelly coat (R.C.), non-immune control sera (C), and with Holtfreter's solution (H). U = normal sperm suspension. lO = sperm mixed for lO minutes with an egg water solution. * = values significantly higher than J-U. + = values significantly different from C-U.

The results of this experiment are shown in figure 4. As seen in the previous experiments, antijelly sera - treated eggs inseminated with normal sperm (J-U) had a significantly lower fertilization rate. Treatment of sperm with the homogenates of upper, middle, or lower thirds or with whole oviducts as well as with homogenates of the six histochemically different oviducal regions described by Shivers and James (1970 A.), or with normal egg water raised the percentage of cleaving eggs treated with antijelly sera. No significant differences between the various capacitating solutions as to their ability to supress the inhibition was noted. The exception to this is a very small significant difference between the capacitating solution from oviducal region 1 (J-1) and the capacitating solution from the whole oviducal extract (J-W). Again the enhancement in cleavage with the treated sperm does not reach the levels shown by the controls.

In the fourth experiment the capacitation activity of the egg water was tested. The results of this experiment are shown in figure 5. Of the four solution used, only the supernatant (J-S) was no longer able to increase the fertilizability of the antijelly sera - treated eggs. The ability of the egg water to effect a higher percentage of fertilizability can thus be used up or removed. The mere spinning of the egg water in the centrifuge did not significantly remove the activity of the egg water (J-Spun). The activity of the egg water also seems to be stable over a 24 hr. period of time. As with the normal egg water, the

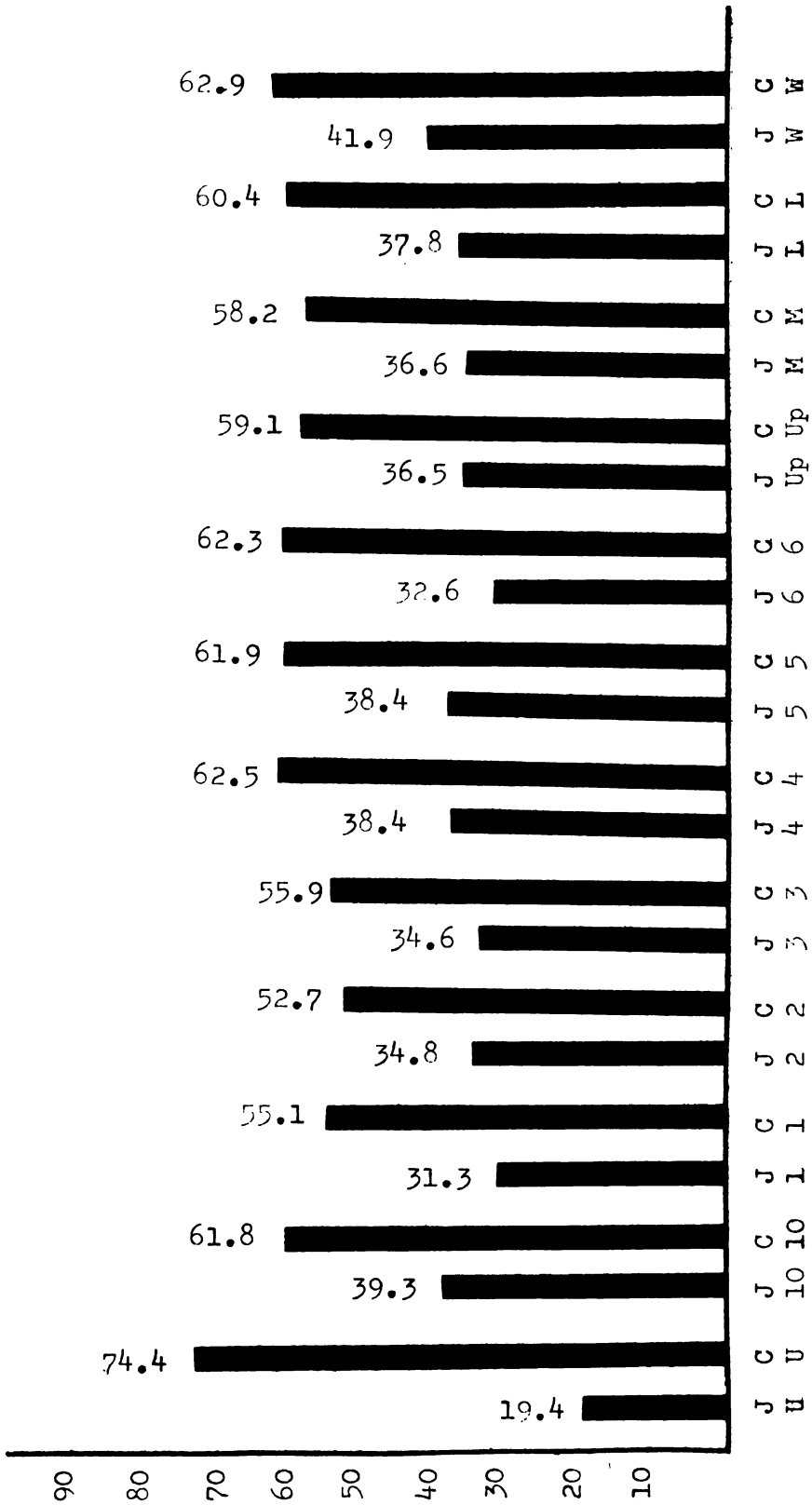

Figure 4. Sperm capacitation experiment # 3: Fertilization values in arc sin equivalents of percentages of cleaving R. pipiens eggs after preinsemination treatment with antisera against the jelly coats (J) or non-immune control sera (C). Sperm capacitation was accomplished by 10 minute exposure to jelly materials exuded from oviducal regions 1 through 6 (1-6), upper third oviduct (Up), middle third oviduct (M), lower third oviduct (L), whole oviduct (W), and egg water (10). All values are significantly higher than J-U.

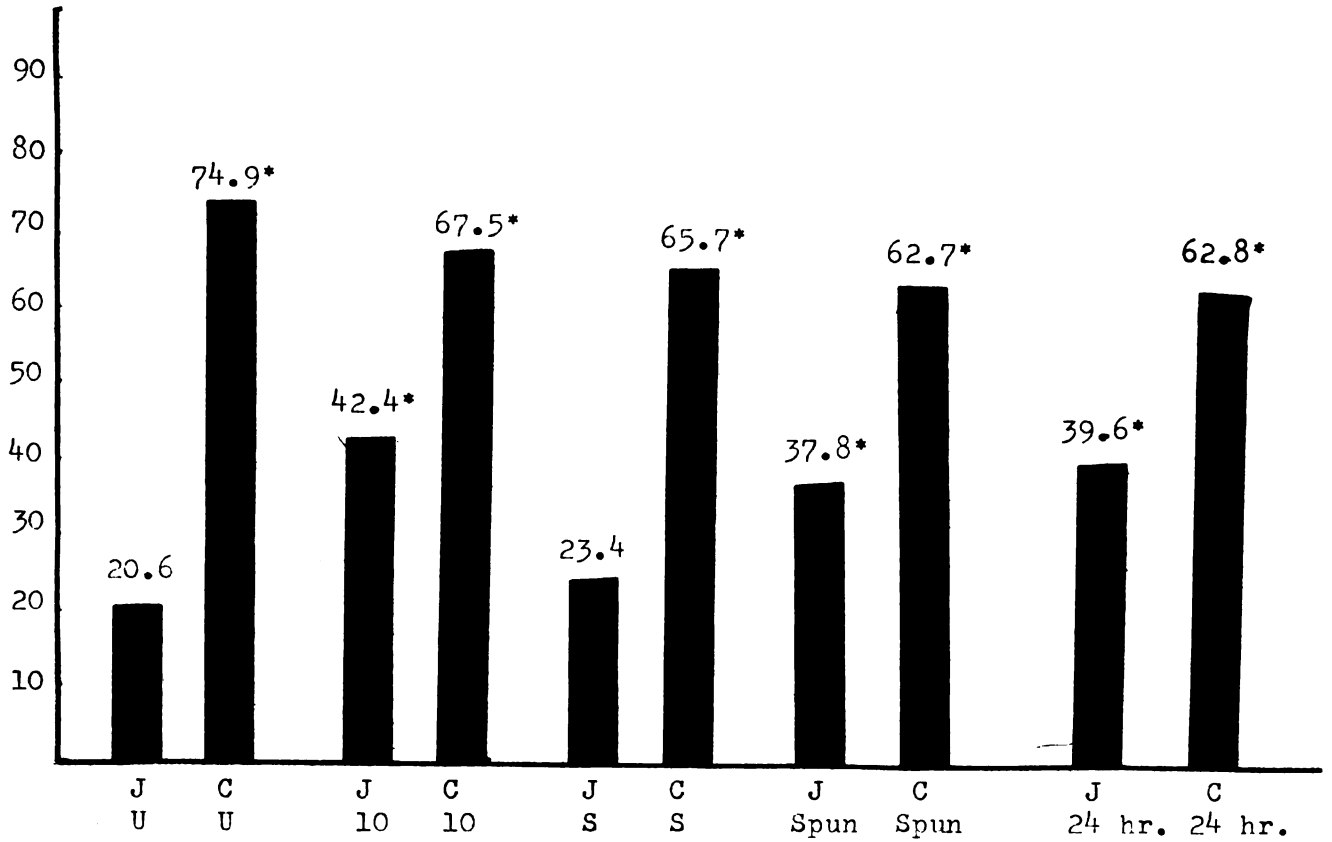

Figure 5. Sperm capacitation experiment # 4: Fertilization values in arc sin equivalents of percentages of cleaving R. pipiens eggs after preinsemination treatment with antisera against the jelly coat (J) or non-immune control sera (C). Sperm capacitation was accomplished by mixing sperm for 10 minutes in egg water (10), 24 hr. old egg water (24 hr.), egg water spun for 15 minutes at 11,500 x g (Spun), and the supernatant of a 10 minute egg water - sperm suspension (S). U = normal sperm suspension. * = values significantly higher than J-U.

increase in percentage of cleavage (J-24 hr.) is the same. In accordance with the other experiments, treatment of sperm with fresh egg water, 24 hr. old egg water, and spun egg water increased the success of fertilization significantly above that of the untreated sperm (J-U) but the level seen in the controls was still not reached.

The results of the protein determinations of all egg water and oviducal extracts used are given in figure 6. Such determinations were used as an indicator to note if jelly or egg substances had diffused into the surrounding media. Note that the egg water - sperm supernatant of experiment # 4 had a tremendous increase in protein content but showed little capacitating ability.

Chemical Composition

The results of Nelson's test, which gives an indication of reducing sugars present in the jelly,is shown in figure 7a. Approximately 25% of the jelly is reducing sugars, a value consistent with other investigators' work. Inorganic phosphate as determined by the Fiske-Subbarow method (figure 7b) is not present. Results from the Mejbaum method of pentose determination as shown in figure 7c disclosed no pentose sugars. Also the deoxypentose determination revealed no amounts of this sugar present in the jelly (figure 7 d). The Lowry method for protein determination, shown in figure 7e, indicated that approximately 47.5% of the egg jelly is protein.

Solution	Optical Density	Protein Concentration (mg/ml)	
Egg water for experiment # 1.	.165	.30	
	.245	.44	
	.400	.70	
	.160	.27	Average .43
Egg water for experiment # 2.	.100	.15	
	.140	.24	
	.195	.32	average .24
Egg water for experiment # 3	.16	.26	
	.14	.24	
	.14	.24	
Extracts used in experiment # 3 from oviducal regions: 1	.21	.36	average .38
1	.15	.26	
2	.15	.26	
3	.14	.24	
4	.12	.18	
5	.13	.22	
6	.64	1.14	
Whole oviduct	.20	.34	
upper "	.28	.50	
middle "	.37	.66	
lower "	.31	.55	
Egg water for experiment # 4	.16	.26	
	.14	.24	
	.14	.24	
	.21	.36	average .38
Sperm-egg water supernatant for experiment # 4	.67	1.2	
	.93	1.7	
	.84	1.5	
	.50	.9	average 1.32
24 hr. old egg water for experiment # 4 O.D. values did not change over the 24 hrs.	.08	.12	
	.165	.30	
	.115	.18	
	.31	.54	average .28

Figure 6. Protein determinations on solutions used to treat or capacitate sperm. A standard curve of bovine albumen was used to convert absorbancy to mg. of protein per ml. All solutions were read at 280 mu on a Hitachi Model 101 UV*Vis Spectrophotometer.

Figure 7 a. Nelson's test

Sample	Concentration	Optical Density 540 mu	% reducing sugar
Standard (galactose)	.07 mg/ml	.61	
Standard "	.10 "	.92	
Jelly	.10 "	.21	25%

Figure 7 b. Fiske-Subbarow inorganic phosphate determination

Sample	Concentration	Optical Density 660 mu	% Pi
Standard	.8 umoles/ml	.93	
Standard	.4 umoles/ml	.489	
Jelly	.1 mg/ml	.046	$\angle$ 1%

Figure 7 c. Mejbaum pentose determination

Sample	Concentration	Optical Density 660 mu	% Pentose sugar
Standard	1 u mole ATP	.492	
Jelly	.1 mg/ml	.020	$\angle$ 1%

Figure 7 d. Deoxypentose determination

Sample	Concentration	Optical Density 595 mu	% Deoxypentose sugar
Standard	1 u mole deoxyadenosine-5' -phosphate	.43	
Jelly	.1 mg/ml	.01	$\angle$ 1%

Figure 7 e. Lowry protein determination

Sample	Concentration	Optical Density 660 mu	% Protein
Standard	50 ug/ml	.20	
Jelly	100 ug/ml	.19	47.5%

The results of the amino acid analysis on various oviducal extracts of R. pipiens, R. clamitans, R. catesbieana, and Bufo fowleri are shown in figure 8. The values of valine, cystine, and methionine are consolidated as one since sugar residue contamination appears at the same time these amino acids come off the column. Therefore it must be kept in mind that the values for these three amino acids also incorporate some sugar contamination in the hydrolyzed sample. The lysine content of R. clamitans oviducal extract was lost due to a mechanical failure of the recording instrument. Those amino acids which absorb at 440 mu and proline were not analyzed.

