ABSTRACT

STUDIES ON THE DISTRIBUTION OF MUCOPOLYSACCHARIDES IN ADULT RANA PIPIENS MALE RUDIMENTARY OVIDUCTS AFTER HORMONAL STIMULATION

By .

Carmen Celia Umpierre

Of a variety of sex hormones tested on adult Rana pipiens males, estradiol and testosterone were effective in inducing hypertrophy of the rudimentary oviducts. Increases in the size, weight and degree of convolution of these oviducts, due to an accumulation of jelly secretion, were observed.

Histochemical techniques were used to detect the kinds of mucopolysaccharides present in the oviducal secretions, both before and after hormonal stimulation. In order to study the distribution of mucopolysaccharides along the length of the oviducts, these were divided into approximately three equal regions: upper, middle and lower. Oviducts from non-ovulated, mature Rana pipiens females served as controls.

Neutral mucopolysaccharides predominated in the three oviducal regions of untreated male rudimentary oviducts. Very little acid mucopolysaccharide was present. No sulfomucins nor sialomucins were detected histochemically in these oviducts.

Upon hormonal stimulation, a preponderance of acid mucopolysaccharides, mainly sulfomucins and sialomucins, was detected in the upper regions of estradiol-treated oviducts. The secretions in the middle and lower regions of estradiol-treated oviducts remained identical to those found in untreated male oviducts, consisting mainly of neutral mucopolysaccharides with very little acid mucopolysaccharide.

The presence of acid mucopolysaccharide (sulfomucins) was detected in the lower region of testosterone-treated male oviducts, thus making this region rich in both neutral and acid mucopolysaccharides. The jelly secretions present in the upper and middle regions of these testosterone-treated oviducts remained also identical to those present in untreated male oviducts, namely, a preponderance of neutral mucopolysaccharides over acidic ones.

On hormonal stimulation, at least using estradiol, the male oviduct has the same kinds of mucopolysaccharides as non-ovulated, mature female oviducts. Also approximately the same mucopolysaccharides are present in corresponding oviducal regions of female oviducts and hormone-treated male oviducts.

Untreated male rudimentary oviducts showed at least eight different antigens as indicated by the number of immunoprecipitation lines obtained in double-diffusion tests. Six of these antigens are common to both untreated male

rudimentary oviducts and non-ovulated, mature female ones. The other two antigens are unique to the untreated male oviduct.

One new antigen appeared in male rudimentary oviducts after either estradiol or testosterone stimulation. This new antigen appears to be identical to one of the antigens found in non-ovulated, mature female oviducts. It is suggested that the "new antigen" line identified by the double diffusion tests in male rudimentary oviducts after hormonal-stimulation may be either the sulfomucins or sialomucins detected histochemically in these oviducts.

A total of seven lines of identity was observed between testosterone- and estradiol-treated rudimentary oviducts and non-ovulated, mature female oviducts.

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INTRODUCTION

The present investigation was undertaken with the purpose of elucidating the effects of sex hormones on the rudimentary oviducts of adult male Rana pipiens, especially with regard to the kinds of mucopolysaccharides produced under hormonal stimulation. As the oocytes descend through the female oviduct, they become coated with several layers of jelly. Since the histochemistry of the female oviduct and egg-jelly coats is relatively well-known at present, it seemed of interest to find if identical or similar mucopolysaccharides are also present in the male rudimentary oviduct of R. pipiens.

For this purpose, a series of experiments was performed in which a variety of sex hormones was tested on adult males for effectiveness in inducing hypertrophy of the oviduct, i.e., increase in size, weight, convolution, and accompanying jelly secretion. Once it was determined which hormones were more effective, both histochemical and immunological techniques were used to detect the presence, kinds of mucopolysaccharides and their distribution in the male rudimentary oviduct after hormonal stimulation. Descriptions of the

morphology and histochemistry of the normal, i.e., untreated, male rudimentary oviduct as well as of the normal, untreated, mature female oviducts, are also given for purposes of comparison.

LITERATURE REVIEW

Morphology and Development of Oviducts in the Frog

Morphology of adult female and male oviducts

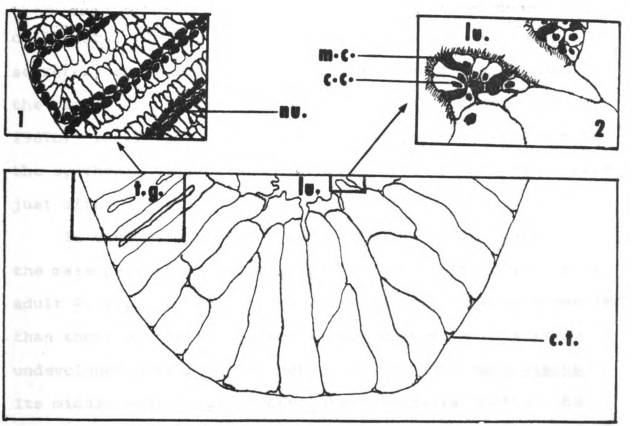
The oviducts of mature female R. pipiens (see Text Figure I), which lie lateral to the kidneys can be divided into three parts (Christensen, 1930): 1. a straight, upper portion beginning at the ostium, called the pars recta, 2. a relatively long and convoluted middle portion, which secretes most of the egg jelly, and is called the pars convoluta, 3. a dilated lower portion, the pars uterus which empties into the cloaca.

Histologically, the oviducts of mature female R. pipiens consist of an inner epithelial lining and tubular jelly-secreting glands surrounded by a connective tissue tunic (Christensen, 1930; Lee, 1969; Kelly et al., 1970; Pereda, 1970a). Two kinds of epithelial cells are present in the single-layered epithelial lining of the oviduct: the more abundant ciliated columnar cells and scattered mucous-secreting cells. The lining epithelium is thrown into longitudinally oblique folds, which often branch, thus forming longitudinal ridges (see Text Figure I).

Text Figure I

Diagrammatic representation of a cross-section of a mature Rana pipiens female oviduct. A single-layered epithelial lining, thrown into folds, surrounds the oviducal lumen (lu), while tubular glands (t.g.) make up the rest of the oviduct. An outer tunica of connective tissue (c.t.) is also present. The tubular jelly-secreting glands consist of tall columnar epithelial cells having basal nuclei (nu.)--(see inset 1). The epithelial lining is made up of 2 kinds of cells (see inset 2): ciliated columnar cells (c.c.) and dark-staining mucous-secreting cells (m.c.).

Text Figure 1



Tubular glands are evident in the pars recta. At a short distance beyond the ostium, these appear as short, simple tubular glands extending from the bases of the folds of the surface epithelium down into the outer connective tissue layer. Such glands are also evident in the pars convoluta, where they become larger and increase in number. These glands are the ones responsible for the synthesis of egg jelly. Individual glands are composed of columnar cells arranged radially around the lumen of the cylindrical tubule. Such columnar cells contain jelly-secretory granules, which exhibit a seasonal variation in the female correlated with the reproductive cycle (Lee, 1967b), increasing in size during the summer months due to the synthesis of secretory granules, and decreasing in size just after ovulation.

In various amphibian species the Müllerian ducts of the male persist as rudimentary oviducts. The oviducts of adult R. pipiens males (see Text Figure II) are much smaller than those of the adult females and possess a relatively undeveloped pars recta (superior region) and pars uterus. Its middle region (pars convoluta) resembles that of the female except that the thickening and coiling are limited to the posterior two-thirds. In this more differentiated portion of the pars convoluta, tubular glands are found. Some of these contain, a few secretory granules, while others contain none (Lee, 1967b). The appearance of the

Text Figure II

A. Diagram illustrating gross appearance of the rudimentaty oviducts (Müllerian ducts) of an untreated adult Rana pipiens male. It consists of 3 regions:
(1) a straight upper portion (pars recta), which ends up blindly,
(2) a middle, slightly convoluted portion (pars convoluta); and
(3) a lower, dilatable portion (pars uterus) emptying into the cloaca.

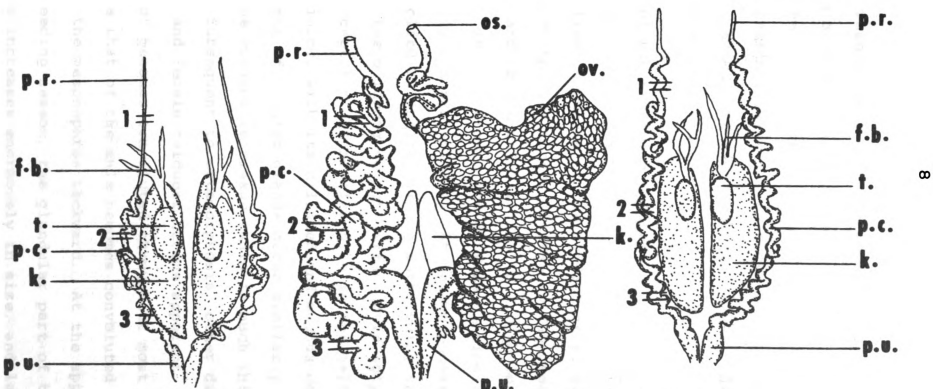
B. Diagram illustrating gross appearance of the left oviduct of a non-ovulated mature Rana pipiens female. It consists of 3 regions:
(1) a straight upper portion (pars recta) opening anteriorly at the ostium (OS)
(2) a middle, highly convoluted portion (pars convoluta) and
(3) a lower, dilatable portion (pars uterus) emptying into the cloaca.

Right oviduct is covered by ovary full of eggs.

C. Diagram illustrating gross appearance of the rudimentary oviducts of hormone-treated (e.g., estradiol, testosterone) Rana pipiens male. Note enlargement of oviduct diameter and increased degree of convolution along the whole length of the oviduct.

Explanation of symbols: p.r.--pars recta; p.c.--pars convoluta; p.u.--pars uterus; f.b.--fat body; t.--testis; k.--kidney; os.--ostium; ov.--ovary. Numbers 1, 2, and 3 indicate approximate levels from which upper (1), middle (2), and lower (3) oviducal sections were taken.

Text Figure II



A. Untreated ♂ B. Non-Ovulated ♀ C. Hormone-treated ♂

male glands resembles that of the female oviduct at an early stage of the oviducal growth cycle, i.e., few secretory granules. The tubular glands of the male oviduct do not exhibit any seasonal variation as those of the female ones (Lee, 1965). Mucous and ciliated cells are also present in the male rudimentary oviduct.

Development of the amphibian oviduct

Morphological changes

The earliest evidence of the oviduct of *R. pipiens* is a thickening of the peritoneum lateral to the mesonephros in both male and female tadpoles 7.5 to 7.0 cm long (Christensen, 1930). Growth and differentiation then proceed anteriorly and posteriorly. At metamorphosis the oviduct has a folded epithelial lining surrounding the lumen. At this time the oviducts do not differ noticeably in the two sexes, except for the smaller size in the male. The epithelial lining, with its ciliated and mucous cells, and the development of tubular glands occur similarly in both sexes, but the connective tissue tunic is much thicker in the female. Subsequent to metamorphosis, other differences between male and female oviducts become apparent. The female oviduct becomes convoluted throughout most of its length, while that of the male becomes convoluted only from the level of the mesonephros backward. At the approach of the first breeding season, the glandular part of the oviduct in the female increases enormously in size, and large amounts

of secretion are formed. The funnel openings (tubae ostia) then become well-differentiated and the ovisac portions broaden in size in the female oviduct. These three changes do not occur in the male oviduct. The anterior end of the pars recta of the male remains blind.

Hormonal influence on oviducal development

On the basis of the developmental studies described above as well as his castration studies on male and female R. pipiens, Christensen (1931), has recognized two stages in the development of R. pipiens oviducts. First, there is a period of self-differentiation up to metamorphosis, in which differentiation occurs autonomously, without hormonal stimulation. This period of differentiation, common to both males and females, is not influenced by exogenous female hormones. Second, in the phase of growth subsequent to metamorphosis, when an increase in the glandular portion of the oviduct due to synthesis of secretory material occurs under the influence of female hormones. This second stage of development occurs only in the female oviduct. The oviduct of the male retains the state of development reached in the self-differentiating period. Androgens, e.g., testosterone, are probably not involved in the initial differentiation of the male oviduct, nor in the maintenance of the tubular glands of the male oviduct. Burns (1939) found that oviducal development could be prevented or caused to regress by treatment of frogs with testosterone, but male oviducts

do not regress after castration of adult males (Wolf, 1928; Christensen, 1931). Androgens, in fact, as will be discussed, do have a stimulatory effect on the growth phase of the oviducts of both male and female adults.

Hormonal Control of the Amphibian Oviduct

Adult females

The seasonal cyclic variation in size of the female oviduct is correlated with the accumulation, growth and secretion of secretory granules in the tubular gland cells (Lee, 1965). Regression of the oviduct after ovulation (DeAllende, 1939a; Gitlin, 1939), observed to be due to marked atrophy of the tubular glands (Houssay, 1947), is the result of expulsion of their secretory product (Lee, 1965). Growth and restoration of full size of the oviduct during the summer feeding season (DeAllende, 1939a) is primarily due to accumulated secretory product. Mucous and ciliated cells do not undergo cyclic changes in size as the glandular cells grow.

This cyclic variation in oviducal size due to the synthesis, accumulation and secretion of jelly material by the tubular gland cells is under control of both the pituitary and ovarian hormones.

Influence of the pituitary

Stimulation of the oviduct to synthesize and secrete jelly may be <u>directly</u> or <u>indirectly</u> mediated by hormones of

the pituitary (Houssay, 1950). It has been suggested that a prolactin-like hormone secreted by the anterior pituitary acts directly on the oviduct. Working with the toad Bufo arenarum, DeAllende (1938) and Houssay (1947) have found that hypophysectomy produces atrophy of the oviduct. Implants of mammalian and toad anterior pituitaries can induce secretion by the oviducal glands of ovariectomized B. arenarum (DeAllende, 1938; Pomerat, 1940). Injections of mammalian prolactin into females of B. arenarum with oviducts at different stages of jelly synthesis, showed in some cases an increase in the amount of jelly present in them (DeAllende, 1939b).

Indirect stimulation of the oviduct by the pituitary appears to be exerted through the ovary. Gonadotropic hormones from the pituitary induce the secretion of ovarian hormones, i.e., progesterone and estrogen, which in turn act on the oviducal glands (Houssay, 1947; Galli-Mainini, 1962; Thornton and Evennett, 1969).