The amino acid content of the different parts of the R. pipiens oviduct has minor variations as can be noted in the lysine, glutamic acid, and threonine values. The middle and lower oviducal extracts consistantly showed almost the same percentages throughout the amino acids tested. In addition some amino acids such as tyrosine, isoleucine, and phenylalanine are present in practically the same amount in the oviduct extracts of R. clamitans, R. catesbieana, and Bufo fowleri as well as R. pipiens. It should be noted, nonetheless, that variations are present between the species tested. This is seen, for example, in the serine, glutamic acid, and threonine percentages. However the differences apparent in the amino acid content have no set pattern.

Amino Acid	W	U	M	L	O	W R. C.	C a t	B. F.
Serine	8.0	9.0	7.5	7.5	7.8	9.7	7.8	12.4
Glycine	8.1	9.0	8.5	9.1	7.8	9.3	8.3	5.2
Asp. Acid	10.0	10.3	9.6	9.3	8.1	11.0	8.5	8.7
Val., Cys., & Meth.	19.5	17.4	19.5	20.5	22.3	16.4	15.5	17.4
Glut. Acid	9.3	11.5	9.4	9.1	7.7	12.2	8.0	9.3
Threonine	17.5	11.0	17.5	16.8	21.6	13.8	16.6	18.2
Tyrosine	2.5	2.7	2.6	2.6	2.3	2.8	1.9	2.7
Alanine	4.3	5.5	4.5	4.3	3.7	6.5	8.4	4.5
Histidine	1.2	1.8	1.6	1.5	1.1	1.6	0.7	1.0
Leucine	4.3	5.1	4.8	4.8	3.5	6.6	3.2	4.9
Phenylalanine	2.0	2.6	2.2	2.1	1.4	3.0	1.5	2.7
Arginine	4.8	2.9	2.4	2.3	3.1	3.2	1.5	2.7
Isoleucine	4.1	4.1	4.3	4.0	4.2	4.0	2.1	3.7
Lysine	4.5	7.2	6.2	6.2	5.5	---	6.9	5.9

Figure 8. Amino acid content of lyophylized homogenate extracts from the whole oviduct of R. pipiens (W), upper ⅓ oviduct (U), middle third oviduct (M), lower third oviduct (L), the outer jelly layers of R. pipiens eggs (O), the whole oviduct of R. clamitans (W R.C.), the whole oviduct of R. catesbieana (Cat), and Bufo fowleri (B.F.). The numbers represent percent of the protein content.

Microscopy

Although sections were taken at the various oviducal levels described in the materials and methods, few if any ultrastructural differences were noted which could be correlated to differences in the jelly secretions. However much information was obtained on the morphology of the oviduct. Sections shown in figures 9 through 22 were taken at various oviducal levels but are representative of the oviduct as a whole.

A major portion of the mature female oviduct was noted to have three basic cell types. First ciliated epithelial cells were present along the folded lining of the lumen. Interspersed with these are mucus - secreting cells which also border the lumen of the oviduct (figure 9). With light microscopy these two cell types were difficult to distinguish due to their common location. They both appear distally to the third cell type commonly called the jelly - secreting or jelly - producing cells. At the time of sacrifice of the females, the oviducts were near the end of glandular growth and so were filled with jelly secretions. The jelly - secreting cells constitute the bulk of the oviduct and are arranged as tubular glands (figure 9).

Starting with the outermost edge of the oviducts, several layers of muscle and connective tissue are seen which anchor the oviducts to the body wall (figure 10). Immediately adjacent are the jelly - producing gland cells which constitute most of the oviduct. They are packed with

secretory material which consists of electron - dense secretory granules surrounded by a white mucus type capsule (figures 10, 11, 12). Due to the large amount of secretory material produced by the cells, many of the normal cell constituents such as the nucleus, mitochondria, golgi, etc. are pushed to the periphery of the cell or crowded into a very small area (figure 12) so that, depending on the plane of section, these cells appear to be filled only with the secretory material (figure 11). Throughout the oviduct there is also evident a rich capillary network (figure 13) which sometimes appears very close to the lumen. Permeating the glandular portion of the oviduct are many small secretory ducts which seem to connect with the lumen (figure 14). The ducts seem to be lined with micro - villi which perhaps aid the movement of the secretory material toward the lumen during ovulation.

Moving inward toward the lumen there is a sharp boundary between the jelly - producing cells and the mucus - secreting cells (figure 15). The mucus - secreting cells are also packed with a secretory substance. This substance, however, lacks the granular structure of the jelly - producing cells. The nuclei and other cytoplasmic constituents also seem to be crowded into a small area by the secretory product (figures 16, 17, 18). There is usually a single layer of this type of cell present. The secretory product seems to be exuded directly into the lumen (figure 18).

The third cell type, the ciliated epithelial cell, does

not appear to have any secretory product. It usually possesses a large and folded nucleus with an abundant amount of ribosomes and mitochondria (figures 19, 20, 21). Numerous cilia are present with anchoring elements extending into the cytoplasm. These cilia display the typical 9 + 2 structure. Projecting between the cilia of these cells are micro - villi.

All three cell types alternately border on the lumen (figure 22). The secretory product of the jelly producing cells seems to change before exiting into the lumen. The electron dense core seems to lose its white mucus envelope (figure 19). This could be due to the time of fixation. The animal was not induced to ovulate and so the cells shown are in a state of jelly production, not jelly secretion.

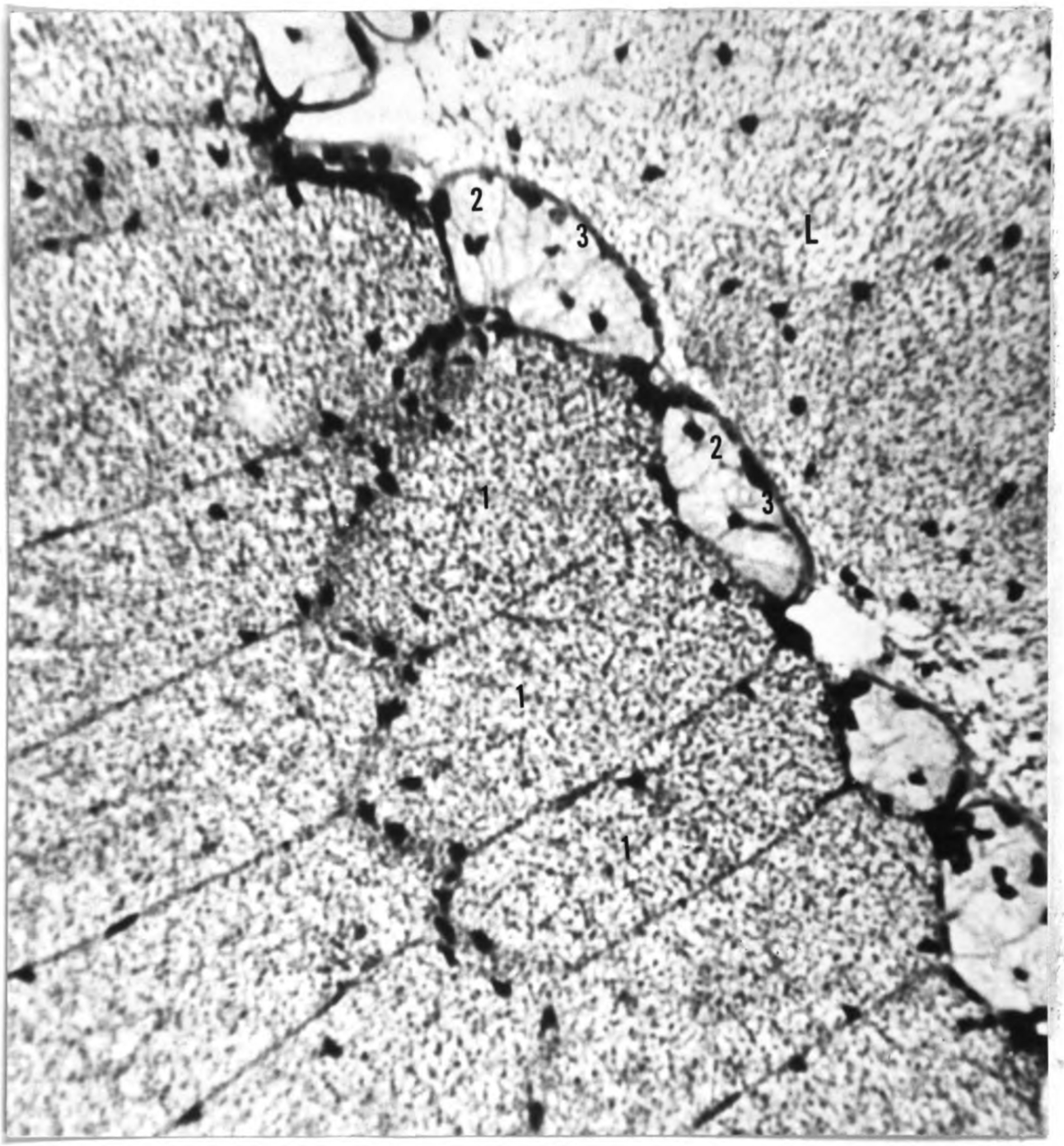

Figure 9. Section of an oviduct of an adult female Rana pipiens. Three cell types are present: (1) jelly-producing, tubular gland cells, (2) mucus-secreting cells, (3) ciliated epithelial cells. Hematoxylin and eosin 150 X

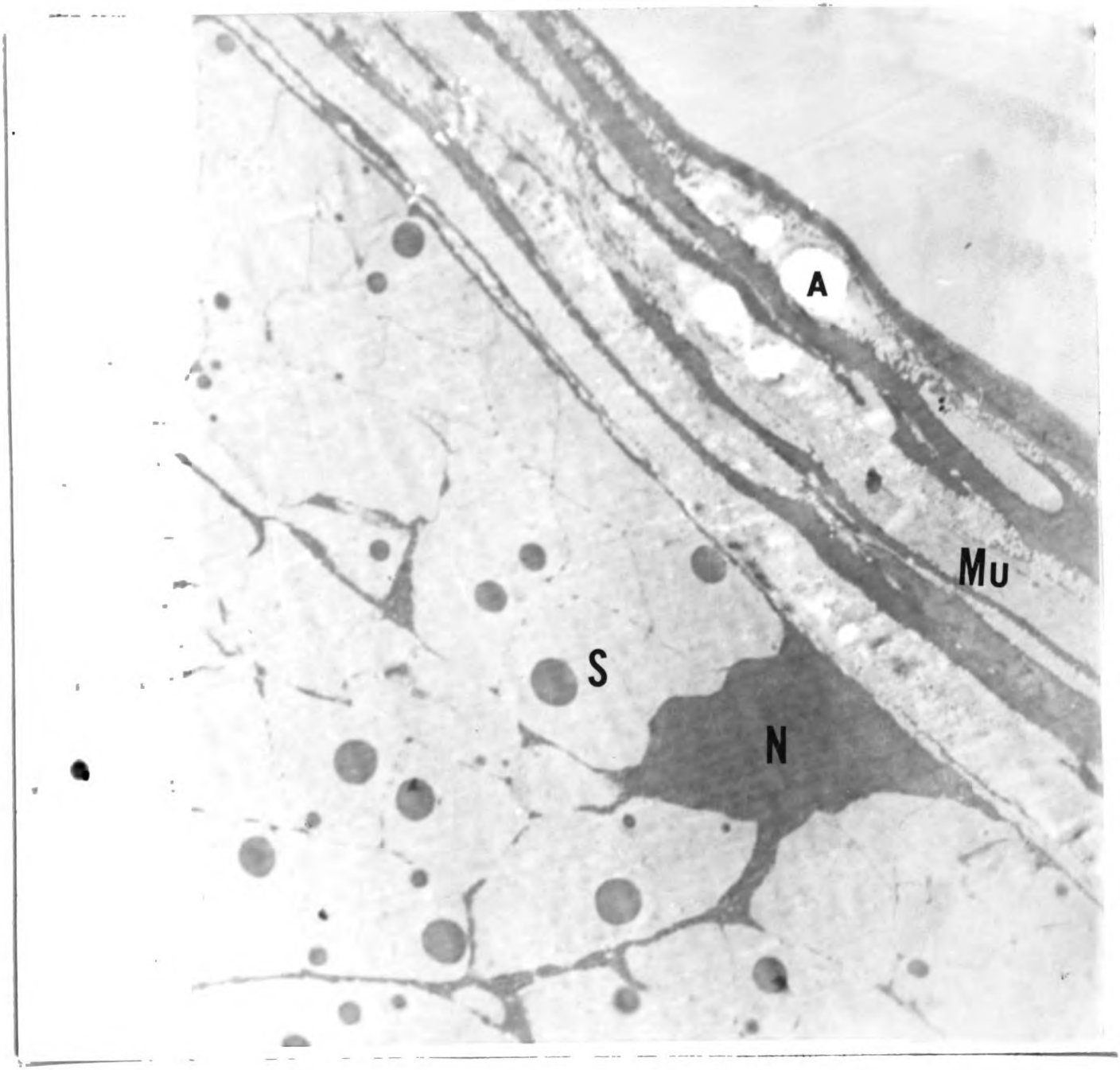

Figure 10. Outermost edge of the oviduct showing muscle (Mu) which anchors the oviduct to the body wall. N = nucleus, S = secretory material, A = artifact 11,000 X

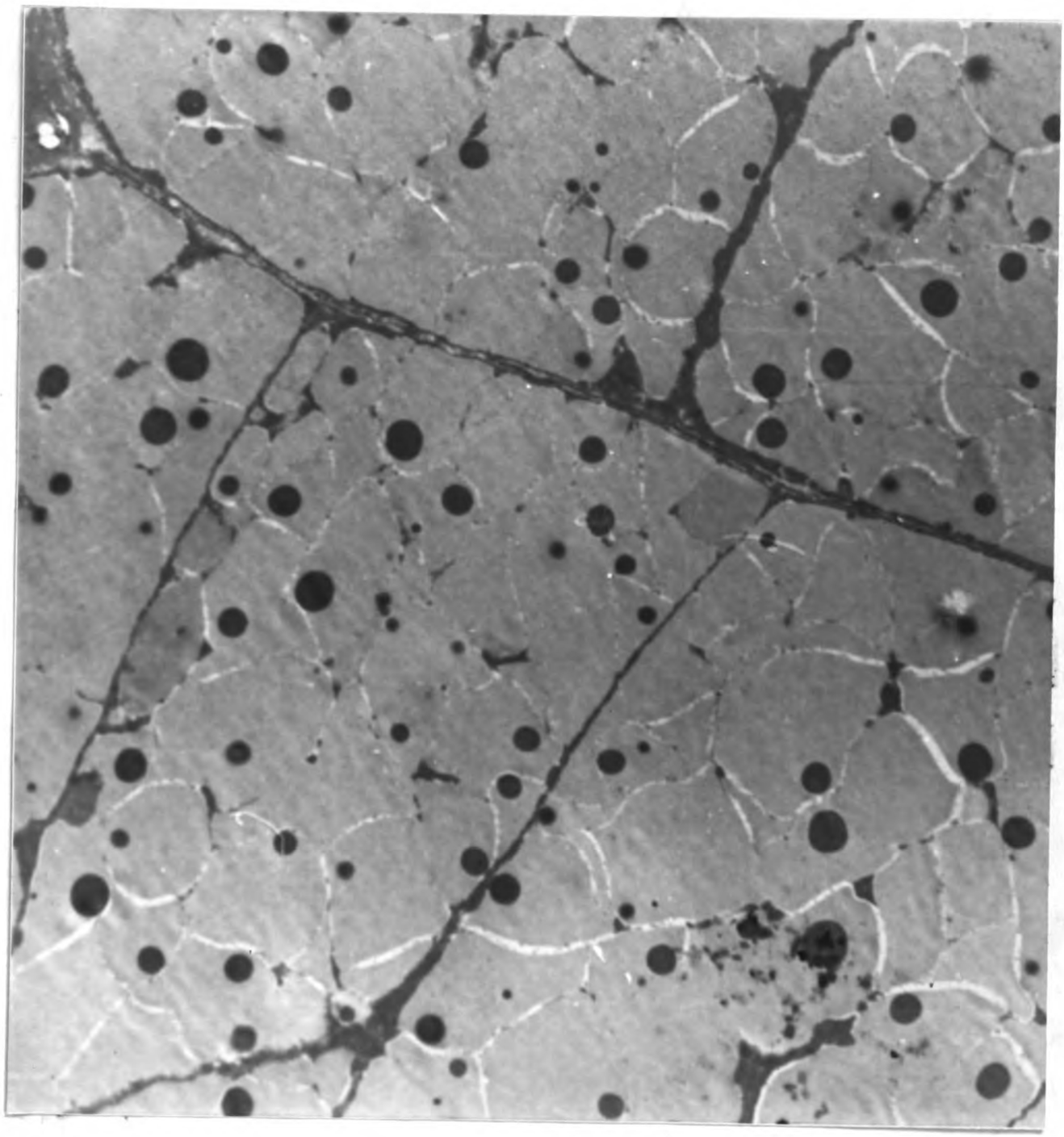

Figure 11. Jelly-producing cells packed with secretory products. Five cells are shown. 9,000 X

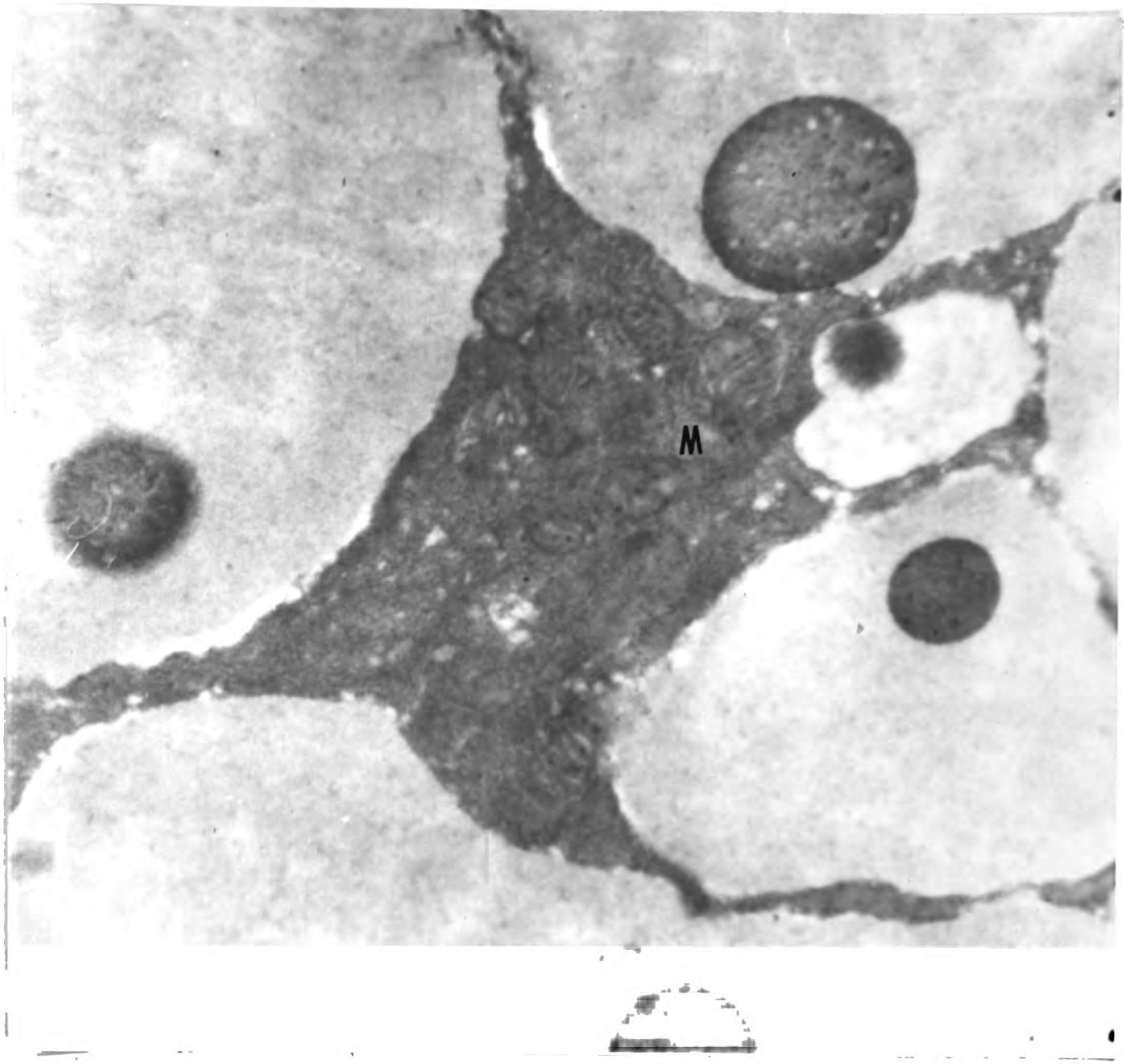

Figure 12. Mitochondria (M) of a jelly-producing cell crowded into a small area by the secretory materials. 41,000 X

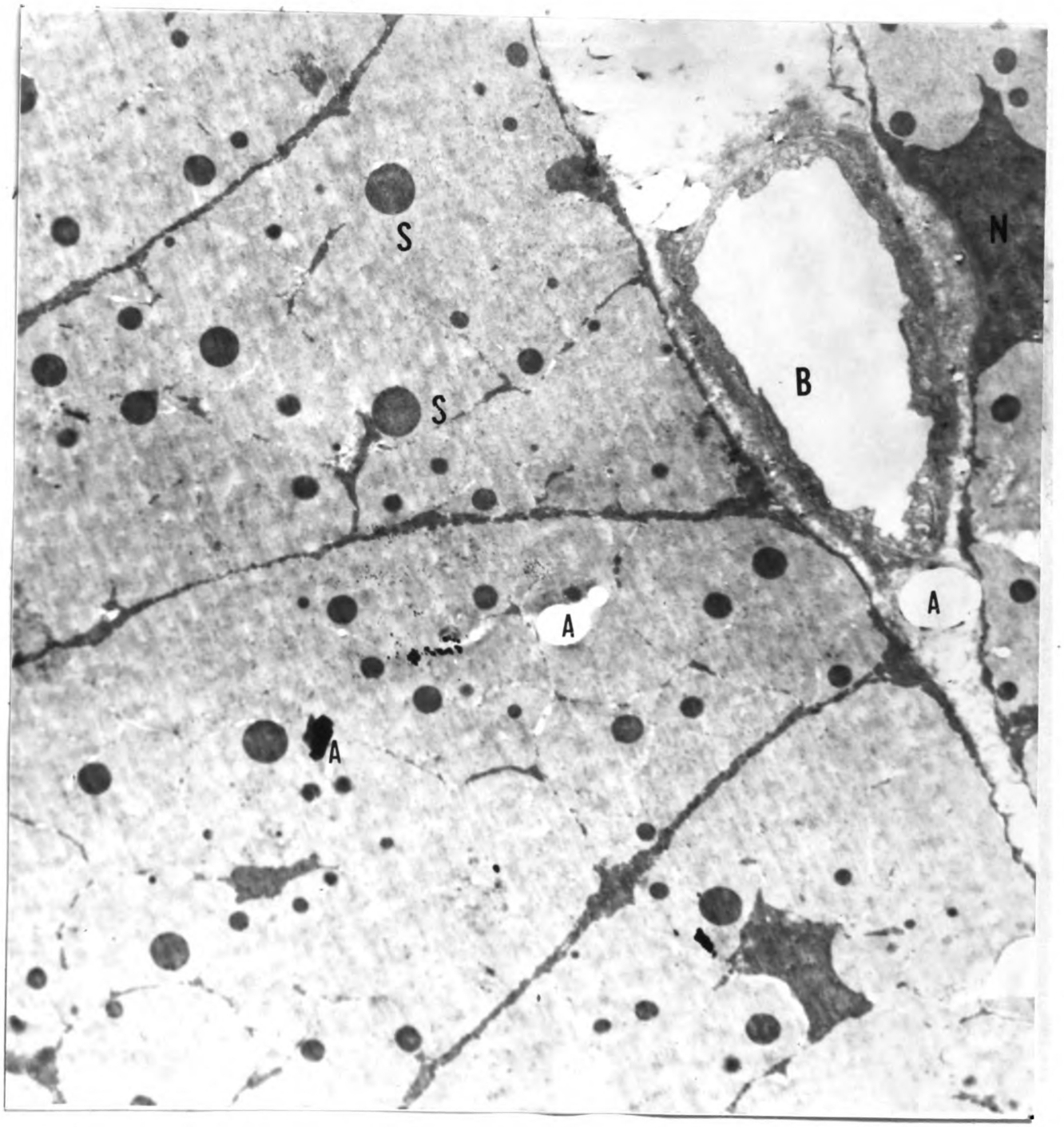

Figure 13. Blood vessel (B) surrounded by jelly-producing cells. Artifacts (A) are holes in the Epon and stain residue. N = nucleus, S = secretory granules. 8,000 X

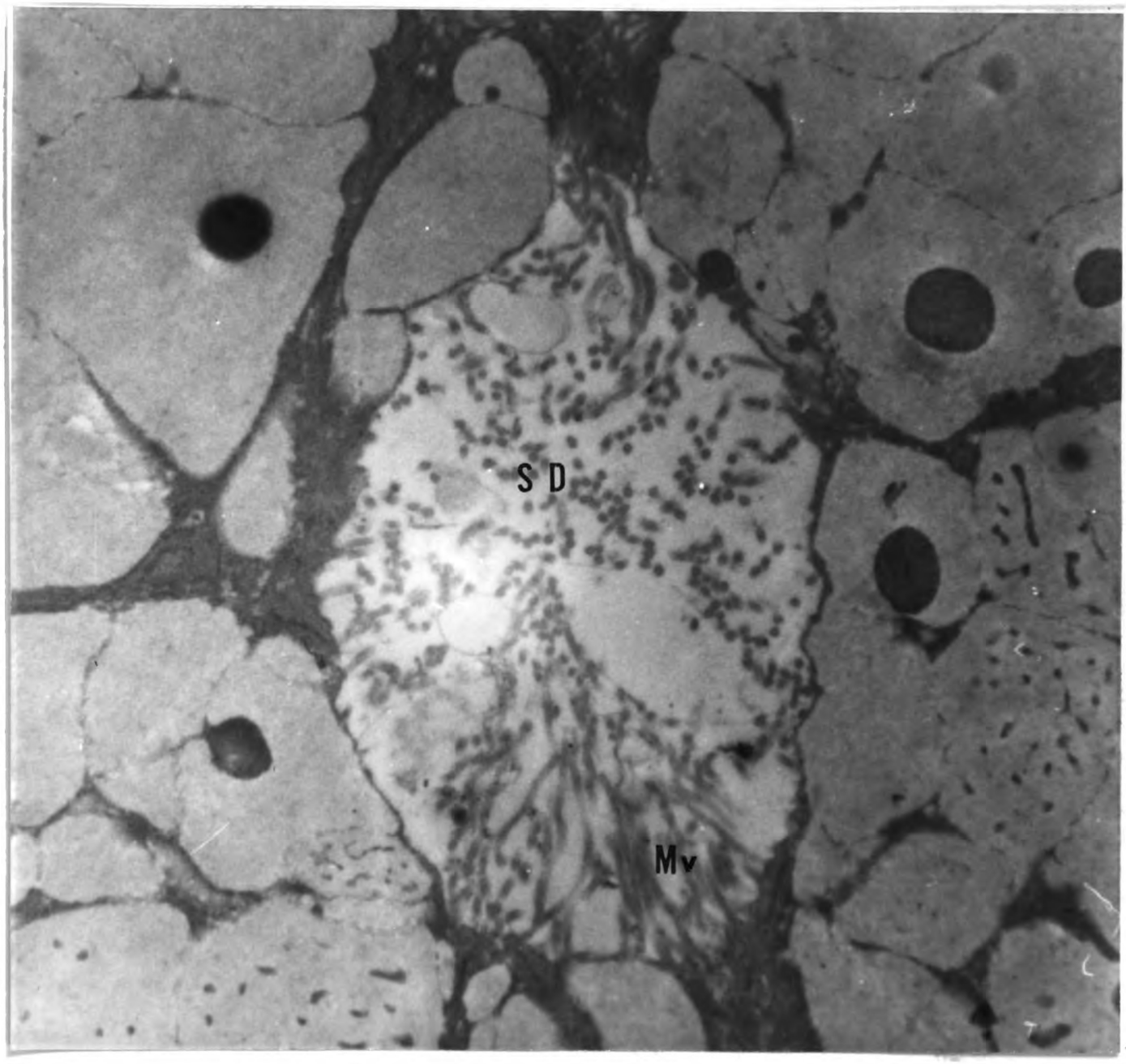

Figure 14. Secretory duct (SD) which connect the jelly-producing cells with the lumen. Microvilli (Mv) are present. 20,000 X