Influence of the ovary

Evidence for the influence of the ovary on the function of the female amphibian oviduct, i.e., synthesis and secretion of jelly, is as follows: a.) Ovariectomy leads to atrophy of the oviduct, i.e., reduction in size of the oviduct due to a diminution in the amount of secretory material in the tubular gland cells of R. pipiens (Christensen, 1929, 1931; Lee, 1965); Xenopus laevis (Gitlin, 1939); B. arenarum

(Houssay, 1947; Galli-Mainini, 1950b; Penhos, 1951). b.) Administration of exogenous ovarian hormones, e.g., estradiol, to ovariectomized toads (B. arenarum) prevents this atrophy (Galli-Mainini, 1950a). c.) Administration of estrogen causes hypertrophy of the oviduct of normal adult females of R. pipiens (Christensen, 1931; Lee, 1965), and B. arenarum (DeAllende, 1939a; Galli-Mainini, 1950a). Histological examination reveals that increase in number and size of granules in the jelly-secreting glands is the principal feature in oviducal hypertrophy (Lee, 1965, 1967b). Similar results have been reported by Galli-Mainini (1950b) for B. arenarum and by Adams (1950) for Triturus viridscens. d.) Administration of progesterone to adult females stimulates secretion of jelly by the oviducal glands of frogs and toads, e.g., R. pipiens (Lee, 1965); Rana temporaria (Lodge and Smith, 1960); Bufo bufo (Lodge and Smith, 1960; Thornton and Evennett, 1969); and B. arenarum (Houssay, 1950; Galli-Mainini, 1962). This action of progesterone was enhanced by administration of estradiol and testosterone, but not by corticosteroids (Penhos and Nallar, 1956).

In summary, the effect of ovarian hormones, i.e., progesterone and estrogen, on the tubular gland cells of the mature female oviduct may be at two levels: the synthesis of jelly and the control of jelly release. Estrogen seems to be needed for the development and maintenance of jelly synthesis (Galli-Mainini, 1950a; Lee, 1965). Progesterone secreted during ovulation seems to be involved in the release

of jelly by the oviducal glands (Lodge and Smith, 1960; Thornton and Evennett, 1969; Galli-Mainini, 1962).

Effect of exogenous androgens

Puckett (1939) found that administration of testosterone propionate stimulated growth of oviducts in both adult males and females of R. pipiens and Bufo americanus. A similar hypertrophic effect was obtained when adult females of T. viridescens (Adams, et al., 1941) were injected with testosterone.

Adult males

Persistence of rudimentary oviducts (Müllerian ducts) in mature males of many species of amphibians, e.g., frogs, toads, and newts, has been observed by several investigators. As previously stated, this oviduct retains the state of development reached in the self-differentiating period, i.e., has tubular glands with few secretory granules. It has been suggested that the lack of ovarian influence rather than an inhibitory effect of the testicular secretion may be the cause of the persistent rudimentary condition of the oviducts of the male. Castration of males had no effect on the structure of the rudimentary oviducal glands (Christensen, 1929, 1931; Lee, 1965). No increase in size was noted in these structures following castration and the appearance of the rudimentary oviduct remained the same (Wolf, 1928).

However, the rudimentary oviduct of adult males seems to be responsive to the administration of both female and male exogenous hormones.

Effect of exogenous female hormones

Estrone of mammalian origin injected into male toads, B. bufo and B. americanus, and into frogs, R. pipiens, induced a marked hypertrophy of the Müllerian ducts (van Oordt and Klomp, 1946; Puckett, 1939). A distinct thickening and convolution of the oviduct walls (see Text Figure I) and secretion of mucous substance by the tubular glands were obtained. Histological observations indicated that estrogens stimulated the growth of the secretory granules in the oviducal glands of normal and castrated males of R. pipiens (Wolf, 1940; Lee, 1965). Administration of diethylstilbesterol to normal and castrated males of the newt, T. viridescens, also induced hypertrophy of the rudimentary oviducts (Adams, 1946).

Effect of exogenous male hormones

Administration of testosterone propionate into normal and castrated male R. pipiens, brought about a great enlargement of the rudimentary Müllerian ducts (Wolf, 1939, 1940; Puckett, 1939). Similar hypertrophic effects were obtained in male B. americanus, following injections of testosterone (Puckett, 1939).

Chemical Nature of Amphibian Egg Jelly

Biochemical analyses

Numerous investigators have revealed the presence of carbohydrates and protein compounds and/or complexes in the egg-jellies or oviducal secretions of various amphibian species, after suitable hydrolysis. For recent reviews see Lee (1967a) and Freeman (1968). Among the substances identified are the following: hexoses (glucose, galactose, mannose, fucose); hexosamines (galactosamine, glucosamine); protein (about 40% dry weight R. pipiens egg-jelly); sialic acid (Pereda, 1970b; Steinke and Benson, 1970) and sulfate (Pereda, 1970a; Humphries, 1970).

Histochemical analyses

In all of the above mentioned biochemical studies, the jelly layers surrounding the egg were analyzed together, i.e., egg-jelly coats were treated as a homogenous material. No attention was given to the possible differences among layers of jelly and along the length of the oviduct. Since the possibility that several secretory products of different chemical constitution may exist and that these may express themselves as distinct jelly envelopes, a new approach to the study of egg jellies was introduced. This consisted in using a variety of histochemical techniques on eggs surrounded by all or some of their jelly layers. These histochemical studies performed on several anuran and urodele species have shown differences in the chemical nature of the

egg-jelly layers as well as in the different levels of the oviduct which secrete them.

Kambara (1956) has shown that epithelial cells of the upper two regions of the oviduct of the newt, Triturus pyrrhogaster, are positive for the PAS reaction and the Bauer-Feulgen method. No glycogen could be detected at any level, i.e., staining reactions of the sections remained unaltered upon saliva treatment. Variable metachromasia in the cells of the first two regions was obtained when sections were stained with toluidine blue or thionin (Kambara, 1959). Strong metachromasia was obtained in the first (infundibular) region while the second secretory region exhibited only a weak reaction. On the basis of the above data, Kambara conjectured that the cytoplasm of the epithelial cells of the first and second regions of the oviduct of this newt contains acid mucopolysaccharide and that of the second region, which is strongly PAS positive but shows only weak metachromasia, must contain neutral mucopolysaccharide or mucoprotein. When oviducal sections were subjected to hyaluronidase digestion before staining with toluidine blue or thionin, their metachromatic stainability was not perceptibly modified. Therefore, he suggested that perhaps no hyaluronic acid is present in the oviduct of this newt.

Humphries and Hughes (1959), working with adult females of T. viridescens, reported that epithelial cells lining the oviduct of this species are all PAS-positive, with no

glycogen indicated. Three of the five secreting regions described in the oviduct of this species (regions A, B and D) showed metachromasia with toluidine blue and a positive alcian blue reaction. Regions C and E gave very weak or negative results with these two techniques. Neither hyaluronidase nor ribonuclease abolished staining with toluidine blue or alcian blue. The authors suggested that the differential staining could be due to the presence of acid polysaccharide in regions A, B and D only; the positive PAS reaction of regions C and E was due perhaps to neutral polysaccharides.

In a comparative study of the jelly envelopes of several species of amphibians, Ghiara (1960) observed three jelly coats in X. laevis eggs. He noted metachromasia in the 2 inner layers after staining living eggs with toluidine blue.

In a more comprehensive study of X. laevis eggs, Freeman (1968) corroborated histochemically the presence of three jelly coats in the eggs of this species. Working with oviducts containing eggs and with uterine eggs, she distinguished, on the basis of differential staining, two secreting regions in Xenopus oviducts: the anterior half of the oviducts, which secretes the first jelly coat (J1) and the second half of the oviducts, which secretes the second and third jelly coats (J2 and J3). Both secreting regions and all three jelly layers were distinctly PAS-positive and diastase-resistant. The second secreting region and J2 and J3 reacted

more intensely than did the first secretory region and J1. The inner layer of jelly (J1) and the glands of the first oviducal region showed alcohol-resistant metachromasia with toluidine blue, intense staining with alcian blue (pH 2.6), and a bright orange-red fluorescence with coriphosphine O. These facts strongly suggest the presence of acid mucopolysaccharide (AMP) with acid groups of the sulfate type. A very weak reaction with Ninhydrin-Schiff reagents was also obtained here. The staining reactions for layers J2 and J3 and the second secreting region were similar. They gave negative results for AMP and a positive reaction for protein. Thus, the author concluded that the 2 outer layers may contain neutral mucopolysaccharide and protein, while the inner layer appears to contain neutral and acidic mucopolysaccharides and possibly some protein.

The five layers of jelly (J1 to J5), which are deposited about the T. viridescens egg as it traverses the oviduct (regions A to E, respectively), differ in their response to various cytochemical tests (Humphries, 1966). All five layers, with the possible exception of layer J5, contain polysaccharide, which is PAS-positive and diastase-resistant. Only layers J1, J2 and J4 seem to contain acid mucopolysaccharide, i.e., showed alcianophilia, metachromasia with toluidine blue and a bright orange-red fluorescence with coriphosphine O. The coincidence of Pas-positivity and metachromasia in these three layers suggested the possibility of the presence of neutral mucopolysaccharides as well as of

acidic ones. Sulfation of the mucopolysaccharides of layer J1 was indicated by the alcohol-resistant metachromasia. Layer J3 probably contains neutral mucopolysaccharide, i.e., it was PAS-positive but lacked metachromatic staining. Layer J5 seems to be composed mainly of protein. It gave strong positive reaction with a variety of protein tests (Millon's, Ninhydrin-Schiff, etc.). Some protein was also detected in J2. This was interpreted as suggesting that layers J2 and J5 may contain mucoproteins. The possibility that the other layers, although giving negative results, contain some protein was not discarded. Histochemical evidence of sialic acid was given only by layers J2 and J5. No hyaluronic acid was detected. The results presented by Humphries (1966) agree well with the findings on the histochemistry of the oviduct of T. viridescens discussed earlier (Humphries and Hughes, 1959).

In a histochemical analysis of sections of female R. pipiens oviducts, Lee (1965, 1967b) compared the staining properties of the secretory material present in the glandular cells of the oviduct at various stages of growth. An intense positive reaction was obtained in the glandular cells of the mature oviduct for proteins, using the mercury bromphenol blue and Ninhydrin-Schiff reactions, and for mucopolysaccharides (i.e., PAS-positive, diastase-resistant). Histochemical demonstration of the presence of sialic acid was also obtained in these glands. Less intense staining reactions were obtained with post-ovulatory oviducts (those with little

secretory material). Tests performed to detect the presence of acid mucopolysaccharide, e.g., methylene blue extinction, binding of toluidine blue, colloidal iron and alcian blue, gave negative results in both kinds of oviducts, i.e., pre- and post-ovulatory ones. The author thus concluded that the jelly glands of the mature oviduct of female R. pipiens contain neutral carbohydrate, a protein moiety and sialic acid.

Steinke and Benson (1970) have reported the presence of five jelly layers in the jelly capsule of R. pipiens eggs that differed clearly in their response to cytochemical tests. These layers were classified as M1 through M5 from the inner to the outermost layer. A sixth layer occasionally could be observed between M3 and M4. All five layers contain neutral mucopolysaccharide or mucoprotein, as indicated by their PAS-positivity which was not altered by either trypsin, chloroform-methanol, or diastase treatments, and acid mucopolysaccharide, as indicated by their affinity for alcian blue at pH 2.0 and manifestation of beta-metachromasia. In addition, layers M1 and M3 contain sulphated mucopolysaccharide as indicated by alcohol-resistant metachromasia and affinity for alcian blue at pH 0.5. Layers M2 and M4 contain non-sulphated acid mucopolysaccharide and layer M5 contains both sulphated and non-sulphated acid mucopolysaccharide. Neither hyaluronic acid nor sialic acid were detected by the histochemical methods employed.

In a study of mature female R. pipiens, Shivers and James (1970) demonstrated that the oviduct of this species

can be divided into six regions designated R1 (anteriorly) to R6 (posteriorly), based on the differential staining of the jelly glands. Furthermore, they found that in general a correspondence exists between the histochemical properties of the jelly glands present in these regions and the six histochemically distinct layers of jelly (designated J1 nearest the egg through J6 farthest from the egg), which are deposited around the egg as it passes down the oviduct. All regions and layers, except R5 and J5, contained a large amount of acid mucopolysaccharide, i.e., stained strongly with alcian blue at pH 2.5. The mucopolysaccharides in regions R1, R3 and R4 and in jelly layers J1, J3 and J4 are sulfated as indicated by binding of alcian blue at pH 0.6 and bright orange-red fluorescence with coriphosphine-O. In the other layers and corresponding oviducal regions (R2, R5 and R6, J2, J5 and J6) acid mucopolysaccharide seems to be non-sulfated. Neutral mucopolysaccharide may be present in R2, J2, R5 and J5, since strong PAS-positivity was seen together with weak protein staining, with the Ninhydrin-Schiff reagent. Although protein seems to be present in all layers and oviducal regions, i.e., give a positive Ninhydrin-Schiff reaction, it is present in higher concentration in R1, J1, 3 and 6 and in lowest concentration in R and J2. These same three layers and corresponding oviducal regions gave also strong PAS staining, thus suggesting that protein here could be in the form of mucoprotein or glycoprotein.

Small amounts of mucoprotein or glycoprotein are present in regions 2, 4, 5 and jelly layers 2, 4, 5.

Histochemical studies have suggested the presence of sulfated polysaccharides in oviducal secretions and egg jelly layers of several species of amphibians (Kambara, 1956, 1959; Humphries and Hughes, 1959; Ghiara, 1960; Humphries, 1966; Freeman, 1968; Kelly et al., 1970; Pereda, 1970a). In general, these showed alcohol-resistant metachromasia with toluidine blue, alcian blue staining at low pH's, bright orange-red fluorescene with coriphosphine-O and astrablau staining at pH 0.2.

Pereda (1970b) reports some histochemical studies on the distribution of sialomucins and proteins in the different regions of the oviduct, i.e., proximal, median and distal zones, as well as in the 3 jelly coats surrounding the oocyte of R. pipiens, i.e., inner, middle and outer layers. Representative sections were digested with neuraminidase followed by conventional staining for acid mucopolysaccharide with alcian blue pH 2.6 and metachromatic staining with toluidine blue. The median zone of the oviduct and the middle jelly layer contain a large amount of sialomucins in contrast to the proximal and distal zones and the respective inner and outer jelly layers, which revealed only a small amount of the sialomucins. The distal zone and the third layer of the jelly coat revealed a preponderance of neutral mucopolysaccharide, i.e., a preponderance of PAS staining over alcian blue staining at pH 2.5.

Protein is present in all three layers and corresponding oviducal regions, although definite positive reactions are evident only in the median oviducal region and in the middle jelly layer, while diffuse reactions are obtained in the other two oviducal regions and their respective jelly layers.

MATERIALS AND METHODS

Animals Used

Adult male and female R. pipiens were obtained from a commercial dealer (Lake Champlain Frog Farms, Alburg, Vermont). Frogs were kept in hibernation (4-6^{o}C) until ready for use. Body weights were recorded at the beginning and end of the experiments.