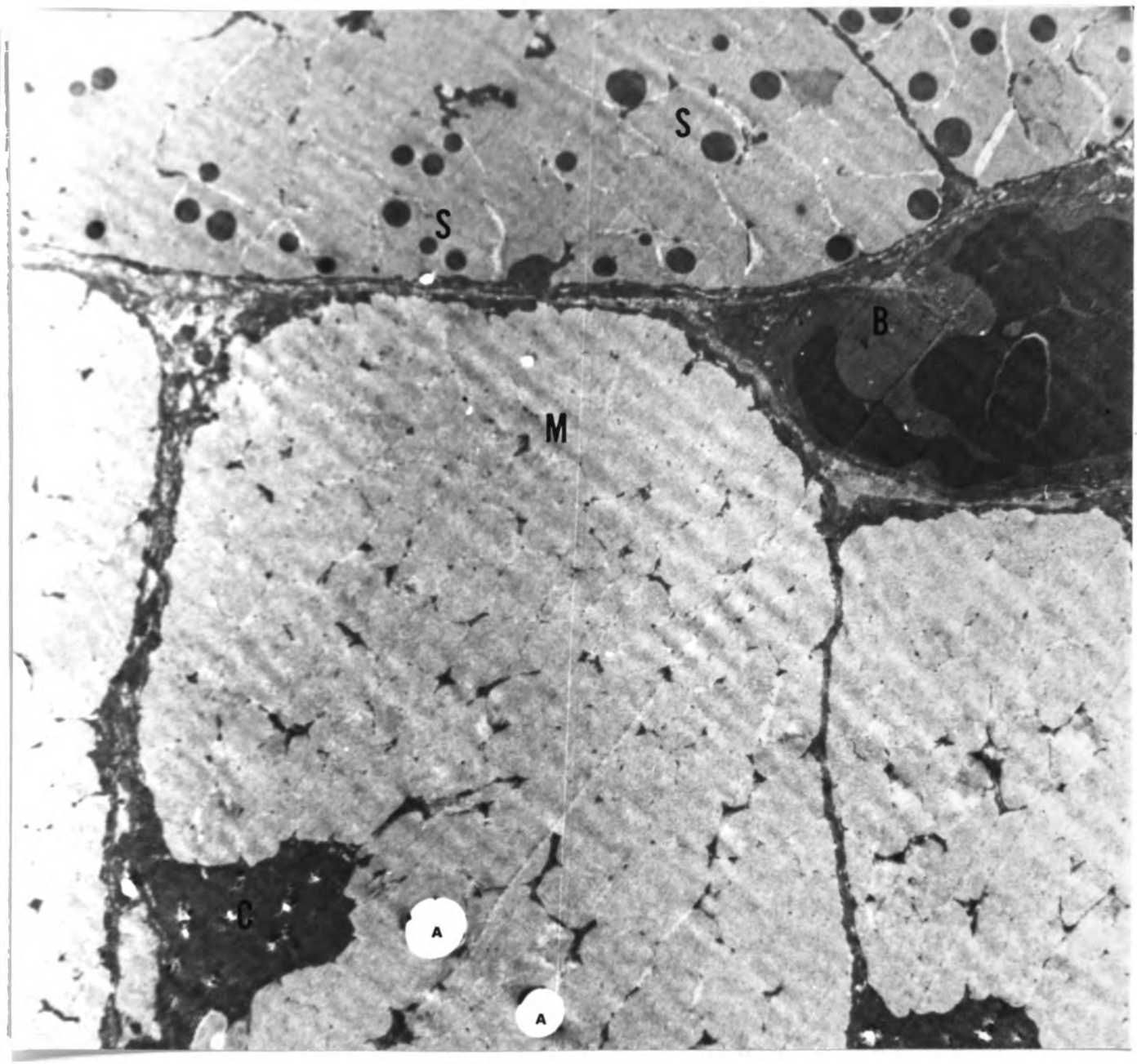

Figure 15. Boundary between the jelly-producing cells and mucus secreting cells. A blood vessel is also apparent (B). S = secretory product of jelly-producing cell, M = secretory product of the mucus secreting cells, C = cytoplasm of mucus cell, A = artifacts. 8,000 X

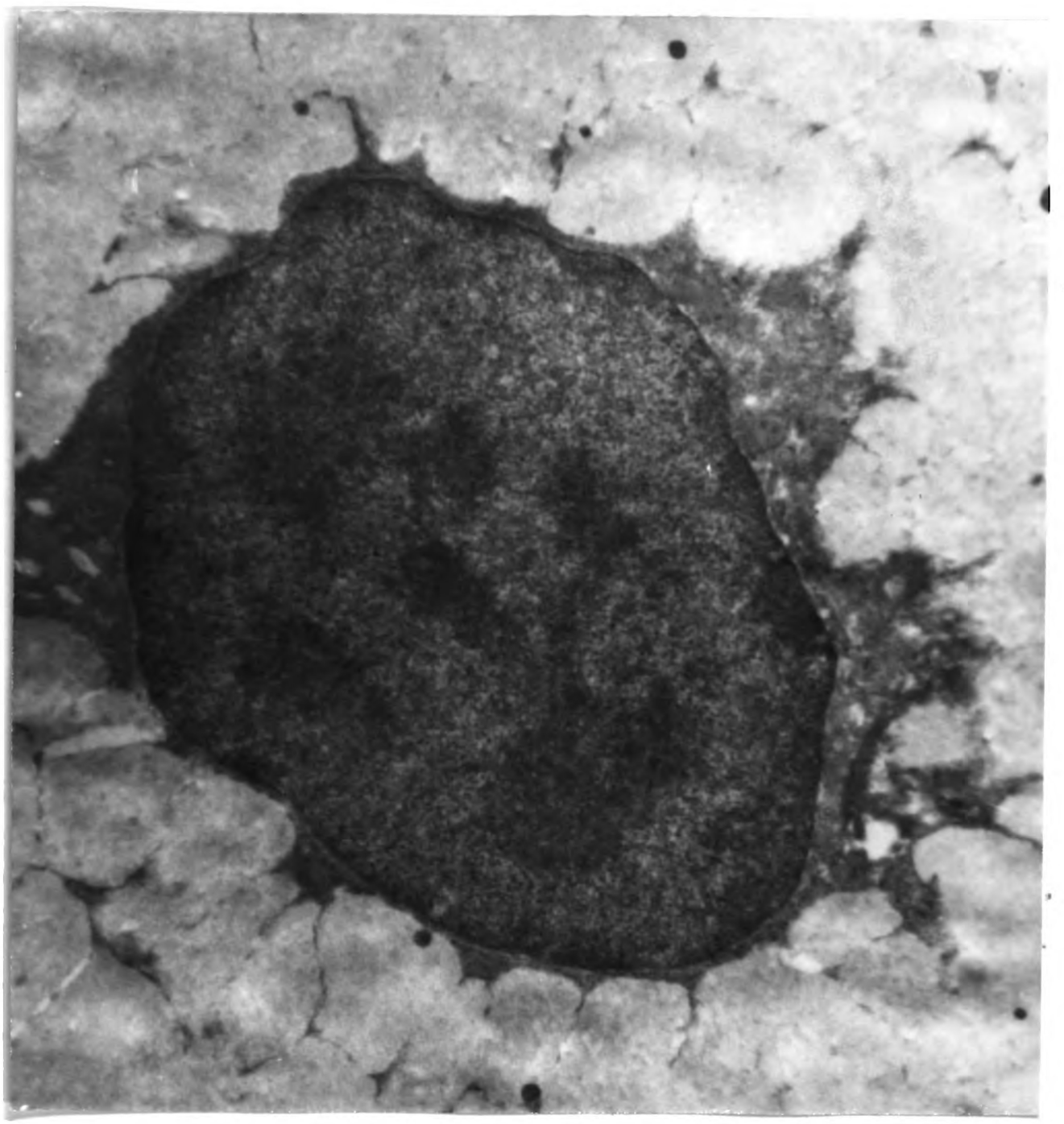

Figure 16. Nucleus of mucus secreting cell surrounded by the cell's secretory product 30,000 X

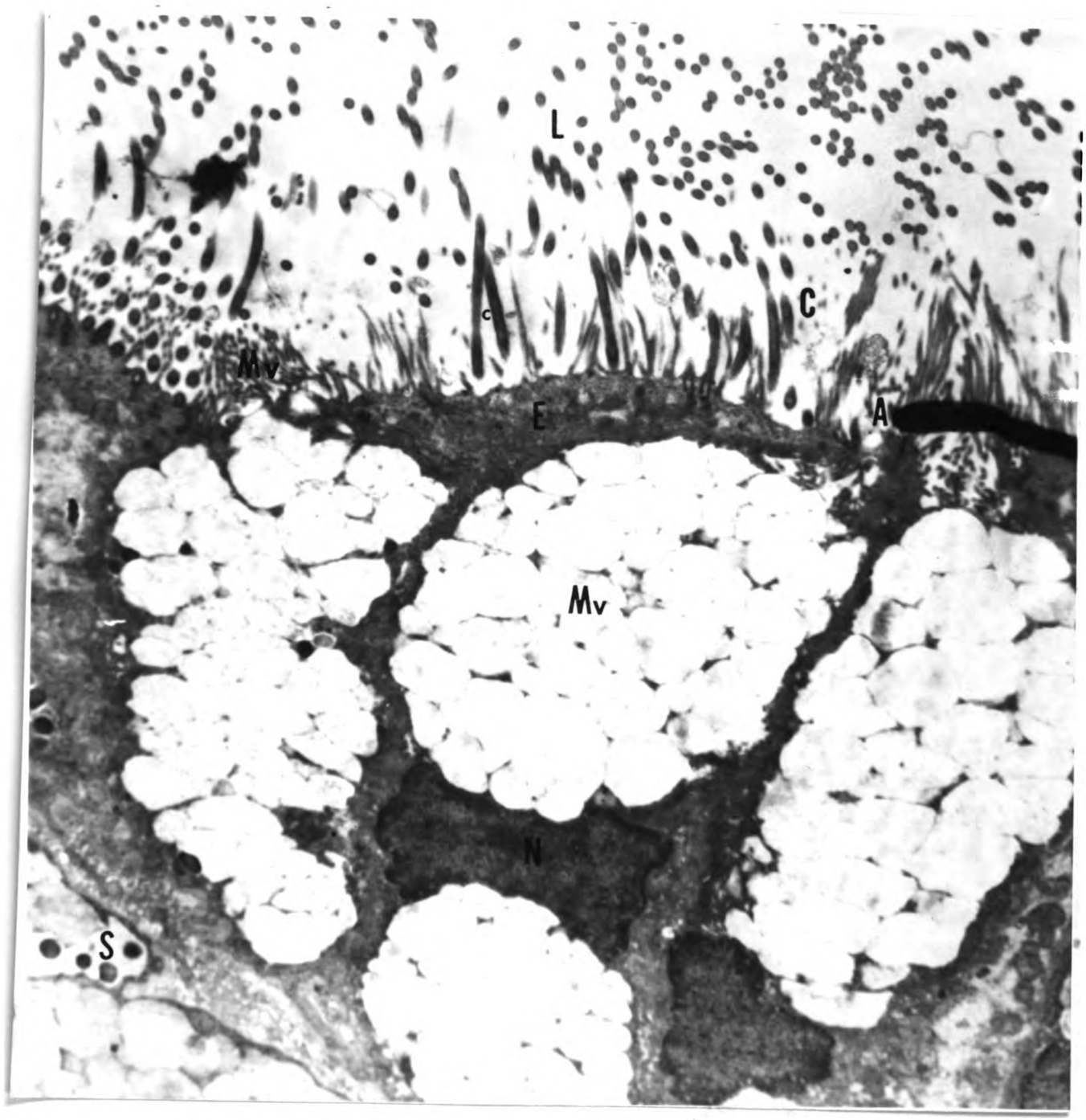

Figure 17. Mucus secretory cells bordering the lumen (L) of the oviduct. Parts of a ciliated epithelial cell (E) are also present. N= nucleus, S= secretory product of jelly-producing cells, Mu= secretory product of mucus cell, C = cilia, Mv = microvilli, A= artifacts. 9,000 X