Hormonal Treatments

A variety of sex hormones (see Table I) (Nutritional Biochemicals) in different dosages was tested for effectiveness in inducing hypertrophy of the male rudimentary oviduct. The dosages used were those reported by other investigators, which are known to induce jelly secretion in male frogs or to induce ovulation in female frogs and toads. Steroid hormones were dissolved in sesame oil and injected into the dorsal lymph sacs of the animals. Non-steroid hormones were dissolved in ten percent full strength Holtfreter's solution and injected intraperitoneally. Three types of controls were used in these experiments: males injected with sesame oil, injected with Holtfreter's solution, and males left untreated.

Table I. Hormones tested for their effectiveness in inducing hypertrophy of Rana pipiens male rudimentary oviducts.

Treatments	Source of Hormone	Dosage Used	Code or Abbreviation
HORMONES			
Testosterone Propionate	Nutritional Biochemical Company	5 mg-0.25 ml oil/frog	Test A
		10 mg-0.25 ml oil/frog	Test B
Estradiol Benzoate	Nutritional Biochemical Company	5 mg/0.25 mml oil/frog	Est
Progesterone	Nutritional Biochemical Company	2.5 mg/0.25 ml oil/frog	Prog A
		5 mg/0.25 ml oil/frog	Prog B
		10 mg/0.25 ml oil/frog	Prog C
Progesterone + Estradiol	Nutritional Biochemical Company	5 mg + 5 mg/0.25 ml oil/frog	Prog + Est
Prolactin	Ovine, NIH-P-S8	2 I.U./0.25 ml Holt/frog	Prol
Chorionic Gonadotropin	Nutritional Biochemical Company	250 I.U./0.25 ml Holt/frog	Chor
CONTROLS			
Sesame oil	--------	0.25 mg/frog	Ses
Untreated	--------	-----	Unt
Holtfreter's	--------	-----	Holt

Since in the first couple of experiments prolactin and chorionic gonadotropin gave a high mortality rate and showed no hypertrophic effect on the male oviduct, these two hormones were not used in subsequent experiments. Progesterone also did not show a hypertrophic effect with the initial dosage tested. However, it was tried at other dosages in subsequent experiments. Each experiment was initiated with 8-10 animals per treatment. However, since some frogs died in each group, the same experiments were repeated several times (see experiments 1 to 5 in Appendix Tables II-VI) in order to obtain an adequate sample of each experimental group. Furthermore, since no significant variation was observed between the results obtained from the individual experiments, they were added and a mean determined for each individual treatment. (See Appendix Table I.) For each frog in each treatment, body weights (initial and final) and oviducal weights are given, as well as the ratio of oviduct weight to final body weight.

At the beginning of each experiment, frogs were brought out of the cold room and allowed to come to room temperature and weighed before the injection of the hormone or control solution was given (see Table I for dosages). Thereafter, frogs were left untreated and without feeding for a period of two weeks, at room temperature (20^{o}C) in glass jars containing approximately 1 inch of water. Water was changed daily.

Dissection of Oviducts

At the end of two weeks the animals were sacrificed by pithing (demedullation), weighed (final body weight) and their oviducts dissected. The wet weight of oviducts was recorded. The oviducts were then rinsed in ten percent full strength Holtfreter's solution. Photographs of some dissected oviducts were taken while in Holtfreter's solution. The right oviduct of each frog was used for histochemical tests, and the other for the preparation of antigens when running double-diffusion tests.

The oviduct to be used for histochemical tests was stretched out on a glass plate and cut into approximately three equal segments: an upper region (up), which includes the pars recta, a middle region (mid), which includes the upper part of the pars convoluta, and a lower region (low), which includes the lower part of the pars convoluta. The pars uterus or ovisac was discarded. A sample of approximately 3 millimeters was cut from the center part of each third (see Text Figure II) and fixed by freeze-substitution. Oviducts from untreated, non-ovulated, mature females were also sampled for histological and histochemical comparison.

Fixation and Embedding Procedures

Fixation by the freeze-substitution method was employed since it is known to give good cytological preservation. Soluble acid mucopolysaccharides are also very well preserved

by this method (Neder and Sidman, 1958). For freeze-substitution, the pieces of oviduct were frozen (quenched) in freon chilled with liquid nitrogen (-170 to -175°C), transferred to 1% alcoholic picric acid cooled in dry ice (-70°C) and kept there for 7 days. The tissues were then transferred into several changes of 100% ethanol at 0°C, cleared in xylene, and embedded in Paraplast. Sections of 4 μ thickness were cut and mounted on albumin-coated slides.

Histochemical Tests

Representative sections from the upper, middle and lower regions of male hormone-treated oviducts (estradiol and testosterone-treated), as well as from untreated male oviducts were employed in the histochemical tests. Sections from the upper, middle and lower regions of untreated, non-ovulated mature female oviducts were utilized for comparison. The following tests were performed to detect carbohydrates and protein.

Carbohydrates

The periodic acid-Schiff (PAS) procedure for neutral mucopolysaccharides, rich in adjacent hydroxyl or equivalent groups (Mowry, 1963) was used both with and without preceding treatments to determine the nature of the PAS-reactive compounds with greater accuracy (Barka and Anderson, 1963). The Schiff's reagent was prepared according to the method

of de Tomasi (Pearse, 1970). PAS-positive protein was removed by incubating sections at 37°C for 1 hour with trypsin (a 0.1% solution in 0.I M sodium phosphate buffer, pH 7.4). The buffer without the enzyme was used as a control (Barka and Anderson, 1963). Treatment of sections with a 1:1 mixture of chloroform and absolute methanol for 4 hours at 60°C prior to the PAS procedure was used to eliminate PAS-reactive lipids (Barka and Anderson, 1963). The specificity of the PAS reaction was tested by subjecting the sections to the Schiff's reagent without prior oxidation by periodic acid, and by acetylation for two hours at 60°C with a 2:3 mixture of acetic anhydride and pyridine. Acetylation blocks both hydroxyl (O-acetylation) and amino (N-acetylation) groups, thus preventing their reactions with periodic acid (Barka and Anderson, 1963). For the removal of glycogen, a 0.1% malt diastase solution in 0.02 M sodium phosphate buffer, pH 6.0, containing 0.65% NaCl was used (Barka and Anderson, 1963). Sections were placed for 1 hour at 37°C in staining jars containing preheated malt diastase solution. Frog liver sections, treated with diastase as well as oviducal sections treated with buffer alone served as controls.

Alcian blue staining (pH 2.6) was employed for the detection of complex carbohydrates with free acidic groups (acid mucopolysaccharides) (Mowry, 1963). A 1% solution of the dye (alcian blue 8GX, Allied Chemicals), in 3% acetic

acid was used alone or followed by a 5 minute treatment with a 0.1% Nuclear Fast Red (Kernechtrot) solution for contrast (Luna, 1968). Mowry's (1968) procedure for the detection of sulfated mucopolysaccharides was also used. This utilized a 0.25% alcian blue solution in 0.5 N HCl (pH 0.5). Counterstaining with Nuclear Fast Red was done in some cases. The specificity of the alcian blue reaction was checked by methylating the acid groups with absolute methanol in 0.1 N HCl for 4 hours at 60°C prior to staining (Thompson, 1966).

The alcian blue (pH 2.6)-periodic acid-Schiff (AB-PAS) sequential procedure for the combined coloration of free acidic and adjacent hydroxyl groups was used for a survey of the distribution of both acidic and neutral mucopolysaccharides in the oviducal sections (Mowry, 1963).

Enzymatic treatments

Bovine testicular hyaluronidase (Nutritional Biochemicals-600 units/mg) was used in a 0.1 M sodium phosphate buffer at pH 6.0, in a concentration of 0.5 mg/ml (or 300 units/ml). Sections were treated for 6 hours at 37°C with 3 drops of solutions containing either the enzyme, boiled enzyme (20 minutes at 100°C), or buffer. The last two treatments served as controls. Frog xiphoid cartilage and Achilles' tendon served also as controls. After enzymatic digestion, sections were stained for acid mucopolysaccharide with alcian blue pH 2.6 or with toluidine blue 0.1% (Barka

and Anderson, 1963). Sections stained with toluidine blue were mounted in water, sealed with nail polish, and examined immediately under the microscope for metachromasia both before and after dehydration with alcohol.

For the detection of sialic acids, sections were incubated with sialidase (neuraminidase, Clostridium perfringens, Nutritional Biochemicals, 125 units/mg) in a concentration of 0.1% (1 mg/ml) in a 0.I M sodium phosphate butter, pH 6.0, for 24 hours at 37°C (Spicer and Warren, 1960). About 0.1 ml (2 drops) of the enzyme solution was used on each slide. Controls were covered with a similar volume of boiled enzyme (20 minutes at 100°C), or with buffer alone. Slides were stained with the AB-PAS sequential procedure. Other sections were deacetylated prior to digestion with sialidase by treatment with an alcohol-ammonia mixture (Lillie, 1954) for 24 hours at 37°C in a closed container. A considerable number of sialomucins contain sialic acid in the N-acetyl-O-diacetyl form, a configuration which imparts a great resistance to the action of neuraminidase (sialidase) (Ravetto, 1968). Deacetylation works by exposing free sialic acid residues on which the enzyme can act.

Slides containing sections exposed to either enzyme were suspended on stirring rods placed in large Petri dishes containing a few mls of buffer solution, which were incubated in an oven at appropriate temperatures.

Proteins

The ninhydrin-Schiff procedure of Yasuma and Itchikawa (1953) was used for the general detection of protein, i.e., αNH_2 groups. Three types of controls were used to test for the specificity of the reaction: 1. Slides in which the ninhydrin oxidation step was omitted, thus not producing the imino groups needed for making the complex with Schiff's reagent, which is responsible for the visible color reaction. 2. Slides subjected to either deamination, i.e., freshly prepared solutions of nitrous acid at $0^{o}C$ for 45 minutes, or acetylation, i.e., solution of 1% acetic acid in acetic anhydride at $60^{o}C$ for one hour. These procedures were employed prior to testing with the ninhydrin-Schiff procedure. Both types of reactions block αNH_2 groups, thus making them unavailable for reaction (i.e., oxidation) with the ninhydrin reagent.

Color photographs of stained sections at 100 X and 430 X magnification were made for permanent record. Kodak Ektachrome High Speed Film (ASA 125) was used with a Canon 35 mm camera.

Immunological Procedures

Production of anti-oviducal jelly sera

Preparation of antigens

Two types of oviducts were utilized as sources of antigens for the production of antisera against oviducal jellies.

These are: 1) untreated, mature, non-ovulated female whole oviducts, and 2) untreated, male whole rudimentary oviducts. Whole oviducts (having no eggs in the case of female oviducts) were dissected from R. pipiens male and female adults, and were cut into small pieces and homogenized in conical glass homogenizers in 10% full strength Holtfreter's solution. For each gm (or mg) of oviducts, 20 ml (or 0.02 ml) of 10 percent full strength Holtfreter's solution was used for homogenization. This is the standard antigen solution.

Injection protocol

The oviducal antigen preparations consisted of 1 ml of the standard antigen solution emulsified in an equal volume of Freund's complete adjuvant. This emulsion was injected, half on each side, into subscapular region of rabbits. One ml of antigen and one ml of incomplete Freund's adjuvant were injected one week later. In 3-4 weeks, if antibodies could be detected on agar-diffusion plates, bleedings from the ear vein were continued every other week. Antigen injections were repeated every six weeks or two months. The antisera thus obtained, were employed in the Ouchterlony plates. Blood, collected from the rabbits prior to the first antigen injections, was used as the source of control serum. Unfractionated antisera were used in all cases.

Ouchterlony double-diffusion tests

The presence of the antigenic components of the various jellies was detected by means of a modification of the Ouchterlony double-diffusion technique (Shaver, 1961). A total of 0.3 mls of antigen preparation or antiserum was used per well. Plates were prepared employing 1% agar (Ionogar) and were developed at room temperature (20-22°C) for 4 days. Photographs or drawings were made.

Preparation of antigens for double-diffusion tests

Whole male rudimentary oviducts were cut into small pieces and homogenized in 0.5 ml of 10% full-strength Holtfreter's solution for the untreated animals, or 1.0 ml for the hormone-treated animals (testosterone and estradiol). In the case of non-ovulated, mature females, the standard antigen solution (1 gm of oviduct per 20 ml of 10% full strength Holtfreter's solution) used for the production of anti-oviducal jelly sera was utilized. Antigen preparations from different animals were tested individually on Ouchterlony plates.

RESULTS

Morphological Effects of Hormonal Stimulation on Male Oviducts

The enlargement of the hormone-treated male rudimentary oviducts is due to increases (both in cellular size and) in the functional activation of the tubular glands. The rudimentary oviducts of untreated male frogs appear as two thread-like tubes paralleling the lateral aspects of the kidneys. They have an average diameter of 1 mm, a small degree of convolution in the pars convoluta, and an average length, when stretched, of approximately 6 cm.

Upon hormonal stimulation, there was an overall enlargement in the average diameter of the oviducts to $\simeq$ 2-3 mm and a large increase in the degree of convolution all along the length of the oviducts. Furthermore, after treatment with individual hormones or with combinations of hormones, the male oviducts grew forward to a level comparable with that of the ostium of the female oviduct, which normally they do not do, but the ostium did not open. However, neither in diameter nor length did the male hormone-treated oviduct ever equal the female, untreated, non-ovulated mature oviduct, with the hormone concentrations used. The diameter of the female oviduct is approximately twice that

of the hormone-treated male oviducts. The lengths of the two types of oviducts also vary (females--33 cm; hormone-treated males--7 cm).

Hormones which induced hypertrophy of the male rudimentary oviduct of R. pipiens are shown in Table II and Text Figure III.

A significant increase in the mean ratio of oviduct weight/body weight over that for untreated, male rudimentary oviduct was observed for estradiol at a concentration of 5 mg/0.25 ml of sesame oil (see Table II and Text Figure III).

The effect of testosterone was tested using two dosages of the hormone (5 mg/0.25 ml = Test A; 10 mg/0.25 ml = Test B). In each case, a significant increase in the mean ratio of oviduct weight/body weight over that of both controls (oil and untreated) was obtained. (Compare Test A and Test B with Unt and Oil.) However, this hypertrophic effect of testosterone on the male rudimentary oviduct, although significant, is less than that of estradiol at an equivalent dosage, i.e., 5 mg/0.25 ml (compare EST and Test A in Table II and Text Figure III). An equivalent hypertrophic effect to that of estradiol is obtained when testosterone is used at a concentration of 10 mg/0.25 ml.

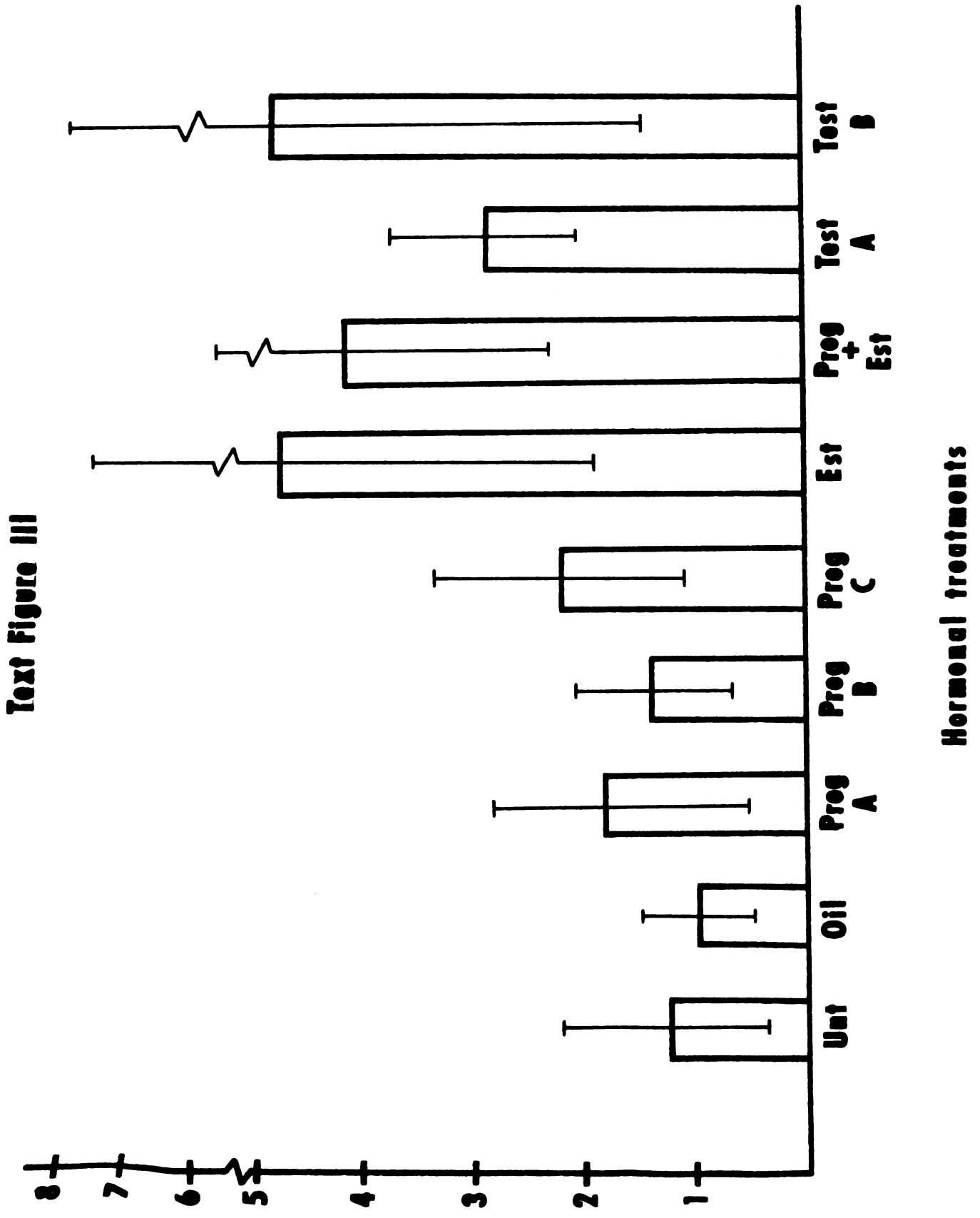
Treatment with progesterone at two concentrations (2.5 mg/0.25 ml = Prog A; 5 mg/0.25 ml = Prog B) had no effect on the male rudimentary oviduct. Progesterone at a concentration of 10 mg/0.25 ml (Prog C) seems to have a

Table II. Hypertrophic effects of hormones tested in mean ratios of oviduct weight to final body weight.

Treatment	Sample size (n)	Mean ratio ($\bar{y}$) of oviduct wt./final body wt.	Standard Deviation (s)
UNT	17	1.2	0.9
OIL	24	0.9	0.5
PROG A	8	1.7	1.0
PROG B	17	1.3	0.7
PROG C	7	2.1	1.1
EST	17	4.6	2.8
PROG + EST	13	4.0	1.8
TEST A	15	2.8	0.8
TEST B	7	4.6	3.2

Text Figure III

Effects of various hormonal treatments on weights of rudimentary male oviducts.



Text Figure III

slight hypertrophic effect on the male oviduct, but no definite conclusions can be made since there is some degree of overlapping between the ranges covered by the oviducal ratios of this hormone treatment with that of the 2 controls.

The combination of progesterone plus estradiol (5 mg of progesterone + 5 mg of estradiol dissolved in 0.25 ml of sesame oil) also induced hypertrophy of the male oviduct.

The mean ratios of oviduct weight/body weight for the 2 types of control used, i.e., untreated and sesame oil-injected frogs, are not significantly different from each other (see Table II and Text Figure III).

Three kinds of epithelial cells are observed microscopically in the upper (pars recta), middle (upper part of pars convoluta) and lower (lower part of pars convoluta) regions of male rudimentary oviducts: 1. tall, columnar cells with basal nuclei, making up the tubular glands, which are found arranged radially along the whole length of the oviduct, 2. the mucous cells and 3. the ciliated cells. The latter two cell types surround the oviducal lumen (see Text Figure II). Very little secretion is observed in the tubular glands cells per se, and none in the lumen of the tubular glands (Figure 6, Plate II of untreated male rudimentary oviducts.

Upon hormonal stimulation (e.g., testosterone, estradiol), there is an increase in tubular gland cell size due

to an accumulation of newly synthesized jelly in the cytoplasm of these cells. This increase in size of the tubular glands is greater in the middle and lower regions than in the upper regions (compare Figure 8, Plate III and Figure 10, Plate IV). The nuclei of the jelly-enlarged tubular gland cells appear displaced from their basal position, and in some cases are masked completely due to the amount of secretory material (see Figure 10, Plate IV). The epithelial lining surrounding the male oviducal lumen becomes thicker and more folded (see Figure 21, Plate X).

The histological appearance of the hormone-treated male rudimentary oviduct is very similar to that of the female, untreated, non-ovulated, mature oviduct (compare Figure 9, Plate IV and Figure 14, Plate VI) with enlarged tubular gland cells, full of jelly material and with virtually no visible nuclei and an oviducal lumen surrounded by epithelial folds.

Injections of sesame oil and progesterone at the various concentrations caused no hypertrophy of male rudimentary oviducts (see Figures 2 and 4, Plate I). The gross morphology of these two types of oviducts is identical to that of untreated males (see Figure 1, Plate I).

Histochemical Tests

Only those hormonal treatments, i.e., estradiol and testosterone, which had a marked hypertrophic effect on

the male rudimentary oviduct of R. pipiens were used in the histochemical tests. In some cases female oviducts were used for comparison.

Periodic acid-Schiff reaction

A strong, positive reaction (magenta color) was obtained in all the tubular glands of the oviducal sections which stained with periodic acid-Schiff (PAS) (see Table III). In all oviducal sections tested mucous cells exhibited variable staining reaction (Figure 11, Plate V). However, differences between hormone-treated and untreated male oviducts and between the different regions of the hormone-treated male oviducts were observed with respect to the amount (color intensity) and distribution of the PAS-positive secretory material present in their tubular glands.

Untreated male oviducts

Cross-sections through either the upper, middle (Figure 5, Plate II) or lower regions of untreated, male rudimentary oviducts showed tubular glands with very little PAS-positive material. Non-staining nuclei can be observed in the basal region of the individual tubular gland cells (Figure 6, Plate II). Mucous cells give a strong PAS-positive reaction. The same pattern of staining was observed in the sesame oil-injected animals.

Hormone-treated male oviducts

Representative sections taken through the upper region

Table III. Oviducal tissues stained by PAS reaction.

Oviducal tissue		PAS normal		W/O Period. Acid Oxid.		After diast. digestion		After acetyl.		After lipid extr.		After Protein extr.	
		MC	TG	MC	TG	MC	TG	MC	TG	MC	TG	MC	TG
MALE													
EST	UP	+++	+++	0	0	+++	+++	0	0	+++	+++	+++	+++
	MID	+++	+++	0	0	+++	+++	0	0	+++	+++	+++	+++
	LOW	+++	+++	0	0	+++	+++	0	0	+++	+++	+++	+++
UNT	UP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	MID	+++	+++	0	0	+++	+++	0	0	+++	+++	+++	+++
	LOW	+++	+++	0	0	+++	+++	0	0	+++	+++	+++	+++
OIL	UP	+++	+++	0	0	+++	+++	0	0	+++	+++	+++	+++
	MID	+++	+++	0	0	+++	+++	0	0	+++	+++	+++	+++
	LOW	+++	+++	0	0	+++	+++	0	0	+++	+++	+++	+++
TEST A	UP	+++	+++	0	0	+++	+++	0	0	+++	+++	+++	+++
	MID	+++	+++	0	0	+++	+++	0	0	+++	+++	+++	+++
	LOW	+++	+++	0	0	+++	+++	0	0	+++	+++	+++	+++
FEMALE													
NO UNT	UP	+++	+++	0	0	+++	+++	0	0	+++	+++	+++	+++
	MID	+++	+++	0	0	+++	+++	0	0	+++	+++	+++	+++
	LOW	+++	+++	0	0	+++	+++	0	0	+++	+++	+++	+++

+++ = Strong color reaction
0 = no color reaction
MC = Mucous cells
TG = Tubular glands
ND = Not done
NO = Non-ovulated

of estradiol-treated male oviducts (Figure 7, Plate III) showed tubular glands staining with different intensities of red. The darker red-staining tubular glands were generally randomly distributed within the upper oviducal regions, although in some cases (Figure 8, Plate III) they were localized in the periphery of the oviduct.

In sections taken from the middle (Figure 9, Plate IV) and lower regions of estradiol-treated male oviducts, all the tubular glands stain magneta with the same intensity and the constituent cells have more secretory material than in the upper region (compare Figure 10, Plate IV, and Figure 8, Plate III). The cells as well as the lumen of the glands are so full of secretory material that nuclei are not always visible in a particular cross-section (Figure 10, Plate IV).

Sections taken from either the upper, middle and lower oviducal regions of testosterone-treated males showed that all the tubular glands gave a strong PAS-positive reaction (see Figure 11, Plate V). Scattered PAS-positive mucous cells are also observed around the lumen of the oviduct.

Controls

The PAS-staining of any of the oviducal sections tested (male and female) was not altered by diastase digestion (compare Figure 9, PIate IV and Figure 12, Plate V), nor after lipid and protein extraction. Rat liver sections, incubated with diastase, prior to PAS staining,

gave a very weak PAS reaction. No staining occurred in sections if the periodic acid hydrolysis (oxidation) step was omitted (Figure 13, Plate VI) or when sections were acetylated prior to PAS staining.

Oviducts from untreated, non-ovulated mature females were used as another type of control. Representative sections from any of the three oviducal regions showed tubular glands full of PAS positive material (see Figure 14, Plate VI). These reactions are very similar to those obtained with hormone-treated male oviducts.

Alcian blue staining at pH 2.6

The presence of acidic mucopolysaccharides in the various oviducal regions of hormone-treated and untreated R. pipiens males, as detected by alcian blue staining at pH 2.6 is indicated in Table IV. Alcian blue was used alone or Nuclear Fast Red was used as a counterstain.

Untreated male rudimentary oviducts

A very weak or a negative reaction was obtained in the tubular glands from upper, middle and lower oviducal regions from untreated males when stained with alcian blue at pH 2.6 (Figure 18, Plate VIII). On the other hand, the mucous cells present in all three oviducal regions gave a strong positive reaction for acid polysaccharides (see same Figure).

Table IV. Alcian blue staining at pH 2.6.

Oviducal Tissue		Tubular Glands	Mucous Cells
MALE			
EST	UP	+++	+++
	MID	+	+++
	LOW	+	+++
TEST A	UP	++	+++
	MID	+	+++
	LOW	++	+++
UNT	UP	+	+++
	MID	+	+++
	LOW	+	+++
FEMALE			
NO UNT	UP	++	+++
	MID	+	+++
	LOW	+	+++

\+ = Weak color reaction
++ = Moderate color reaction
+++ = Strong color reaction, i.e., turquoise blue

Female control

Acid mucopolysaccharides were detected in all three oviducal levels of untreated, non-ovulated, mature females, by a moderately positive reaction in the upper level (Figure 15, Plate VII), and a very weak reaction was obtained in the middle (Figure 16, Plate VII) and lower oviducal regions. The mucous cells present in all three oviducal regions stained strongly with alcian blue at pH 2.6.

Methylation of sections, prior to staining with alcian blue at pH 2.6 eliminated the staining reaction of all oviducal tissues.

Hormone-treated male oviducts

A strong positive reaction, i.e., turquoise blue, for acid mucopolysaccharides was obtained in the vast majority of the tubular glands present in the upper oviducal region of estradiol-treated males (see Figures 19 and 20, Plate IX). Representative sections from the middle and lower levels of these oviducts showed a very weak staining reaction with alcian blue at pH 2.6 (see Figure 21, Plate X).

Moderate staining reaction with alcian blue at pH 2.6 was obtained in sections of testosterone-treated male rudimentary oviducts from the upper and lower oviducal regions (see Figure 17, PIate VIII), and a very weak reaction in the middle oviducal region, similar to that obtained in the upper level of estradiol-treated tissues.

In both hormone-treated oviducts (estradiol and testosterone) a strong positive reaction for acid mucopolysaccharide was obtained in the mucous cells present in all three oviducal regions (see Figure 22, Plate X).

Alcian blue staining at pH 0.5

A summary of alcian blue staining reactions at pH 0.5 for sulfated mucopolysaccharides present in the male rudimentary oviducts of R. pipiens appears in Table V.

Untreated male rudimentary oviducts

No sulfate was detected histochemically in the tubular glands of untreated or sesame oil-injected male oviducts in any of the three oviducal levels (Figure 23, Plate XI). However, scattered alcianophilia at pH 0.5 was observed in the mucous cells of the three oviducal regions of these two control oviducts (see same figure).

Hormone-treated male rudimentary oviducts

A cross-section through the upper region of an estradiol-treated male rudimentary oviduct showed that some tubular glands contained sulfated mucopolysaccharides, and some did not. In the latter only the nuclei stained red due to the counterstaining with Nuclear Fast Red (Figure 25, Plate XII). These tubular glands appear in higher magnification in Figure 26, Plate XII, and Figure 27, Plate XIII. No sulfated mucopolysaccharides were detected in the middle and lower oviducal regions of estradiol-treated oviducts.