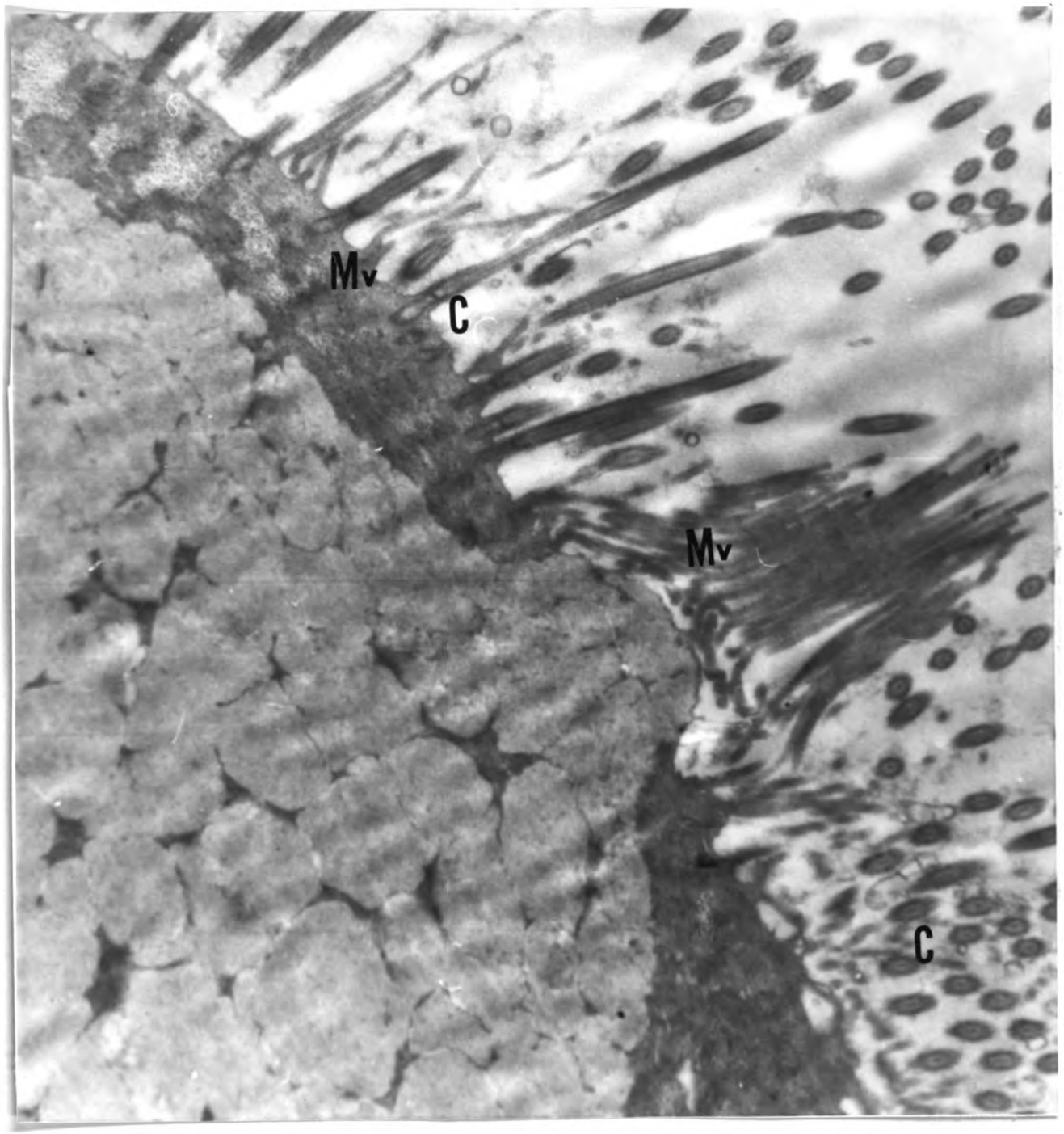

Figure 18. Secretory product of mucus cells being exuded into the lumen of the oviduct. C = cilia, Mv = microvilli. 19,000 X

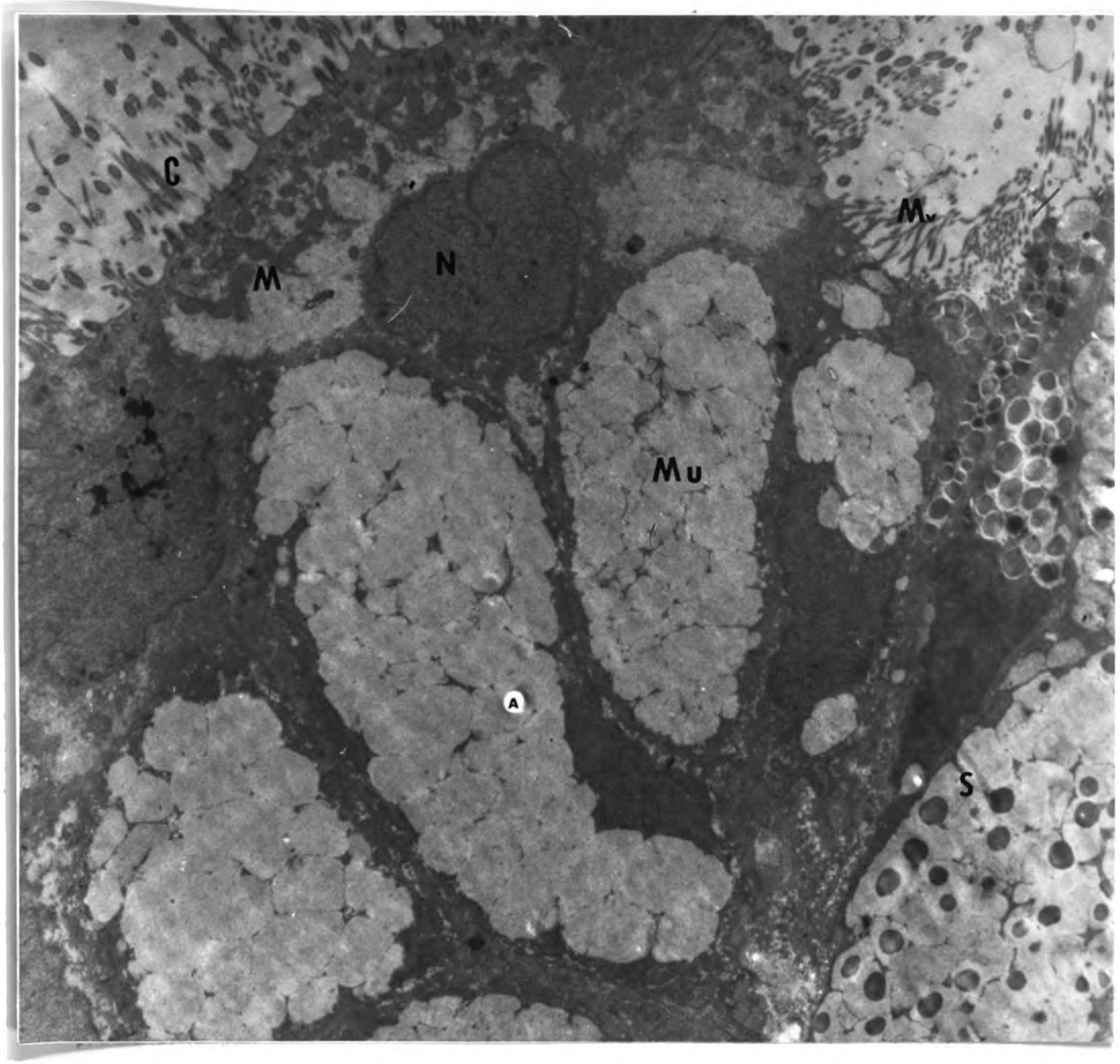

Figure 19. Section shownig all three cell types of the oviduct. Products of both secretory cell types are being exuded into the lumen. N= nucleus of ciliated epithelial cell, M= mitochondria, C= cilia, Mv = microvilli, S = secretory product of jelly producing cells, Mu = secretory product of mucus cells. A = artifacts. 8,500 X

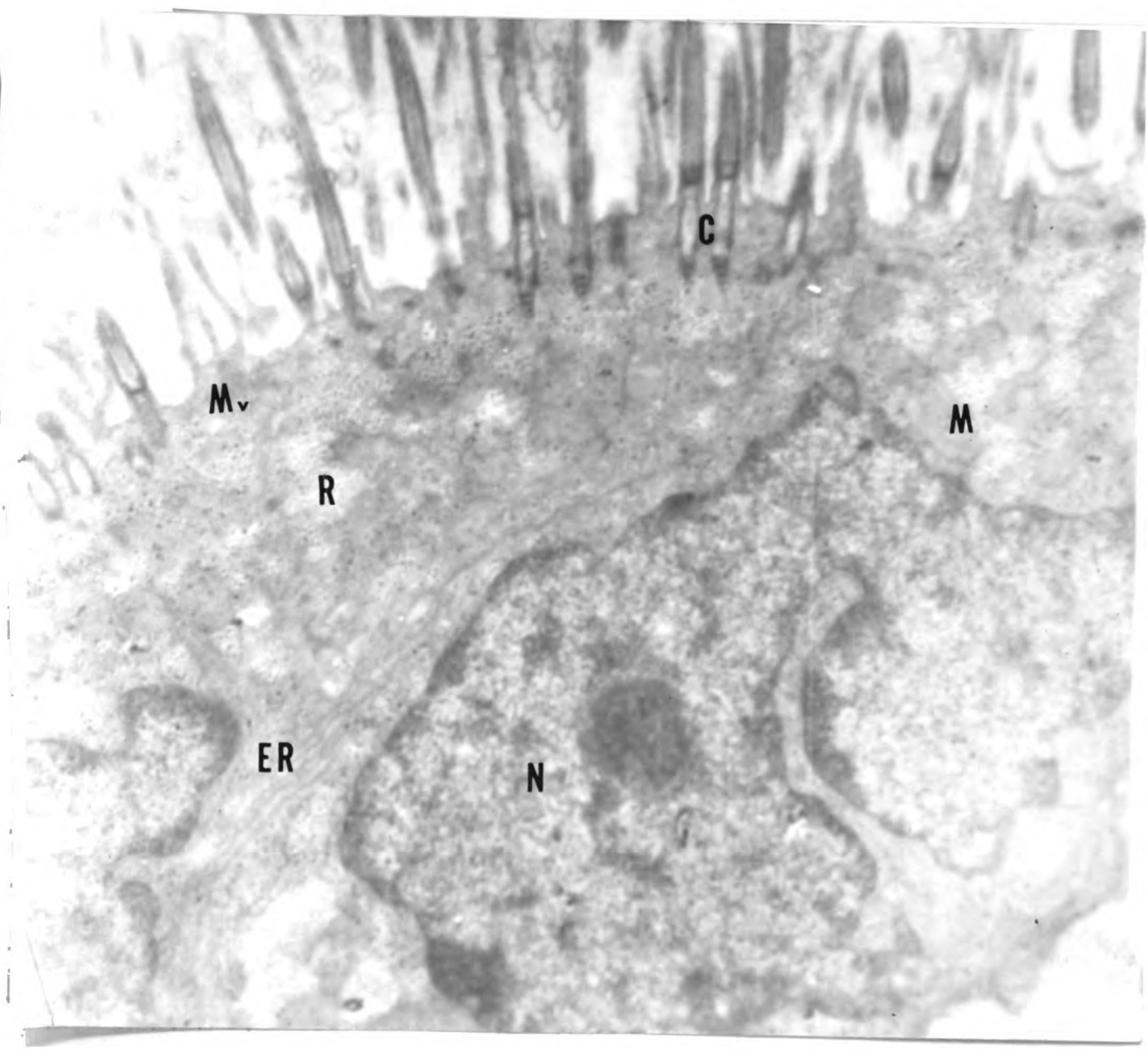

Figure 20. Ciliated epithelial cell. N = nucleus, C = cilia, M = mitochondria, R = ribosomes, Mv = microvilli, ER = endoplasmic reticulum. 30,600 X

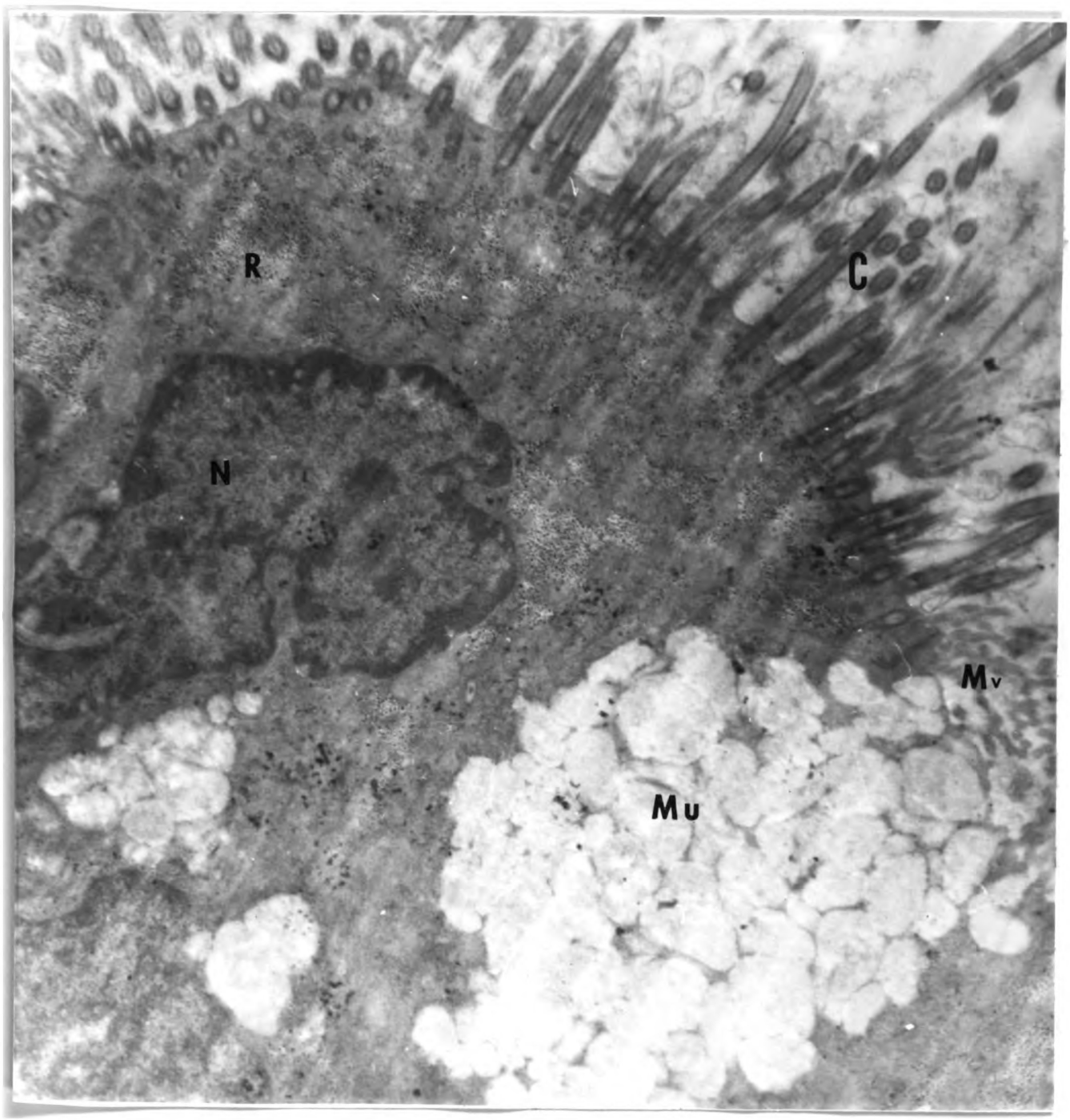

Figure 21. Ciliated epithelial cell and secretion of mucus cell material. N= nucleus, R= ribosomes, Mu = secretory product of mucus cell, C = cilia, Mv = microvilli. 20,000 X