Table V. Alcian blue staining at pH 0.5.

Oviducal Tissue		Tubular Glands	Mucous Cells
MALE			
UNT	UP	0	+++
	MID	0	+++
	LOW	0	+++
OIL	UP	0	+++
	MID	0	+++
	LOW	0	+++
EST	UP	+++	+++
	MID	0	+++
	LOW	0	+++
TEST A	UP	0	+++
	MID	0	+++
	LOW	+++	+++

0 = No color reaction
\+ = Weak color reaction
++ = Moderate color reaction
+++ = Strong color reaction

A similar pattern of staining was observed in the lower oviducal region of testosterone-treated males (Figure 28, Plate XIII). However, in this case the number of glands containing sulfated mucopolysaccharides is less than that present in the upper oviducal region of estradiol-treated males. No sulfated mucopolysaccharides were detected in the upper and middle regions of testosterone-treated oviducts.

Mucous cells present in the three regions of rudimentary oviducts treated with estradiol and testosterone gave scattered staining for sulfated mucopolysaccharides (Figure 24, Plate XI).

When oviducal sections were subjected to methylation to test for the specificity of the alcian blue reaction, no staining reaction was observed.

Toluidine blue staining

The results obtained when oviducal sections are stained with toluidine blue appear in Table VI.

Untreated male oviducts

The tubular glands present in the three oviducal regions of untreated male oviducts showed a very weak metachromatic reaction (faint pink) when stained with toluidine blue. The mucous cells stained dark pink.

Female control oviducts

A strong positive metachromatic reaction (dark pink) was obtained in both the tubular glands and the mucous cells

Table VI. Toluidine blue metachromasia.

Oviducal tissue		W/O alcohol dehydration		Alcohol dehydrated	
		Tubular glands	Mucous cells	Tubular glands	Mucous cells
MALE					
TEST A	UP	++	+++	0	0
	MID	++	+++	0	0
	LOW	+++	+++	0	0
OIL	UP	+	+++	0	0
	MID	+	+++	0	0
	LOW	+	+++	0	0
FEMALE					
NO UNT	UP	+++	+++	0	0
	MID	+++	+++	0	0
	LOW	+++	+++	0	0

0 = No color reaction
\+ = Very weak metachromasia
++ = Moderate metachromasia
+++ = Strong metachromasia

of the three oviducal levels of untreated, non-ovulated, mature females (see Figure 29, Plate XIV). The presence of metachromatic granules is evident in the enlarged tubular glands found in these oviducal tissues.

Hormone-treated male oviducts

Staining with toluidine blue yielded a metachromatic reaction in the tubular glands of testosterone-treated male rudimentary oviducts. A cross-section through the upper or middle oviducal regions of such specimens showed tubular glands staining from a pinkish-orange to a dark pink. Mucous cells always stained dark pink (see Figure 30, Plate XV). The presence of dark pink-staining tubular glands was evident also in the lower oviducal regions of testosterone-treated oviducts.

In all the oviducal sections tested, metachromatic staining disappeared subsequent to dehydration in alcohol.

Sialidase digestion

The presence of sialomucins in the jelly secretions of oviducal tissues was indirectly assessed by digesting representative sections with sialidase and then staining for the presence of acidic mucopolysaccharides. If sialomucins were present, the enzyme would remove the sialic acid moieties, thus exposing free carboxyl groups on the molecules which would then react with basic dyes such as alcian blue at pH 2.6. Furthermore, since the possibility

exists that the sialic acid moieties may be in the N-acetyl-O-diacetyl form, a configuration which imparts to the sialomucins a great resistance toward the action of sialidase (Ravetto, 1968), some sections were deacetylated, prior to digestion with the enzyme. Table VII gives a summary of the protocol for the enzyme digestion and the results obtained.

Four sets of slides were tested for each oviducal tissue being assayed for sialomucins (see Table VII): 1) slides that were first deacetylated, sialidase-digested and then stained with the alcian blue-PAS sequential procedure (AB-PAS), 2) slides that were first deacetylated, but left undigested, and stained with AB-PAS, 3) slides that were not deacetylated, but were sialidase-digested and stained with AB-PAS, 4) slides that were not deacetylated nor sialidase-digested, but only stained with AB-PAS (see Table VIII).

Representative sections from the upper, middle and lower regions of untreated, non-ovulated, mature female oviducts were also assayed for comparison (as a control).

Untreated male rudimentary oviducts

A representative section through the lower oviducal region of an untreated male stained by the alcian blue-PAS sequential procedure appears in Figure 32, Plate XVI. Tubular gland cell cytoplasm stained red, and the nuclei stained turquoise blue while mucous cells stained purple. This same pattern of staining was also obtained in the upper

Table VII. Sialidase digestion of oviducal tissues. Stained with alcian blue (pH 2.6)-PAS procedure.

Oviducal tissue		Undeacetylated		Deacetylated	
		Digested	Undigested	Digested	Undigested
MALE					
EST	UP	0	0	+++	0
	MID	0	0	0	0
	LOW	0	0	0	0
UNT	UP	0	0	0	0
	MID	0	0	0	0
	LOW	0	0	0	0
FEMALE					
NO UNT	UP	0	0	+++	0
	MID	0	0	0	0
	LOW	0	0	0	0

0 = No alteration in normal staining reaction (AB-PAS) was observed (see text for details).

+++ = Staining reaction was altered. Either a decrease in intensity of alcian blue staining or a change from reddish-purple to red was observed.

Table VIII. Alcian blue (pH 2.6)-periodic acid-Schiff sequential procedure.

Oviducal tissue		Tubular glands	Mucous cells
MALE			
EST	UP	Blue	Purple
	MID	Red	Purple
	LOW	Red	Purple
TEST A	UP	Red	Purple
	MID	Red	Purple
	LOW	Purple	Purple
UNT	UP	Red	Purple
	MID	Red	Purple
	LOW	Red	Purple
OIL	UP	Red	Purple
	MID	Red	Purple
	LOW	Red	Purple
FEMALE			
No UNT	UP	Purple	Purple
	MID	Red	Purple
	LOW	Red	Purple

and middle oviducal regions of untreated males. Furthermore, when representative sections from any of these 3 oviducal regions from untreated males were digested with sialidase, with or without the deacetylation step, the above mentioned staining pattern remained the same (compare Figure 33, Plate XVI and Figure 34, Plate XVII).

Female oviducts

Representative sections from the upper, middle and lower regions of untreated, non-ovulated, mature females were tested for the presence of sialomucins. In all three oviducal regions, large tubular glands are observed which are full of secretory material and have sparse turquoise-blue staining nuclei. In the upper oviducal region, this secretory material stained reddish-purple (mixture of red and blue) and the mucous cells purple (see Figure 35, Plate XVII).

Upon deacetylation and sialidase digestion, a certain degree of color removal was observed only in the upper oviducal regions. Tubular glands which normally stained reddish-purple now stained light pink (compare Figure 35, Plate XVII and Figure 38, Plate XIX). The alcian blue-positive material was removed by the sialidase treatment and only the PAS-positive material remained.

If the deacetylation step was omitted, sialidase digestion had no effect on the staining (see Figure 36, Plate XVIII). Sections through the middle and lower oviducal regions of non-ovulated females showed tubular glands

staining red (magenta) and mucous cells staining light purple with the AB-PAS procedures. This staining pattern remained the same in sections that were sialidase-digested, with or without the deacetylation step.

All control sections that were deacetylated but not digested, and then stained with AB-PAS had essentially the same staining pattern as that of untreated normal sections (compare Figure 37, Plate XVIII and Figure 35, Plate XVII). In other words, the deacetylation step by itself has no effect on the future stainability of the oviducal sections.

Estradiol-treated male rudimentary oviducts

A representative cross-section through the upper oviducal region of estradiol-treated males stained with the AB-PAS procedure shows that the tubular glands surrounding most of the oviducal lumen stain blue, i.e., they are positive for acid mucopolysaccharide (see Figure 39, Plate XIX). Furthermore, a decrease in the staining intensity of these glands, i.e., stain very light blue, was observed when sections were deacetylated prior to sialidase digestion (see Figure 41, Plate XX). However, if this deacetylation step (prior to the sialidase digestion) is omitted, the alcian blue staining reaction remained unaltered, i.e., blue (see Figure 40, Plate XX).

The tubular glands present in the middle and lower oviducal regions of estradiol-treated males stained red (PAS-positive) with the alcian blue-PAS sequential procedure,

while the mucous cells stained reddish-purple (this is a mixture of red and blue) (see Figure 42, Plate XXI). The staining reactions of both the tubular glands and mucous cells of these two oviducal regions remained unaltered when representative sections were sialidase-digested, with or without the deacetylation step (compare Figure 43, Plate XXI and Figure 44, Plate XXII).

Hyaluronidase digestion

The presence of hyaluronic acid moieties in the acid mucopolysaccharides present in oviducal jelly secretions was indirectly assayed by subjecting representative sections to hyaluronidase digestion prior to staining for acid mucopolysaccharide with alcian blue at pH 2.6 or toluidine blue.

Hyaluronidase treatment did not alter the reactions of the oviducal tissues to alcian blue at pH 2.6, tissue coloration remaining turquoise blue after digestion. The patterns of alcian blue staining characteristic of the tubular glands and mucous cells present in the three oviducal regions were the same in untreated males, in untreated, non-ovulated, mature females and in estradiol-treated males (compare Figures 45 and 46, Plate XXIII).

Toluidine blue metachromasia or testosterone-treated males was also not altered by treatment with hyaluronidase (compare Figures 30 and 31, Plate XV).

After treatment of sections of tendon or cartilage (controls) with hyaluronidase, a decrease in the toluidine blue or alcian blue (pH 2.6) staining was observed.

Ninhydrin-Schiff Reaction for Protein

Representative sections from the three oviducal levels of untreated males (Figure 47, Plate XXIV) showed a slightly darker pink coloration than sections from hormone-treated males. Connective tissue in the periphery of the oviduct stained dark pink.

Representative sections from the three oviducal levels (upper, middle and lower) of both estradiol-treated males (Figures 49 and 50, Plate XXV) and untreated, non-ovulated, mature females (Figure 48, Plate XXIV) gave a very weak positive reaction, i.e., light pink, when stained with the ninhydrin-Schiff procedure. This pinkish coloration is uniformly distributed over all the tubular glands and mucous cells with no indication of localized areas of different intensities.

No staining was obtained when sections were either acetylated or deaminated prior to the ninhydrin-Schiff reaction. Furthermore, no staining was obtained when the ninhydrin hydrolysis (oxidation) step was omitted.

Immunological Tests

The Ouchterlony gel diffusion tests were performed to answer two questions: 1. How does the untreated male rudimentary oviduct compare antigenically with hormone-treated oviducts? 2. How do male oviducts both untreated and hormone-treated compare antigenically with oviducts of non-ovulated mature females? It was hoped that a correlation could be made between the different types of mucopolysaccharides identified by histochemical procedures and the number of antigens identifiable in the gel-diffusion tests in male oviducts both before and after hormonal stimulation.

The top portion of Text Figure IV shows a gel diffusion plate where antiserum against untreated R. pipiens male rudimentary oviduct (UNT ♂-Ab) was reacted with untreated male rudimentary oviduct antigen (UNT ♂-Ag) and non-ovulated, mature female oviduct antigen (NO ♀-Ag). Untreated male rudimentary oviducts have at least 8 different antigens, six of which are shared between untreated male rudimentary oviduct and non-ovulated, mature female oviduct. Two precipitin lines are unique to untreated male oviducts (see arrow).

No bands were obtained when non-immune serum was used.

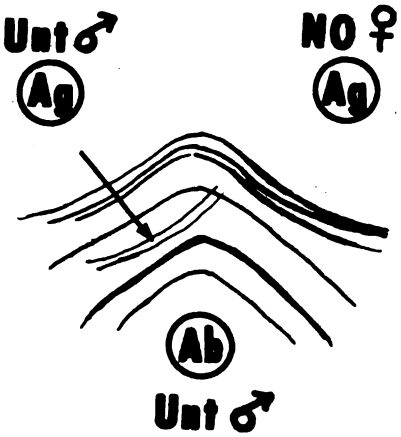
The bottom portion of Text Figure IV shows a gel diffusion plate in which antiserum against non-ovulated, mature R. pipiens female oviducts (NO ♀-Ab) was reacted with untreated male rudimentary oviduct antigen (UNT ♂-Ag),

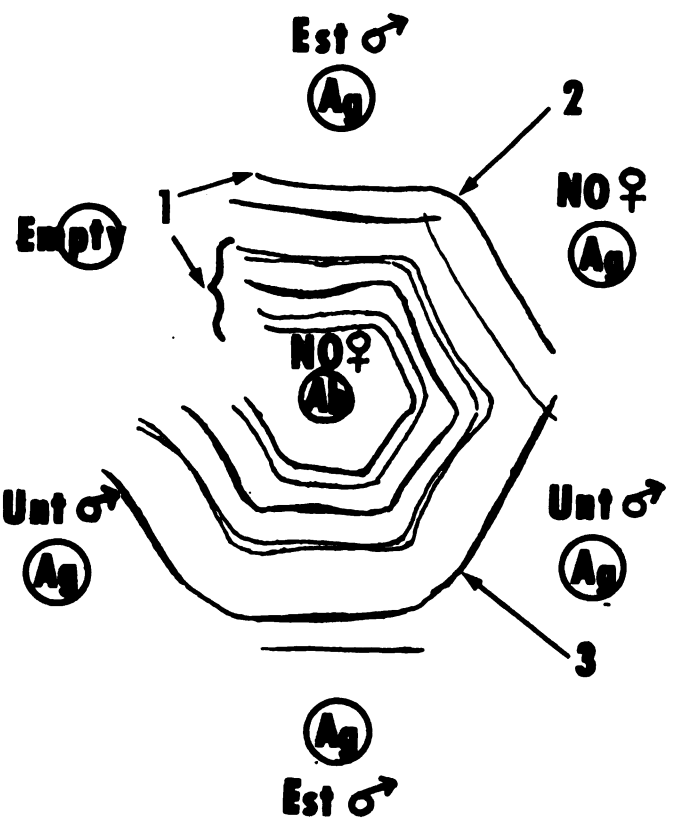
Explanation of Text Figure IV

The top portion of figure IV illustrates a gel diffusion plate where antiserum against untreated Rana pipiens male rudimentary oviduct (UNT ♂ -Ab)--(center well) was reacted with untreated male rudimentary oviduct antigen (UNT ♂ -Ag) and non-ovulated, mature female oviduct antigen (NO ♀ -Ag)--(2 outer wells).

The bottom portion of this figure shows a gel diffusion plate in which antiserum against non-ovulated, mature Rana pipiens female oviducts (NO ♀ -Ab)--(center well) was reacted with 3 different antigens (outer wells): untreated male rudimentary oviduct antigen (UNT ♂ -Ag), estradiol-treated male oviduct antigen (EST ♂ -Ag) and non-ovulated, mature female oviduct antigen (NO ♀ -Ag). No antigen was put in one well (empty).