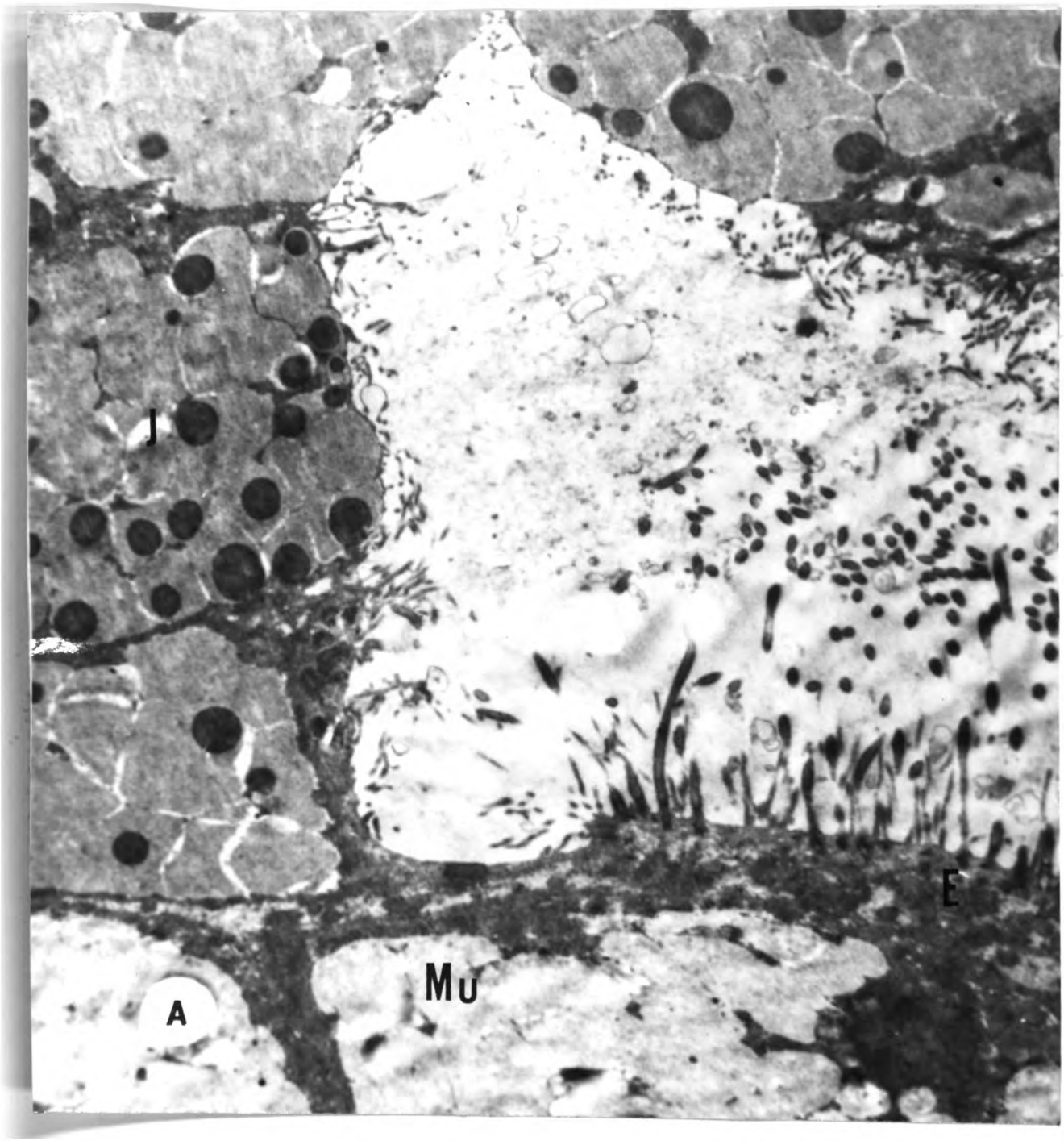

Figure 22. Section showing all three cell types of the oviduct bordering the lumen. J = jelly-producing cells, Mu = mucus secreting cell, E = ciliated epithelial cell, A = artifacts. 8,000 X

DISCUSSION

Capacitation is a vague term usually describing the effect the female reproductive tract has on spermatozoa. This term was first used in studies with mammalian spermatozoa (Austin, 1951) and has since become a general term encompasing the many complex processes of the initial sperm - egg interactions. Only recently has this term been applied to Amphibian sperm. As soon as the sperm cell leaves the testis, it becomes subject to environmental conditions many of which are harmful, but some of which actually aid the sperm in fertilization.

Many factors seem to be involved in preparing the spermatozoa for fertilization after it has left the adult male. The exact processes that render the spermatozoa capable of fertilization (capacitation) are unknown. In this report the term capacitation was used in a general sense to encompass various unknown factors. Thus, no specific processes or mechanisms are implied by the use of this term. Capacitation conveniently describes the treatment of sperm which in some unknown way helps to eliminate or decrease the inhibition of fertilization produced by treatment with antisera.

The nature of the antiserum effect on fertilization may now be taken into account. Just how the antibodies against the jelly materials inhibit fertilization is not

certain. Do the antibodies occupy specific sperm receptor sites in the jelly? If such sites do exist and are necessary to interact with and capacitate the spermatozoa (perhaps *via* an acrosomal reaction) then any type of obstruction to the sperm - jelly receptor interaction would be harmful. Thus a precipitation lattice network which non-specifically blocks important macromolecular interactions that may be a necessary prerequisite to successful sperm - egg fusion would inhibit fertilization. The use of univalent (non-precipitating and non-lattice forming) antibodies would indicate if specific receptor sites are occupied by the antibodies or whether inhibition is an accident of lattice formation and thus a type of physical obstruction.

The fact that the antibodies do not occupy specific sites may be demonstrated by noting the effects of antisera against other frog tissue. Although all eggs were thoroughly washed after a two minute exposure to sperm antisera, there was still inhibition of fertilization (figure 3). Antibodies were thus present in the egg jelly and were able to interact with the sperm thereby blocking fertilization. Antibodies against the sperm should have had no complementary sites in the egg jelly per se with which they could attach. However, egg water material in the jelly could interact with sperm antibodies

Even if the exact nature of the antiserum blockage is not clear, it is certain that with antijelly sera treatment there is significant inhibition of fertilization. This

barrier is partially by-passed by treatment of spermatozoa with egg water or extracts from the female oviduct. Since the sperm - egg water (or oviducal extract) mixtures were applied directly to the eggs which had been treated with antibodies, it is not definitely clear whether the egg water affected only the spermatozoa or whether the mixture acted in some way on the eggs or the jelly coats. Perhaps the sperm - egg water solutions contained some enzymes from the sperm whose acrosome was induced, by factors in the egg water, to rupture. These enzymes then could have digested away the antibody blockage and allowed other sperm to interact with the jelly and hence fertilize the eggs. This is only a hypothetical mechanism which assumes a typical acrosomal reaction. In addition, one must consider in the fourth capacitation experiment the ability of the supernatant of the sperm - egg water mixture to cause enhancement of fertilization. Here the supernatant possibly contained acrosomal and other sperm enzymes but no increases in fertilization was noted (figure 5). If the enzymes alone were able to penetrate the blockage caused by antijelly sera, then one should see an increase in fertilizability. It appears, instead, that all the factors present in the egg water which caused capacitation or enabled the sperm to by-pass the antiserum blockage were used up or removed. This experiment indicates that perhaps the factors bind in some way to the sperm and were removed when the sperm were removed. Another possibility is that the factors, once contact with the spermatozoa has been made, are

themselves changed and so cannot react with several sperm but with only one. The protein determinations on the supernatant showed that proteins (and possibly enzymes) are present. The mere centrifugation of normal egg water did not affect its ability to capacitate sperm and so it seems unlikely that the spinning in the centrifuge adversely affected the released proteins. What the spinning probably did remove is all the sperm structures to which the egg water factors (macromolecules) could be bound.

What the exact effect the egg water or oviducal extract substances had on the spermatozoa it is impossible to state. No acrosomal reaction has been seen in Rana species although an acrosome is present (Poirier, 1970). To say that the egg water substance(s) initiated acrosomal reactions would be mere speculation. Likewise it is not known if one or several substances in the egg water or oviducal extracts is responsible for the enhanced fertilization of the antijelly sera - treated eggs. It must be kept in mind that these substances can originate from the egg as well as from the jelly coat. Washing the sperm after exposure to egg water and then inseminating antisera treated eggs with them would determine if the sperm was directly affected or capacitated by the substances in the egg water. However, the fragile nature of the sperm means that the washings would have to be done in a manner which would not injure the sperm. Capacitated sperm might be more suseptible to washing and be inactivated or killed.

If the egg water substances do change or alter the spermatozoa in any way, the changes did not affect the fertilizing capacity for normal untreated eggs. By capacitating spermatozoa prematurely, one might expext normal fertilization to be hindered. An experimentally capacitated spermatozoa might no longer need the egg jelly and in fact might be impeded rather than helped by the egg jelly. Preliminary evidence (Shaver,1966) indicated that this was the case. The experiments reported here, however, show that the treated (capacitated) spermatozoa were just as capable as normal spermatozoa in fertilizing normal eggs. This apparent conflict may be resolved if one considers the amount of capacitating factor(s) present in the egg water. It could be that enough sperm in the sperm - egg water mixtures were not affected by the egg water and so a normal fertilization rate was apparent. The sperm that were affected by the egg water perhaps could not fertilize normal eggs due to a premature capacitation but were able to by-pass the blockage of the antisera treated eggs. However, the possibility that some sperm were not affected due to a lack of enough egg water substances to capacitate all the sperm is only speculation.

Nevertheless the treated sperm were able to by-pass completely the blockage of the antibodies as is evident by the differences in percentages of cleavage of the antisera treated eggs and the control eggs. Under these conditions, perhaps not all the sperm were being capacitated. Perhaps the necessary sperm - jelly interactions blocked by the

antibodies were supplied by the egg water substances. The sperm, therefore, no longer needed the jelly blocked by the antibodies and so were able to circumvent the necessary jelly reactions or reaction sites. The jelly was then possibly used merely as a supporting medium until the egg surface was reached.

The many components present in the egg jelly indicate a complex series of sperm - jelly interactions. It has been suggested (Shaver, 1966) that different molecular components in the jelly not only mediate a series of sperm - jelly interactions but are responsible also for the species - specificity of fertilization. In any case, to fully understand the mechanisms involved in the sperm - jelly reactions, one will have to examine the molecular composition of the jelly and the substances bound to and released by the sperm during fertilization.

SUMMARY

1. Eggs of Rana pipiens were treated with antisera against the jelly coat materials. Such treatment inhibits fertilization to a significant degree. This inhibition was by-passed by capacitation of the sperm. This capacitation was accomplished by mixing sperm with egg water and using this mixture to inseminate eggs. The sperm were exposed to egg water for various time intervals in order to determine an optimal exposure time.

2. The ability of capacitated sperm to inseminate eggs treated with 10 different types of antisera was tested. In all cases the inhibition of fertilization due to the various antisera was by-passed by capacitating the sperm with egg water.

3. The ability of oviducal materials to capacitate sperm was demonstrated by exposure of sperm to extracts from various levels of the oviduct. The sperm were then used to inseminate antijelly sera treated eggs. With each extract used, the inhibition of fertilization due to the antisera treatment was by-passed.

4. Egg water was tested for its ability to capacitate sperm after a 24 hr. period. Results showed that 24 hr. old egg water was still capable of allowing sperm to by-pass the inhibition of the antijelly sera treatment. In addition this

ability of the egg water could be removed by centrifugation in the presence of excess sperm.

5. A biochemical analysis of the egg jelly revealed a composition of approximately 50% protein and 50% carbohydrate. The amino acid composition of various oviducal materials shows minor variations which could be a reflection of antigenic and histochemical differences found in the oviduct.

6. A cytological study revealed the fine ultrastructure of the jelly producing organ, the oviduct. However, no structural differences were noted which could be correlated to antigenic and histochemical differences of the oviduct as reported by Shivers and James(1970 A.).

BIBLIOGRAPHY

BIBLIOGRAPHY

Arakelian, H.K. 1971. Effect of univalent antibody fragments on the fertilizability of Rana pipien's eggs. Masters thesis Michigan State University, E. Lansing, Mich.

Austin, C.R. 1951. Fertilization of the rat egg. Austral J. Sci Res. B,4, 581.

Austin, C.R., and M.W.H. Bishop. 1958. Some features of the acrosome and perforatorium in mammalian spermatozoa. Proc. Roy. Soc. London, B. 148: 234-240.

Barch, S.H., and J.R. Shaver. 1959. The effects of enzymes on prolongation of fertilizability of frog eggs. Exptl. Cell Res., 17: 114-120.

Barch, S.H., and J.R. Shaver. 1963. Regional antigenic differences in frog oviduct in relation to fertilization. Am. Zool., 3: 157-165.

Barrows, C., and C.R. Austin. 1967. In vitro fertilization and sperm acrosomal reaction in the hamster. J. Exp. Zool., 166: 317.

Bataillion, E. 1919. Analyse de l'activation par le technique des oeufs nus et la polyspermie experimentale chez les Batraciens. Ann des Sciences Nat. (Zool.) 10: 1-38.