Text Figure IV





estradiol-treated male oviduct antigen (EST ♂ -Ag), and non-ovulated, mature female oviduct antigen (NO ♀ -Ag). Non-ovulated, mature female oviduct has at least 7 different antigens, six of which (see arrow 1) are shared between estradiol-treated male rudimentary oviducts and non-ovulated, mature female oviducts. This is an indication that the number and kinds of antigens present in male oviducts treated with estradiol are similar to those present in non-ovulated, mature female oviducts.

One <u>new</u> precipitation line is evident between estradiol-treated male rudimentary oviducts and non-ovulated, mature female oviducts (see arrow 2). An identity line shared between untreated and estradiol-treated male oviducts (see arrow 3) does <u>not</u> extend to the non-ovulated, mature female oviduct antigen well.

When testosterone-treated male oviducts were tested in a similar set-up to that illustrated in the bottom part of Text Figure IV, precipitation patterns similar to that obtained with estradiol-treated male oviducts were observed.

No bands were obtained when non-immune serum was used.

Figure 1. Gross appearance of untreated Rana pipiens male rudimentary oviducts (Ov) lying laterally to the kidneys (Kid). The three regions of the oviducts are: (1) the straight upper portion (pars recta), (2) the middle portion showing a slight degree of convolution (pars convoluta) and (3) the lower, dilatable portion (pars uterus).

Figure 2. Gross appearance of oviducts from a Rana pipiens male that had been given an initial injection of 0.25 ml sesame oil and kept for two weeks at room temperature before dissection. Note that gross appearance is identical to that of oviducts from untreated males.

Figure 3. Gross appearance of estradiol-treated (5 mg/0.25 ml sesame oil) Rana pipiens male rudimentary oviducts. Illustrates hypertrophy observed after hormonal stimulation. Oviducts exhibit an enlargement in diameter and increased convolution along the whole length of the oviduct.

Figure 4. Gross appearance of progesterone-treated (2-5 mg/0.25 ml sesame oil-Prog A) Rana pipiens male rudimentary oviducts. Note that no enlargement of the oviducts was observed (compare Figures 3 and 4). Gross appearance is similar to that of oviducts from untreated or sesame-oil injected males.

PLATE I

Figure 1
UNT

Figure 2
OIL_2

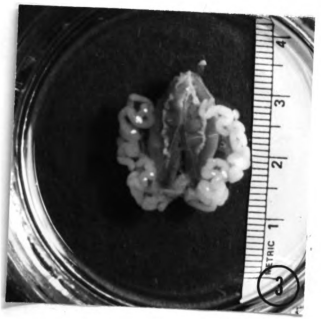


Figure 3
EST_2

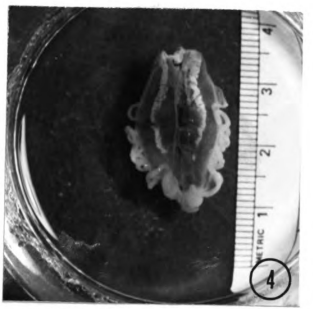


Figure 4
PROG A_2

Figure 5. Representative cross-section through the middle region of untreated male rudimentary oviducts of Rana pipiens fixed by freeze-substitution, stained with periodic acid-Schiff (PAS) (100 X magnification). Note strong PAS-positive reaction in mucous cells (mc) surrounding the lumen (lu) and in the tubular glands (tg).

Figure 6. Higher magnification (430 X) of same section illustrated in Figure 5. Tubular glands (tg) show very little PAS-positive secretion (se). Non-staining muclei (nu) can be observed in basal region of the individual tubular gland cells. Strong PAS-staining mucous cells (mc) are observed surrounding the oviducal lumen (lu).

PLATE II

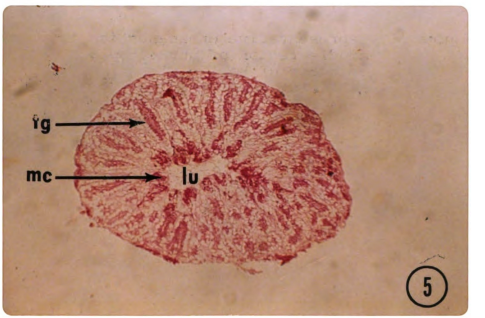


Figure 5. PAS UNT, MID 3/5/70 FS 100 X

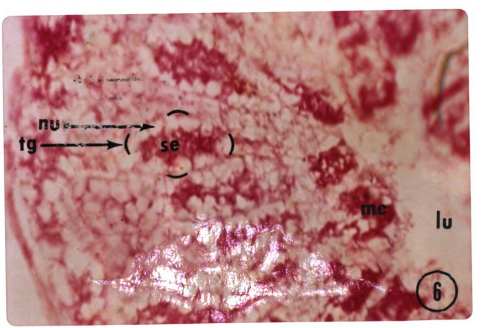


Figure 6. PAS UNT, MID 3/5/70 FS 430 X

Figure 7. Representative cross-section through the upper oviducal region of an estradiol-treated Rana pipiens male fixed by freeze-substitution, and stained by the PAS reaction (100 X magnification). Note that tubular glands (tg) are full of secretory material which stains from pink to dark red. The dark red-staining tubular glands are localized mainly in the periphery of the oviduct.

Figure 8. Higher magnification (430 X) of section shown in Figure 9. Illustrates a region close to the periphery of the oviduct where adjacent tubular glands stain with different intensities of red. Basal, non-staining nuclei (nu) and secretory (se) material in the apical portion of the individual tubular bland cells are also evident.

PLATE III

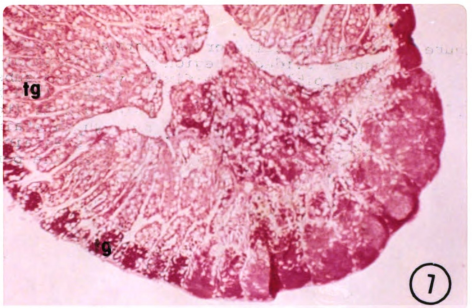


Figure 7. PAS EST_5 UPPER 3/5/70 FS 100 X

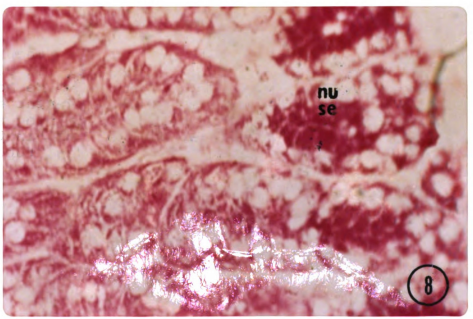


Figure 8. PAS EST_5 UPPER 3/5/70 FS 430 X

Figure 9. Representative cross-section through the middle oviducal region of an estradiol-treated R. pipiens male fixed by freeze-substitution and stained by the PAS reaction (100 X magnification). Tubular glands (tg) shown both in cross and longitudinal sections stain with the same intensity of red. Note scattered PAS-staining of mucous cells (mc) surrounding the oviducal lumen (lu).

Figure 10. Higher magnification (430 X) of the same section shown in Figure 9. The tubular glands (tg) shown in cross-section (outlined in black) are so engorged with jelly secretion (se) that their nuclei (nu) and lumena are not always evident.

PLATE IV

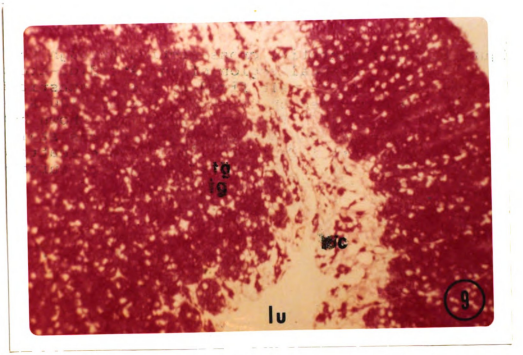


Figure 9. PAS (normal) EST_5 MID 3/5/70 FS 100 X

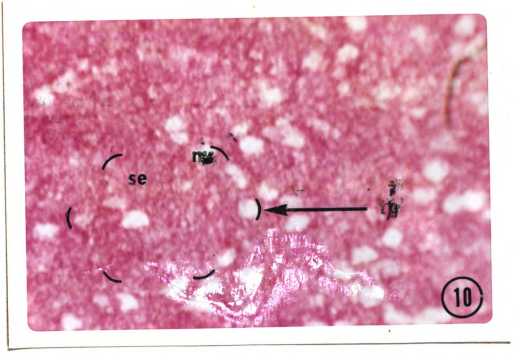


Figure 10. PAS EST_5 MID 3/5/70 FS 430 X

Figure 11. Representative cross-section through the upper region of a testosterone-treated R. pipiens male rudimentary oviduct fixed by freeze-substitution and stained by PAS reaction (100 X magnification). Note that all the tubular glands (tg) gave a strong PAS-positive reaction due to secretion. Scattered PAS-positive mucous cells (mc) are also observed around the lumen (lu) of the oviduct.

Figure 12. Representative section through the middle oviducal region of an estradiol-treated R. pipiens male fixed by freeze-substitution, diastase digested, and then stained by the PAS reaction (100 X magnification). Note that the red coloration of the PAS-staining was unaltered by digestion.

PLATE V

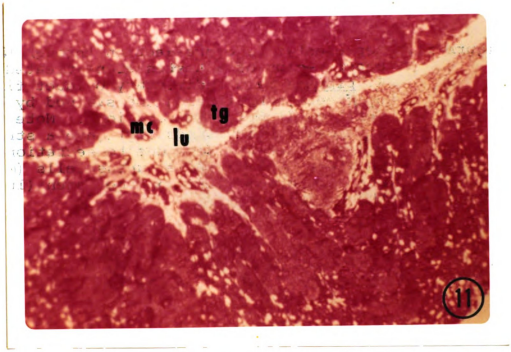


Figure 11. PAS (normal) $TEST_1$ UP 3/5/70 FS 100 X

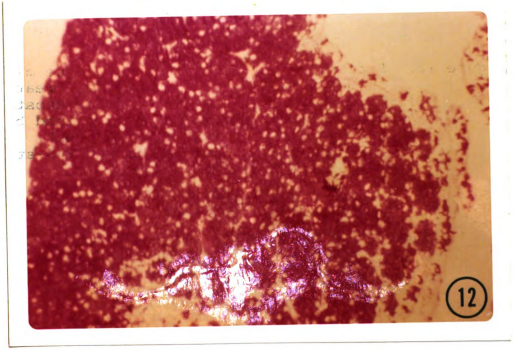


Figure 12. PAS (Diastase) EST_5 MID 3/5/70 FS 100 X

Figure 13. Representative cross-section through the upper region of an estradiol-treated R. pipiens male rudimentary oviduct fixed by freeze-substitution and stained by PAS reaction without prior periodic acid hydrolysis. No color reaction was obtained (100 X magnification).

Figure 14. Representative section through the lower oviducal region of an untreated, non-ovulated, mature R. pipiens female fixed by freeze substitution and stained by the PAS reaction (100 X). Note that the tubular glands (tg) are engorged with a strong PAS positive secretion. PAS-positive epithelial folds (ef) containing mucous cells protrude into the oviducal lumen (lu).

PLATE VI

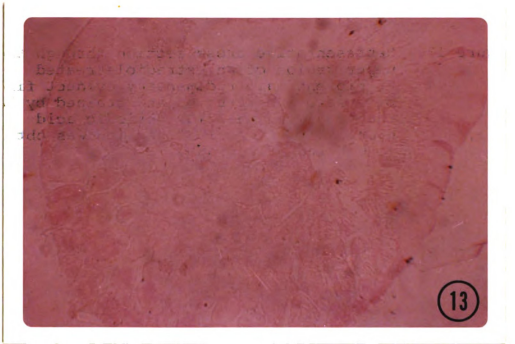


Figure 13. PAS (w/o periodic acid hydrolysis) EST_5 UP 3/5/70 FS

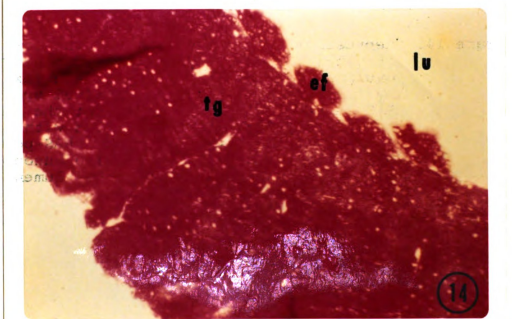


Figure 14. PAS (normal) No UNT LOW 8/20/70 100 X

Figure 15. Representative section through the upper oviducal region of non-ovulated, mature R. pipiens female, fixed by freeze substitution and stained with alcian blue (AB) at pH 2.6 (100 X magnification). Tubular glands (tg) shown here in longitudinal sections exhibit moderate alcianophilia at this pH. The mucous cells present in the epithelial folds (ef) protruding into the oviducal lumen (lu) exhibit a strong reaction (stain turquoise blue). Nuclei (nu) stain dark turquoise blue.

Figure 16. Representative section through the middle oviducal region of a non-ovulated, mature R. pipiens female fixed by freeze substitution and stained with AB at pH 2.6 (100 X magnification). Tubular glands (tg) shown here in longitudinal sections exhibit weak alcianophilia at this pH. Mucous cells present in the epithelial folds (ef) protruding into the oviducal lumen (lu) exhibit a strong positive reaction.

PLATE VII

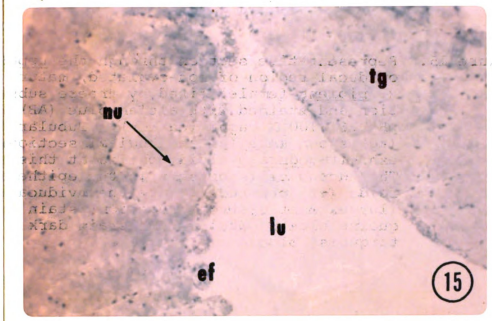


Figure 15. No ♀ UP 8/20/70 FS 100 X
Alc. Blue pH 2.6 (not counterstained)
undigested

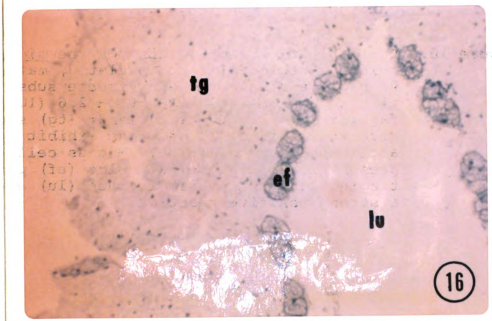


Figure 16. No ♀ MID 8/20/70 FS 100 X
Alc. Blue pH 2.6 (undigested)
not counterstained

Figure 17. Representative section through the lower oviducal region of testosterone-treated R. pipiens male fixed by freeze substitution and stained with Alcian blue at pH 2.6 (100 X magnification). Tubular glands (tg) show a moderate staining reaction with some glands staining darker than others. Mucous cells (mc) surrounding the oviducal lumen (lu) show a strong positive staining reaction.