Bray, H.G., and S.P. James. 1949. Examination of sugars produced by hydrolysis of jelly-like mucin of frog spawn. First Int. Congr. Biochem. Abstr. p 225.

Chang, M.C. 1951. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature, 168: 697.

Colwin, A.L., and L.H. Colwin. 1964. Role of the gamete membranes in fertilization. p. 233-279. In M. Locke (ed.), Symposium on cellular membranes in development. Academic press, New York.

Dan, J.C. 1956. The acrosome reaction. Internatl. Rev. Cytol. 5: 365-393.

Dische, Z. 1955. In "The Nucleic Acids." E. Chargaff and J.N. Davidson ed. Vol I p. 285.

Fiske, C.H. and Y. Subarrow. 1925. The colorimetric determination of phosphorous. J. Biol. Chem. 66: 375.

Flood, H.E., E.L. Hirst, and J.K. Jones. 1948. Quantative analysis of mixtures of sugars by the methods of preparative chromatography. J. Chem. Soc. 1679-1683.

Folkes, B.F., R.A.Grant, and J.K. Jones. 1950. Frog spawn mucin. J. Chem. Soc., 8: 2136.

Giacosa, P. 1883. Etudes sur la composition chemique de l'oeuf et des envelopes chez la grenouille commun. I. Sur l'envelopes muqueuse de l'oeuf. Z. Physiol. Chem., 7: 40-56.

Glick, R.N., and J.R. Shaver. 1963. The fertilizability of frog eggs from different levels of the female reproductive tract. Exptl. Cell Res. 32: 615-618.

Gusseck, D.J., and J.L. Hedrick. 1971. Disulfide bonds in Xenopus laevis jelly coat and a molecular hypothesis for fertilization. For publication in Dev. Biol.

Hartree, E.F., and A.C. Allison. 1970. Lysosomal enzymes in the acrosome and their possible role in fertilization. J. Reprod. Fert., 22: 501.

Hiyama, N. 1945. Biochemical studies on carbohydrates. Tohoku J. Exp. Med. 50: 373-387.

James, S.P. 1951. An examination of frog jelly. Biochem. J., 49: 54.

Kambara, S. 1953. Role of jelly envelopes of toad eggs in fertilization. Annotat. Zool. Japon., 26: 78-85.

Katagiri, C. 1963. Fertilizability of the egg of Hyla arborea japonica, with special reference to the change of jelly envelopes. Zool. Mag. (Tokyo), 72: 23-28.

Kirton, K.T., and H.D. Hafs. 1965. Sperm capacitation by uterine fluid or beta-amylase in vitro. Science, 166:317.

Le Brun, H. 1981. Recherches sur l'appareil genital femelle de quelques batraciens indigenes. La Cellule, 7: 417-484.

Lee, P.A. 1965. Histochemical and biochemical analysis of the annual cycle of growth and secretion in the oviduct of Rana pipiens. Doctoral dissertation. University of Mich, Ann Arbor, Michigan.

Leloir, L.F., and C.E. Cardini. 1957. Characterization of phosphorous compounds by acid lability. In "Methods in Enzymology", S.P. Colowick and N.O. Kaplan ed., Vol III p. 843.

Lowry, O.H., N.J. Rosenbrough, A.L. Farr, and R.J. Randal. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 256.

Luft, J.H. 1961. Improvement in epoxy resin embedding methods. J. Biophys. Biochem. Cytol., 9: 409-414.

Luna, L.G. (ed.) 1968. "Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology". 3rd edition McGraw-Hill Book Co., New York.

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

Minganti, A., and T. D'Anna. 1957. Recherche sulla mucina ovulare di Triton cristatus. Ric. Scientifica 27: 3052.

Minganti, A., and T'Anna. 1958. Sulla composizione sella mucina ovulare di Discoglossus pictus. Ric. Scientifica 28: 2090-2094.

Monroy, A. 1965. "Chemistry and Physiology of Fertilization". Holt, Rinehart, and Winston, New York.

Nobel, G.K. 1931 "The Biology of the Amphibia". McGraw-Hill, New York.

Nace, G.W., T. Suyama, and N. Smith. 1961. Early development of specialized proteins. Symp. Intern. Inst. Embryol., Pallanza, Italy.

Nace, G.W., and Lavin. 1963. Heterosynthesis and autosynthesis in the early stages of anuran development. Am. Zool. 3: 193-207.

Metz, C.B., and A. Monroy. 1967. "Fertilization; comparative morphology, biochemistry, and immunology Vol I." Academic Press, New York.

Ouchterlony, O. 1949. Antigen-antibody reactions in gels. Acta. Path. Microbiol. Scandinav. 26: 507-515.

Poirier, G.R. 1970. An ultrastructural study of spermatogenesis in two species of Rana. Doctoral dissertation. Michigan State University, East Lansing, Michigan.

Pereda, J. 1970. Histochemical study of the distribution of sialomucins in the oviduct and jelly coat of the oocytes of Rana pipiens. Behavior of the different layers of the coat in water. J. Embryol. Exp. Morph. Vol 24: 1-12.

Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron - opaque stain in electron microscopy. J. Cell Biol. 17: 208-212.

Rugh, R. 1935. Ovulation in the frog. J. Exptl. Zool., 17: 163.

Schneider, W.C. 1957 Determination of nucleic acids in tissues by pentose analysis. In "Methods in Enzymology". S.P. Colowick and N.O. Kaplan ed., Vol III p. 680.

Schultz, F.N., and M. Becker. 1935. Uber die Kohlenhydrate der Eiweissdruse von Rana esculenta. Biochem. Z., 280: 217-266.

Schultz,F.N., and F. Ditthorn. 1900. Galaktosamin, ein neur Amidozucker, als Splatungsprodukt des Glykeproteids der Eiweissdruse des Frosches. Z. Physiol. Chem., 29: 373.

Shaver, J.R. 1961. A simple demonstration of antigen antibody reactions. Metropol. Detroit Science Rev. 22: 18-20.

Shaver, J.R. 1966. Immunobiological studies of the jelly coats of Anuran eggs. Am. Zoologist. 6: 75-87.

Shaver, J.R., and S.H. Barch. 1960. Experimental studies on the role of jelly coat material in fertilization in the frog. Acta Embryol et Morphol. Exp., 3: 180-189.

Shaver, J.R.. S.H. Barch, and C.S. Shivers. 1962. Tissue specificity of frog egg jelly antigens. J.Exptl. Zool. 151: 95-103.

Shaver, J.R., S.H. Barch, and C.C. Umpierre. 1970. Interspecific relations of oviducal materials as related to fertilization in Amphibians. J. Embryol. Exp. Morph. 24: 209-225.

Shivers, C.A. 1961. Immunobiological studies of the species-specificity of egg jellies of the frog. Doctoral Dissertation. Michigan State Univ.,E. Lansing, Mich.

Shivers, C.A. 1962. Localization of the inhibitory effect of antijelly serum on fertilization in the frog eggs by fluorescein-tagged antibody. Am. Zool. 2: 448.

Shivers, C.A. 1965. The relationship of antigenic components in egg jellies of various amphibian species. Biol. Bull. 128: 328-336.

Shivers, C.A., and C.B. Metz. 1962. Inhibition of fertilization in frog eggs by univalent fragments of rabbit antibody. Proc. Exptl. Biol. and Med. 110: 385-387.

Shivers, C.A., and J.N. James. 1970 A. Morphology and histochemistry of the oviduct and egg-jelly layers in the frog, Rana pipiens. Anat. Rec. 166: 541-556.

Shivers, C.A., and J.N. James. 1970 B. Capacitation of frog sperm. Nature, 227: 183-184.

Snedecor, G.W. 1956. "Statistical Methods." Iowa State College Press.

Stambough, R.N., and J.C. Buckley. 1969. Identification and subcellular localization of enzymes affecting penetration of the zona pellucida by rabbit spermatozoa. J. Reprod. Fert., 19: 423.

Tchou-Su, and Y.Wang. 1956. Etudes sxperimentales sur le role du mucus des oviductes dans la fecondation chez le crapaud, et la consideration generale sur la mecanisme de la penetration spermatique. Acta Experimentalis Biologica Sinica, 5: 75-122.

Wolf, D.P., and J.L. Hendrick. A molecular approach to fertilization. II Viability and artificial fertilization of Xenopus laevis gametes. For publication in Dev. Biol.

Wright, P.A., and N.R. Flathers. 1961. Facilitation of pituitary-induced frog ovulation by progesterone in early fall. Proc. Soc. Exp. Bio. Med., 106: 346-347.

Yanagimachi, R., and M.C. Chang. 1964. In vitro fertilization of golden hamster ova. J. Exp. Zool., 156: 361.

APPENDIX

Table Ia Fertilization values in arc sin equivalents of percentages of cleaving Rana Pipiens eggs after preinsemination treatment with antisera against the jelly coat (J), non-immune control sera (C), or Holtfreter's solution (H). Sperm capacitation was accomplished by exposure of sperm to egg water for 0 minutes (0), 5 minutes (5'), 10 minutes (10'), or 15 minutes (15').

	Treatments														
Amimal #	H-U	H-0	H-5'	H-10'	H-15'	C-U	C-0'	C-5'	C-10'	C-15'	J-U	J-0	J-5'	J-10'	J-15'
1	54.2	55.3	50.8	53.1	37.5	53.5	18.4	57.2	53.7	59.5	9.5	29.3	43.9	35.2	39.2
2	22.8	41.0	65.5	40.9	37.2	47.9	52.2	51.3	44.1	44.2	25.3	28.9	41.6	38.4	43.1
3	65.7	64.4	48.6	50.1	21.4	61.2	50.5	50.8	56.8	45.0	27.3	16.0	12.9	38.4	53.1
4	59.3	58.9	65.9	60.4	61.3	70.0	46.0	60.0	64.8	57.0	14.1	23.7	36.5	47.7	67.2
5	66.4	54.3	39.2	60.7	61.0	70.3	60.7	53.1	60.4	55.9	23.3	43.3	38.9	56.8	48.0
6	48.6	44.3	34.3	47.1	61.9	49.1	60.7	31.2	56.8	63.9	00.0	29.8	38.7	26.6	49.5
7	56.4	60.4	59.7	60.3	58.1	61.8	57.5	56.3	50.8	58.8	00.0	28.9	8.1	24.7	46.6
8	53.9	59.4	53.7	62.0	59.5	51.4	67.8	52.1	68.6	71.9	00.0	33.2	39.0	33.7	49.4
9	57.0	45.0	53.6	63.4	58.6	57.0	61.9	58.8	67.6	67.6	20.7	41.1	26.6	50.5	56.0
10	59.5	59.7	55.1	66.7	68.3	64.2	90.0	73.9	56.8	70.5	10.3	30.8	42.0	45.7	43.9
11	53.1	59.4	44.2	62.4	58.8	67.8	73.5	51.3	62.0	69.3	19.7	32.6	15.3	41.7	57.7
12	54.3	65.9	73.7	52.1	64.5	59.7	65.0	62.0	55.9	64.4	13.0	53.9	51.3	49.7	45.0
13	49.5	66.2	64.9	61.3	62.9	55.3	55.9	48.7	65.2	58.1	22.5	46.6	60.0	62.9	50.8
14	81.5	73.0	75.7	75.0	66.9	77.3	90.0	73.9	90.0	75.0	18.8	63.9	67.8	71.0	53.0
15	51.7	39.6	64.8	58.0	54.0	55.3	62.5	64.4	55.7	62.3	00.0	53.3	35.2	55.2	45.0
16	72.0	63.4	70.2	77.3	74.2	71.8	69.7	63.4	73.7	67.8	30.7	55.1	56.4	61.8	49.8
$\bar{X}$	56.8	56.9	57.6	59.4	56.6	60.9	61.6	56.8	61.4	61.9	14.4	38.2	38.4	46.2	49.8

Table Ib. Three way analysis of variance on the effect of egg water on sperm used to inseminate eggs of Rana pipiens treated with antisera against the jelly coat, non-immune control sera, or Holtfreter's solution.

	df	Sum of Squares	Mean Square	F
Animals	15	13143.11	876.2	9.9*
Treatment	2	25234.91	12617.5	143.2*
Time	4	4564.95	1141.2	13.0*
Interaction	8	8018.35	1002.3	11.4*
Error	210	18493.70	88.1	
Total	239	69455.02		

* values significant to the 1% probability level

Table II a Fertilization values in arc sin equivalents of percentages of cleaving Rana pipiens eggs after preinsemination treatment with antisera against the jelly coat (J), upper oviduct (Up), middle oviduct (M), lower oviduct (L), sperm (S), kidney (K), spleen (Sp), ovary (O), R.clamitans jelly coat (RC), control sera (C), and Holtfreter's solution (H). U = normal sperm suspension. 10 = sperm mixed for 10 minutes with an egg water solution.