Figure 18. Representative section through the lower oviducal region of untreated R. pipiens male fixed by freeze substitution, and stained with AB at pH 2.6 (100 X magnification). A very weak or negative reaction is observed in the tubular glands (tg). Mucous cells (mc) give a strong positive reaction.

PLATE VIII

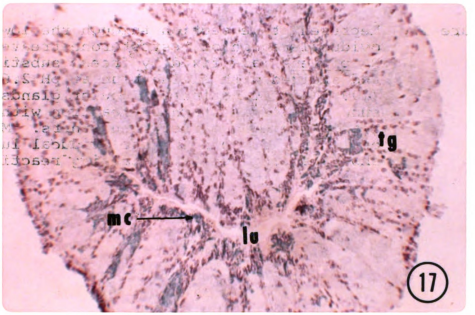


Figure 17. $TEST_1$ LOW 3/5/70 FS
Alc. Blue pH 2.6 NFR 100 X

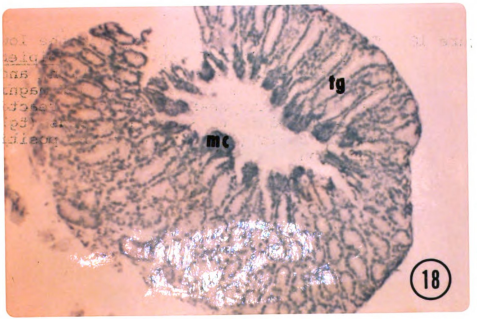


Figure 18. UNT_7 LOW 3/5/70 FS
Alc. Blue pH 2.6 (undigested) 100 X
not counterstained

Figure 19. Representative section through the upper oviducal region of an estradiol-treated R. pipiens male fixed by freeze substitution and stained with AB at pH 2.6 and counter-stained with Nuclear Fast Red (NFR) (100 X magnification). The vast majority of the tubular glands (tg) shows a strong positive reaction (dark turquoise blue). Nuclei (nu) of tubular gland cells stain purple.

Figure 20. Higher magnification (430 X) of the same section shown in Figure 19. Shows tubular gland cells having purple-staining basal nuclei (nu) and cytoplasm full of turquoise-blue staining secretory material (se).

PLATE IX

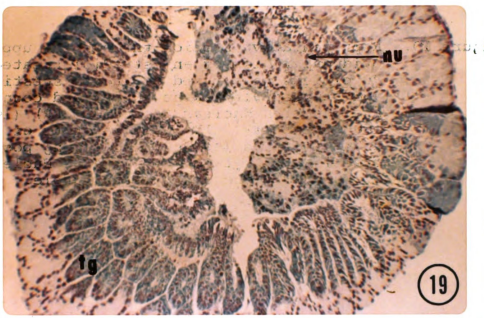


Figure 19. Alc. Blue pH 2.6 NFR
EST_5 UP 3/5/70 FS 100 X

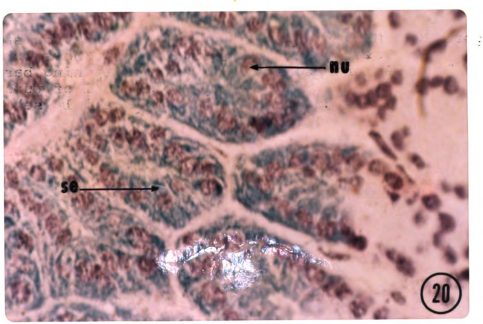


Figure 20. Alc. Blue pH 2.6 NFR
EST_5 UP 3/5/70 FS 430 X

Figure 21. Representative section through the lower oviducal region of an estradiol-treated R. pipiens male fixed by freeze substitution stained with AB at pH 2.6 and counterstained with NFR (100 X magnification). Tubular glands (tg) shown here mostly in cross-section give a very weak reaction. Mucous cells (mc) present in the epithelial folds (ef) protruding into the oviducal lumen (lu) show a strong positive reaction. Nuclei (nu) stain purple.

Figure 22. Higher magnification (430 X) of the same section shown in Figure 21. Shows in more detail the 2 types of cells present in the epithelial folds (ef) protruding into the oviducal lumen, namely; ciliated cells (cc) having purple nuclei and mucous cells (mc) staining dark turquoise blue.

PLATE X

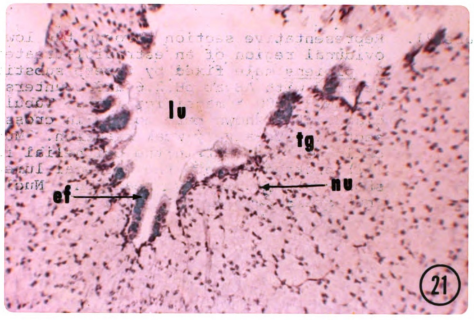


Figure 21. EST_5 LOW 3/5/70 FS
Alc. Blue pH 2.6 NFR 100 X

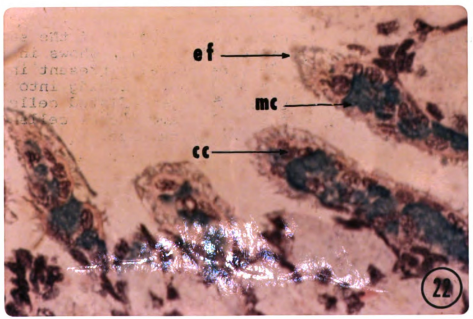


Figure 22. EST_5 LOW 3/5/70 FS
Alc. Blue pH 2.6 3/5/70 430 X

Figure 23. Representative section through the middle oviducal region of an untreated R. pipiens male fixed by freeze-substitution, stained with AB at pH 0.5 and counterstained with NRF (100 X magnification). Tubular glands did not stain at this pH. Mucous cells (mc) exhibited scattered alcianophilia.

Figure 24. High magnification (430 X) of an epithelial fold (outlined in black) found in the lower oviducal region of sesame oil-injected R. pipiens male. Tissue was fixed by freeze substitution, stained with AB at pH 0.5 and counterstained with NFR. Epithelial fold is composed of alternate blue-staining mucous cells (mc) and red-nucleated ciliated cells (cc).

PLATE XI

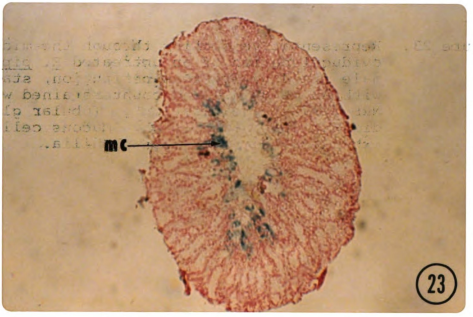


Figure 23. Alc. Blue pH 0.5 NFR
UNT, MID 3/5/70 FS 100 X

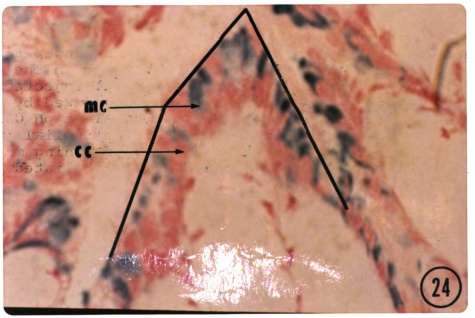


Figure 24. Alc. Blue pH 0.5 NFR
OIL LOW 3/5/70 FS 430 X

Figure 25. Representative section through the upper oviducal region of an estradiol-treated R. pipiens male fixed by freeze substitution stained with AB at pH 0.5 and counterstained with NFR (100 X magnification). A strong positive reaction was obtained in the majority of the tubular glands (tg). Some tubular glands showed only red-staining nuclei (nu)

Figure 26. Higher magnification (430 X) of the same section shown in Figure 25. Sulfated tubular glands (stg) shown dark blue staining cytoplasm and basal red-staining nuclei. Non-sulfated glands (nstg) show only red-staining nuclei.

PLATE XII

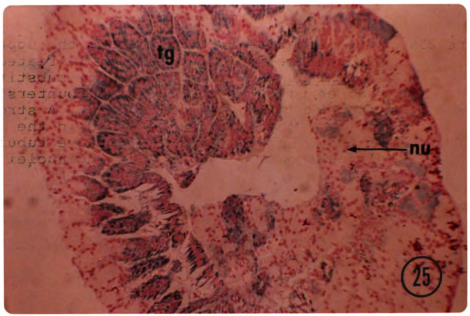


Figure 25. Alc. Blue pH 0.5 NFR
EST_5 UP FS 3/5/70 100 X

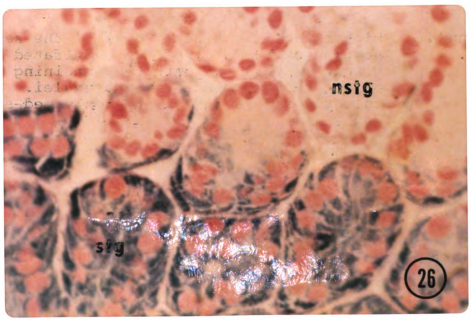


Figure 26. Alc. Blue pH 0.5 NFR
EST_5 UP 3/5/70 FS 430 X

Figure 27. Another high magnification of the same section shown in Figure 25. Illustrates an area where only sulfated tubular glands (stg) are found.

Figure 28. Representative section through the lower oviducal region of a testosterone-treated R. pipiens male fixed by freeze-substitution, stained with AB at pH 0.5 and counterstained with NFR (100 X magnification). Tubular glands (tg) and mucous cells (mc) in this section show scattered staining for sulfated mucopolysaccharides.

PLATE XIII

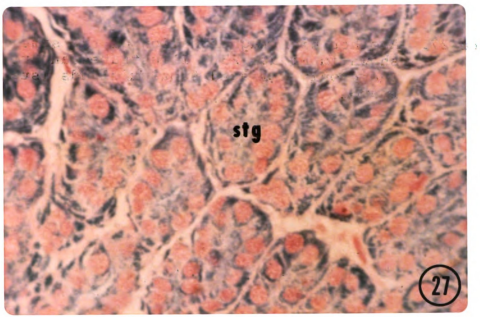


Figure 27. Alc. Blue pH 0.5 NFR
EST_5 UP 3/5/70 FS 430 X

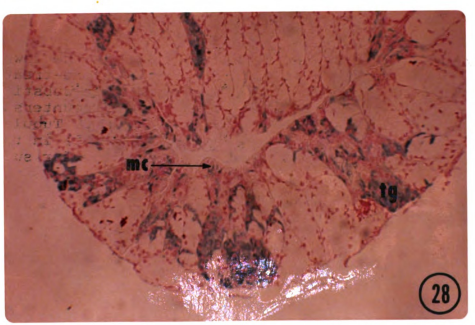


Figure 28. Alc. Blue pH 0.5 NFR
$TEST_1$ LOW 3/5/70 FS 100 X

Figure 29. Representative section through upper oviducal region of a non-ovulated, mature R. pipiens male fixed by freeze substitution, and stained with toluidine blue (100 X magnification). Both the tubular glands (tg) and mucous cells (mc) present in this section give a strong metachromatic reaction (dark pink). Metachromatic granules are evident in the enlarged tubular glands.

PLATE XIV

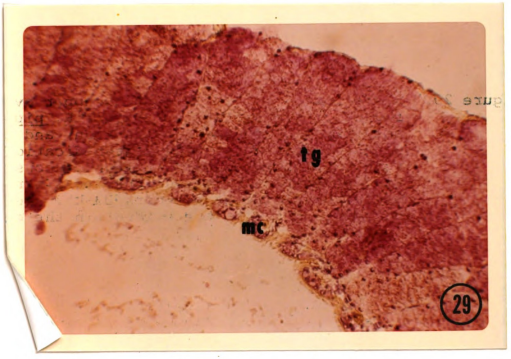


Figure 29. No ♀ UP 8/20/70 FS 100 X
Tol. Blue 0.1%
w/o Hyal. digested

Figure 30. Representative section through middle oviducal region of a testosterone-treated R. pipiens male fixed by freeze-substitution and stained with toluidine blue (100 X magnification). Tubular glands (tg) show a variable metachromatic reaction (i.e., stained from pinkish orange to dark pink). Mucous cells (mc) surrounding the oviducal lumen (lu) gave a strong metachromatic reaction (i.e., dark pink) Brown coloration is due to oxidation of dye.

Figure 31. Adjacent section to that shown in Figure 30. Fixed by freeze-substitution, hyaluronidase digested and stained with toluidine blue (100 X magnification). Note that toluidine blue metachromasia was not altered by the enzyme treatment.

PLATE XV

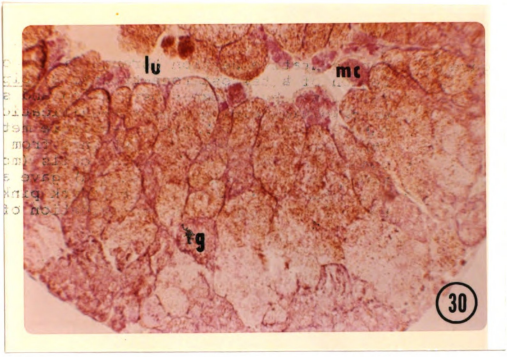


Figure 30. $TEST_1$ MID 3/5/70 FS 100 X
Tol. Blue 0.1% w/o Hyal. digested

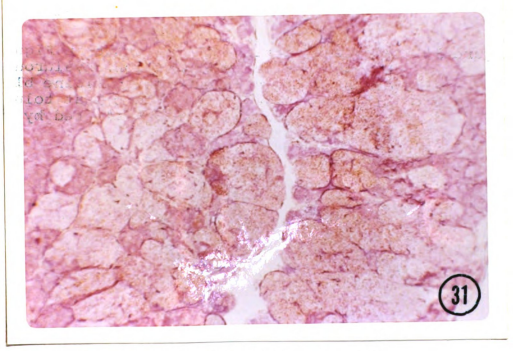


Figure 31. $TEST_1$ MID 3/5/70 FS 100 X
Tol. Blue 0.1% Hyal. digested

Figure 32. Representative section through the lower oviducal region of an untreated R. pipiens male fixed by freeze substitution and stained with the alcian blue (pH 2.6)-periodic acid-Schiff sequential procedure (AB-PAS) (100 X magnification). Tubular glands (tg) cell cytoplasm stain red and their nuclei (nu) turquoise blue. Mucous cells (mc) surrounding oviducal lumen (lu) stain purple.