	Treatments											
Amimal #	H-U	H-10	C-U	C-10	J-U	J-10	Up-U	Up-10	M-U	M-10	L-U	L-10
1	62.5	71.1	64.8	66.4	35.2	45.0	44.3	65.9	21.8	59.7	35.2	47.8
2	56.8	58.1	55.2	54.8	15.2	44.0	33.6	45.8	25.3	39.9	26.6	44.2
3	54.8	62.7	41.3	49.5	16.5	42.4	29.5	39.2	37.1	48.0	10.6	29.3
4	67.3	65.7	49.9	54.8	22.2	39.8	34.2	50.8	24.4	38.9	30.7	43.1
5	65.3	64.2	66.6	61.6	9.9	42.2	18.4	23.4	9.3	30.0	13.1	30.0
6	50.2	60.4	54.3	67.1	30.7	50.5	20.7	58.9	22.6	34.6	23.4	39.2
7	59.3	61.6	60.0	58.9	13.1	43.1	31.7	45.0	30.0	42.5	29.5	39.8
8	61.7	55.5	64.2	61.3	21.3	39.2	36.6	42.6	36.3	43.7	33.1	40.9
9	61.6	56.1	58.4	55.7	18.7	36.8	38.2	44.3	34.0	42.7	24.5	40.6
10	58.1	55.5	57.2	47.5	18.4	39.8	30.0	37.8	36.0	49.1	30.6	40.9
11	54.8	60.0	53.4	55.7	20.3	35.2	32.6	40.1	34.5	46.3	32.8	43.3
12	29.0	16.2	42.0	45.0	17.9	32.3	33.4	36.7	32.3	35.2	19.5	36.2
13	56.8	56.0	45.0	56.5	12.5	51.9	25.7	45.0	13.1	43.1	8.9	52.9
14	77.9	69.0	59.3	65.0	27.8	56.8	49.7	55.3	17.9	42.4	29.1	39.2
15	50.2	71.8	56.6	61.1	26.2	50.0	29.7	58.5	22.6	41.5	27.7	58.9
16	72.2	67.8	46.6	76.3	10.3	46.8	36.3	65.0	00.0	50.1	16.1	52.7
17	74.3	70.7	65.5	66.6	23.6	48.2	37.5	61.3	38.5	47.2	38.1	38.0
18	58.3	64.8	63.4	55.7	10.3	40.9	20.2	50.2	00.0	39.5	24.1	37.9
19	61.9	61.9	72.7	75.0	28.3	48.0	46.1	50.4	35.7	50.7	30.5	51.5
20	61.2	60.0	62.3	66.3	24.1	46.2	26.1	46.2	43.5	50.7	13.7	52.2
21	66.9	65.2	57.5	61.6	28.1	52.9	33.2	42.5	24.0	52.4	31.1	54.2
22	66.4	67.5	60.0	73.4	21.1	57.9	34.8	56.8	18.8	49.4	18.2	56.5
23	68.6	64.9	56.9	70.5	31.2	56.3	24.4	47.4	33.2	42.8	32.3	48.2
$\bar{X}$	60.7	61.2	57.1	61.1	21.0	45.5	32.5	48.2	25.7	44.4	24.8	44.2

Table II a (Continued)

Treatments

Animal #	S-U	S-10	K-U	K-10	Sp-U	Sp-10	O-U	O-10	RC-U	RC-10
1	8.7	23.7	32.5	44.4	60.0	70.5	37.4	41.8	38.8	48.4
2	0.0	13.8	00.0	22.2	29.2	40.2	13.3	30.0	10.6	35.4
3	16.5	21.6	16.7	36.8	31.2	57.9	28.6	39.5	32.9	45.0
4	14.3	25.0	28.6	36.5	42.8	42.4	35.2	41.5	27.1	43.6
5	00.0	21.1	19.5	55.7	47.8	44.3	18.4	29.4	20.4	33.2
6	12.7	19.5	36.2	35.7	57.4	60.8	25.8	41.7	14.1	39.2
7	15.5	30.0	30.4	52.7	58.9	48.2	33.2	45.6	32.3	45.0
8	9.3	14.1	57.9	58.4	60.0	54.5	40.7	62.3	29.5	40.5
9	14.2	15.9	53.5	57.5	61.4	61.0	40.5	56.8	22.2	32.8
10	00.0	12.3	49.5	45.0	58.9	49.8	36.9	43.1	32.6	42.4
11	00.0	16.7	56.8	57.0	55.3	61.1	37.8	56.5	38.6	37.2
12	8.1	20.1	43.1	35.2	34.2	41.8	35.2	45.0	26.9	27.1
13	00.0	12.7	37.5	53.6	50.8	43.1	18.2	42.1	35.2	39.8
14	00.0	14.8	50.8	41.1	48.2	55.2	28.5	41.8	26.0	60.9
15	8.5	13.7	45.9	42.8	44.1	52.2	30.0	37.1	25.2	39.2
16	00.0	21.1	16.5	54.7	60.0	54.8	32.0	56.6	38.2	46.8
17	9.3	00.0	31.6	40.9	56.3	66.5	47.4	52.5	34.2	50.3
18	00.0	12.0	29.2	39.2	37.8	53.4	35.2	40.9	27.8	44.1
19	25.3	10.1	36.7	50.4	59.4	53.6	25.8	50.8	25.7	32.8
20	00.0	10.6	47.2	63.4	45.0	64.8	25.8	64.4	16.2	59.6
21	18.4	19.8	18.0	41.5	47.2	45.0	31.6	60.0	40.1	35.7
22	00.0	12.8	24.7	43.1	45.0	59.4	27.1	55.5	24.7	38.7
23	15.0	21.3	25.6	45.0	47.2	65.1	29.5	57.0	19.1	42.7
$\bar{X}$	7.6	16.6	34.3	45.8	49.5	54.2	31.0	47.5	27.8	41.6

Table II b. Three way analysis of variance on the effect egg water on sperm used to inseminate eggs of Rana pipiens treated with different antisera, non-immune control sera, and Holtfreter's solution.

	df	Sum of Squares	Mean Square	F
Animals	22	9817.01	446.2	6.8*
Treatment	1	85060.09	85060.09	1300.6*
Sera	10	19997.56	1999.8	30.6*
Interaction	10	6314.80	631.5	9.7*
Error	462	30194.96	65.4	
Total	505	151384.42		

* values significant to the 1% probability level

Table III a Fertilization values in arc sin equivalents of percentages of cleaving R. pipiens eggs after preinsemination treatment with antisera against the jelly coat (J) or non-immune control sera (C). Sperm capacitation was accomplished by 10 minute exposure to jelly materials exuded from oviducal regions 1 through 6 (1 - 6), upper third oviduct (Up), middle third oviduct (M), lower third oviduct (L), whole oviduct (W), and egg water (10). U = normal sperm.

	Treatments											
Animal #	C-U	J-U	C-10	J-10	C-1	J-1	C-2	J-2	C-3	J-3	C-4	J-4
1	63.9	00.0	44.2	41.7	40.1	36.0	41.4	38.9	43.7	38.7	65.9	31.0
2	73.9	17.9	55.9	40.9	50.8	40.6	42.9	43.1	49.1	24.5	58.2	28.7
3	75.7	17.6	49.9	28.6	33.0	37.1	27.1	28.5	75.2	30.6	68.4	25.3
4	73.1	23.7	12.0	00.0	00.0	00.0	00.0	00.0	17.3	18.6	41.8	30.8
5	55.1	14.7	65.9	35.2	54.8	25.8	41.3	26.1	60.9	24.1	67.5	22.8
6	56.2	00.0	64.0	33.3	49.5	18.6	29.0	19.3	30.9	20.4	29.5	28.0
7	71.3	26.1	62.1	40.9	66.6	32.6	56.2	35.3	52.9	44.5	59.2	32.0
8	78.3	15.8	64.2	48.9	54.2	47.1	53.1	36.0	57.4	23.1	71.0	46.3
9	90.0	16.4	60.9	37.9	74.8	35.9	61.0	39.2	62.3	48.2	70.3	45.0
10	59.6	22.6	74.3	47.2	64.6	36.0	60.0	48.7	59.0	44.3	75.0	50.8
11	68.4	23.4	61.9	47.4	57.0	39.6	55.6	51.7	59.4	42.4	79.1	65.9
12	90.0	25.8	81.5	33.8	61.0	25.0	74.3	41.3	56.8	28.5	53.7	40.6
13	90.0	[illegible]	73.7	46.2	76.6	31.[illegible]	72.9	44.4	72.7	[illegible]7.8	67.1	41.2
14	61.6	25.0	56.4	[illegible]8.8	55.2	14.5	52.6	30.0	64.4	23.0	54.0	37.3
15	[illegible]3	21.6	67.1	53.4	58.5	33.9	66.5	42.0	62.9	41.1	61.0	36.8
16	[illegible].0	[illegible]3.8	90.0	43.8	71.8	41.1	90.0	35.9	70.9	37.6	77.1	51.5
17	90.0	23.4	52.5	34.1	40.9	43.1	48.7	27.1	56.8	32.6	63.5	31.5
18	75.4	25.3	71.6	37.3	73.0	29.0	67.4	35.2	63.7	44.5	67.8	44.0
19	69.1	21.8	66.7	57.7	64.8	27.2	61.0	38.7	46.5	42.1	56.6	40.3
$\bar{X}$	74.4	19.4	61.8	39.3	55.1	31.3	52.7	34.8	55.9	34.6	62.5	38.4

Table III a (Continued)

	Treatments											
Animal #	C-5	J-5	C-6	J-6	C-Up	J-Up	C-M	J-M	C-L	J-L	C-W	J-W
1	62.3	33.0	64.5	28.5	51.4	30.0	53.3	31.9	49.8	34.0	47.2	42.0
2	58.9	43.1	60.6	26.6	53.3	28.0	57.2	36.5	60.0	35.2	58.5	44.3
3	75.9	35.2	67.3	23.7	76.6	35.2	61.4	34.7	63.4	36.6	71.3	46.6
4	21.5	25.3	31.9	7.7	29.5	8.1	28.9	00.0	27.9	22.1	65.4	23.4
5	59.1	30.0	54.8	32.6	55.6	25.7	60.0	31.8	58.9	23.4	55.4	41.1
6	51.7	32.3	43.7	30.9	32.3	27.0	27.0	55.9	27.8	30.0	47.6	36.5
7	56.0	54.1	65.7	30.7	61.6	39.6	57.0	53.4	55.3	38.6	69.0	36.0
8	55.7	30.7	71.3	34.5	54.4	34.4	57.5	27.6	81.7	30.0	66.4	42.4
9	63.4	39.2	65.4	41.4	90.0	48.4	90.0	50.8	58.5	46.1	61.3	39.5
10	68.0	43.4	66.9	33.3	59.3	55.3	54.0	34.4	54.2	43.5	56.7	44.2
11	65.4	36.0	70.9	45.6	62.4	46.1	64.6	39.6	60.8	30.0	57.9	34.7
12	72.4	21.1	56.8	29.0	62.4	37.4	69.3	32.0	60.0	34.5	65.9	38.1
13	68.8	45.7	76.7	39.0	73.7	39.8	70.8	40.5	59.7	36.6	61.9	42.9
14	66.3	36.2	60.6	34.8	61.4	37.3	61.7	32.7	58.8	34.6	61.3	45.6
15	59.2	35.9	64.1	31.5	57.4	44.3	54.8	41.6	65.6	45.0	58.0	39.2
16	75.2	62.1	77.2	47.8	71.6	38.5	75.9	49.5	77.8	45.0	90.0	59.0
17	74.6	47.5	65.9	38.7	53.6	35.3	55.6	45.5	69.7	45.3	61.1	39.8
18	61.1	30.0	59.5	33.7	57.5	33.7	48.2	34.7	71.3	66.9	79.1	59.3
19	60.9	48.6	60.8	30.0	61.6	43.2	57.7	50.8	58.5	46.8	61.9	40.9
$\bar{X}$	61.9	38.4	62.3	32.6	59.1	36.5	58.2	36.6	60.4	37.8	62.9	41.9

Table III b. Three way analysis of variance on the effect of oviducal extracts and egg water on sperm used to inseminate eggs of Rana pipiens treated with antisera against the jelly coat or non-immune control sera.

	df	Sum of squares	Mean square	F
Animals	18	33450.61	1858.37	22.2*
Treatment	1	74049.47	74049.47	885.1*
Sera	11	3527.89	320.72	3.8*
Interaction	11	6914.78	628.62	7.5*
Error	414	34633.71	83.66	
Total	455	152576.46		

* Values significant to the 1% probability level

Table IV a Fertilization values in arc sin equivalents of percentages of cleaving *R. pipiens* eggs after preinsemination treatment with antisera against the jelly coat (J) or non-immune control sera (C). Sperm capacitation was accomplished by mixing sperm for 10 minutes in egg water (10), 24 hr. old egg water (24hr.), egg water spun for 15 minutes at 11,500 x g (Spun), and the supernatant of a 10 minute egg water sperm suspension (S). U = normal sperm suspension.

	Treatments									
Animal #	C-U	J-U	C-10	J-10	C-24hr.	J-24hr	C-Spun	J-Spun	C-S	J-S
1	51.1	14.7	65.9	35.2	60.0	23.2	60.4	42.1	61.6	25.3
2	56.2	00.0	64.0	33.3	49.0	17.2	38.6	30.0	45.3	15.3
3	71.3	26.1	62.1	40.9	57.3	00.0	52.9	37.1	52.7	17.1
4	78.3	15.8	64.3	48.9	42.3	14.7	56.4	31.3	76.3	27.6
5	90.0	16.4	60.9	37.9	69.0	34.1	78.8	41.4	75.7	26.2
6	59.6	22.6	74.3	47.2	59.3	34.3	77.2	38.5	69.6	24.1
7	68.4	23.4	61.8	47.4	49.8	50.1	49.4	34.6	59.4	26.6
8	90.0	25.8	81.5	33.4	78.9	38.1	54.7	32.1	73.9	25.3
9	90.0	23.5	73.7	46.2	73.7	45.8	59.4	32.0	64.5	18.7
10	61.6	25.0	56.4	38.8	70.8	40.1	65.9	36.2	59.2	19.2
11	82.3	21.6	67.1	53.4	66.2	37.4	56.3	32.8	63.4	29.0
12	90.0	23.8	90.0	45.0	90.2	48.0	90.0	49.1	77.1	19.5
13	90.0	23.4	52.5	34.1	74.8	67.8	68.2	44.0	53.8	24.0
14	75.4	25.3	71.6	37.3	53.4	41.1	72.7	37.3	68.9	29.2
15	69.1	21.3	66.7	57.7	60.4	60.8	61.1	50.5	74.6	29.1
$\bar{X}$	74.9	20.6	67.5	42.4	62.8	39.6	62.7	37.3	65.7	23.4

Table IV b. Three way analysis of variance on the effect of various egg water preparations on sperm used to inseminate eggs of Rana pipiens treated with antisera against the jelly coat or non-immune control sera.

	df	Sum of Squares	Mean Square	F
Animals	14	6885.95	491.9	6.4*
Treatments	1	43139.45	43139.45	560.9*
Sera	4	1809.24	452.3	5.9*
Interaction	4	5705.75	1426.4	18.5*
Error	127	9766.24	76.9	
Total	150	67306.63		

* values significant to the 1% probability level