Figure 33. Adjacent section to that shown in Figure 32. Fixed by freeze substitution, sialidase-digested and stained with the AB-PAS sequential procedure (100 X magnification). Note that the above mentioned staining pattern remains the same after enzyme digestion.

PLATE XVI

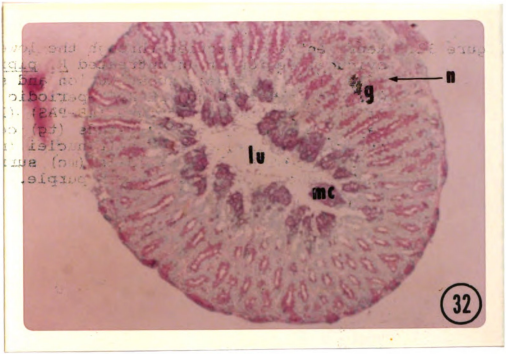


Figure 32. UNT$_7$ LOW ?/5/70 FS 100 X Sialidase control (undig., un-deacatyl) PAS-AB 2.6.

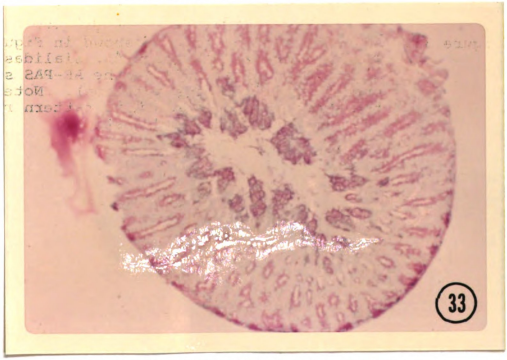


Figure 33. UNT$_7$ LOW 3/5/70 FS 100 X Sialidase digested, un-deacetylated PAS/AB 2.6.

Figure 34. Adjacent section to that shown in Figure 33. Fixed by freeze-substitution, deacetylated sialidase digested and stained by the AB-PAS sequential procedure (100 X magnification). Note that staining pattern remains the same as that of figures 32 and 33.

Figure 35. Representative section through the upper oviducal region of a non-ovulated mature R. pipiens female fixed by freeze-substitution, and stained by the AB-PAS sequential procedure (100 X magnification). Large tubular glands (tg) full of reddish-purple secretory material and showing sparse turquoise-blue staining nuclei (nu) are evident in this section.

PLATE XVII

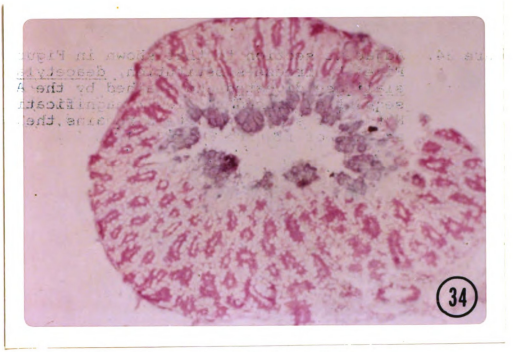


Figure 34. UNT$_7$ LOW 3/5/70 FS 100 X
Sialidase digested, deacetylated
PAS-AB 2.6.

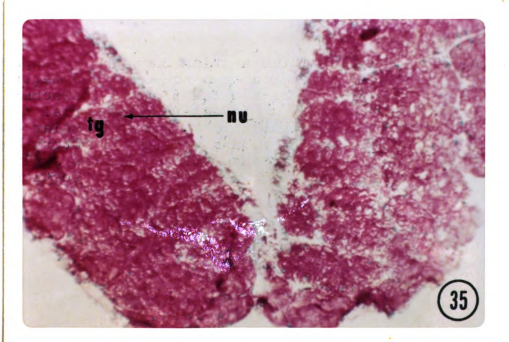


Figure 35. No ♀ UP 8/20/70 FS 100 X
Sialidase control (undig., un-deacetyl.)
PAS-AB 2.6.

Figure 36. Adjacent section to that shown in Figure 35. Fixed by freeze-substitution, sialidase digested, un-deacetylated and stained with the AB-PAS sequential procedure (100 X magnification). Note that staining pattern remains identical to that shown in Figure 35.

Figure 37. Adjacent section to that shown in Figure 36. Fixed by freeze-substitution, deacetylated, and stained with the AB-PAS sequential procedure (100 X magnification). Staining pattern remains the same as that shown in Figures 35 and 36.

PLATE XVIII

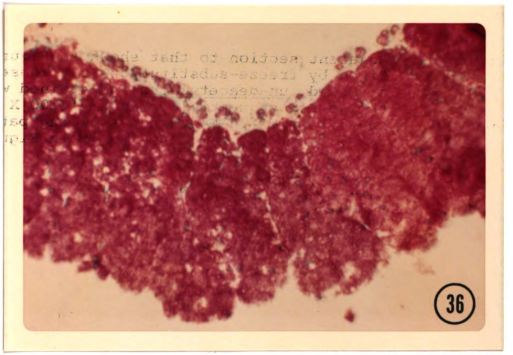


Figure 36. No ♀ UP 8/20/70 FS 100 X
Sialidase digested, un-deacetylated
PAS-AB 2.6.

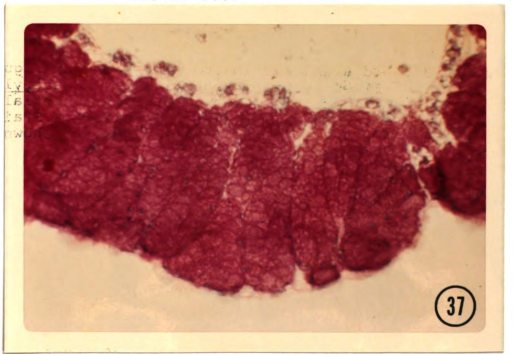


Figure 37. No ♀ UP 8-20-70 FS 100 X
Deacetylated only
PAS-AB 2.6.

Figure 38. Adjacent section to that shown in Figure 37. Fixed by freeze-substitution, deacetylated, sialidase-digested and stained with the AB-PAS sequential procedure (100 X magnification). Note that a certain degree of color removal is observed in the tubular glands of this region when sections are deacetylated prior to digestion. Glands which normally (Figure 35) stained reddish-purple now stain light pink.

Figure 39. Representative section through upper oviducal region of an estradiol-treated R. pipiens male fixed by freeze-substitution, and stained with the AB-PAS sequential procedure (100 X magnification). The tubular glands (tg) surrounding most of the oviducal lumen (lu) stain blue, i.e., are positive for acid mucopolysaccharides. The red-staining tubular glands in the periphery of the oviduct are positive for neutral mucopolysaccharide.

PLATE XIX

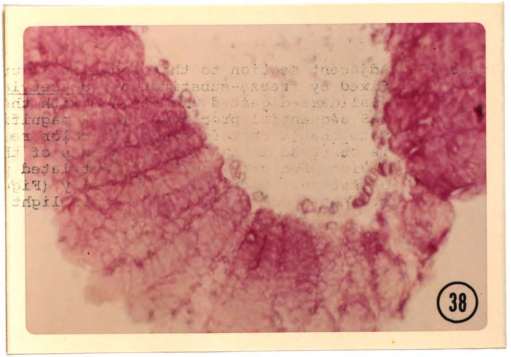


Figure 38. No ♀ UP 8/20/70 FS 100 X
Sialidase digested, deacetylated
PAS-AB 2.6

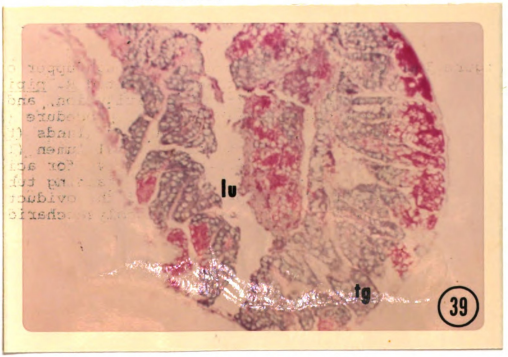


Figure 39. EST_5 UP 3/5/70 FS 100 X
Sialidase control (undig., un-deacetylated)
PAS-AB 2.6.

Figure 40. Adjacent section to that shown in Figure 39. Fixed by freeze-substitution, sialidase-digested, undeacetylated, and stained by AB-PAS sequential procedure (100 X magnification). Note that staining pattern remains identical to that shown in Figure 39.

Figure 41. Adjacent section to that shown in Figure 40. Fixed by freeze-substitution, deacetylated, sialidase-digested and stained with the AB-PAS sequential procedure (100 X magnification). Note that a decrease in the staining intensity of the tubular glands (tg) is observed when sections are deacetylated prior to enzyme digestion.

PLATE XX

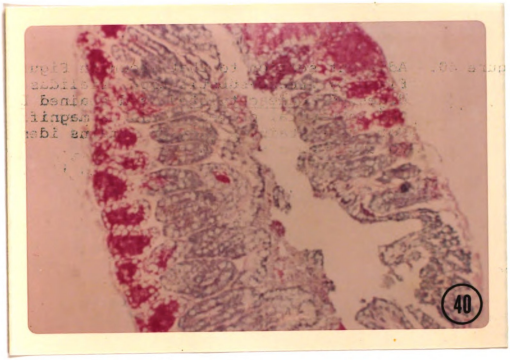


Figure 40. EST_5 UP 3/5/70 FS 100 X
Sialidase dig., un-deacetyl.
PAS-AB 2.6.

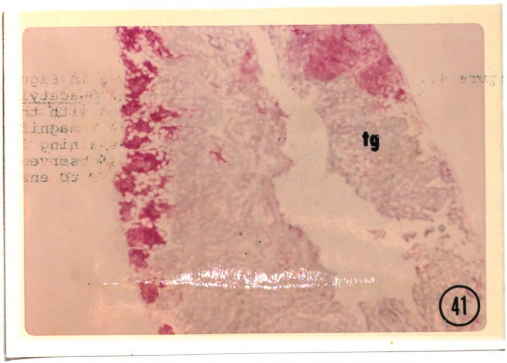


Figure 41. EST_5 UP 3/5/70 FS 100 X
Sialidase digested, deacetylated
PAS-AB 2.6.

Figure 42. Representative section through the lower oviducal region of an estradiol-treated R. pipiens male fixed by freeze substitution and stained with the AB-PAS sequential procedure (100 X magnification). All the tubular glands (tg) stain red, while the mucous cells (mc) surrounding the oviducal lumen (lu) stain reddish-purple.

Figure 43. Adjacent section to that shown in Figure 42. Fixed by freeze-substitution, sialidase-digested, undeacetylated, and stained with the AB-PAS sequential procedure (100 X magnification). Note that the staining reactions of both the tubular glands (tg) and the mucous cells (mc) remain identical to those obtained in Figure 42.

PLATE XXI

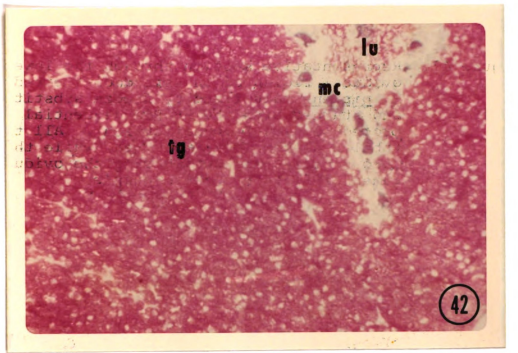


Figure 42. EST_5 LOW 3/5/70 FS 100 X
Sialidase control (undig., un-deacetyl.)
PAS-AB 2.6.

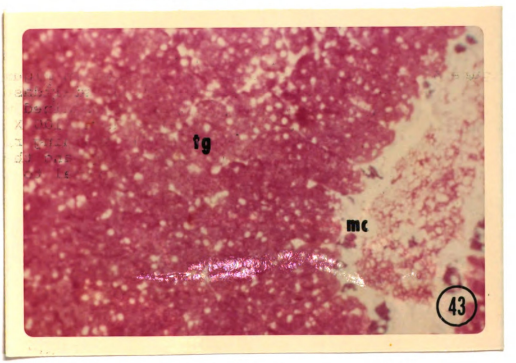


Figure 43. EST_5 LOW 3/5/70 FS 100 X
Sialidase digested, un-deacetylated
PAS-AB 2.6.

Figure 44. Adjacent section to that shown in Figure 43. Fixed by freeze-substitution, deacetylated, sialidase-digested, and stained with AB-PAS sequential procedure (100 X magnification). Note that staining reactions of both the tubular glands (tg) and the mucous cells (mc) surrounding the oviducal lumen (lu) remain identical to those obtained in Figures 42 and 43.

PLATE XXII

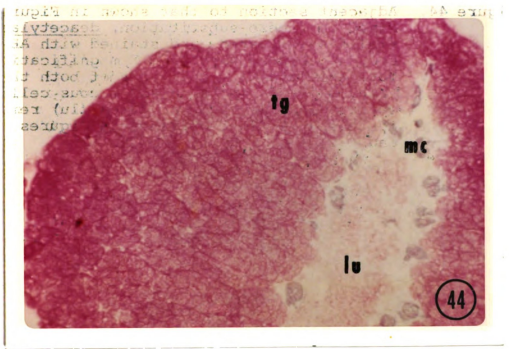


Figure 44. EST_5 LOW 3/5/70 FS 100 X Sialidase digested, deacetylated PAS-AB 2.6.

Figure 45. Representative section through the middle oviducal region of an estradiol-treated R. pipiens male fixed by freeze substitution and stained with alcian blue at pH 2.6, (100 X magnification). A weak staining reaction is observed in all the tubular glands (tg). Mucous cells (mc) surrounding the oviducal lumen (lu) stain a darker blue.

Figure 46. Adjacent section to that shown in Figure 46. Fixed by freeze-substitution, hyaluronidase-digested and stained with alcian blue at pH 2.6 (100 X magnification). Note that the pattern of alcian blue staining characteristic of the tubular glands (tg) and mucous cells (mc) are the same as in Figure 45.

PLATE XXIII

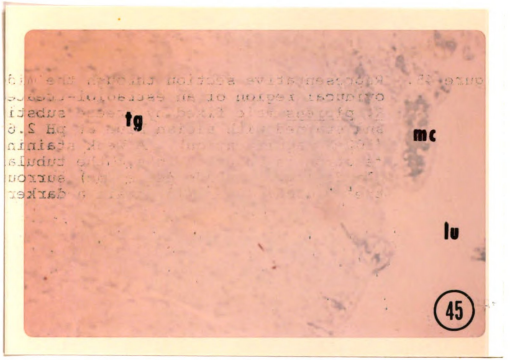


Figure 45. EST_5 MID 3/5/70 FS 100 X
Alc. Blue pH 2.6 undigested

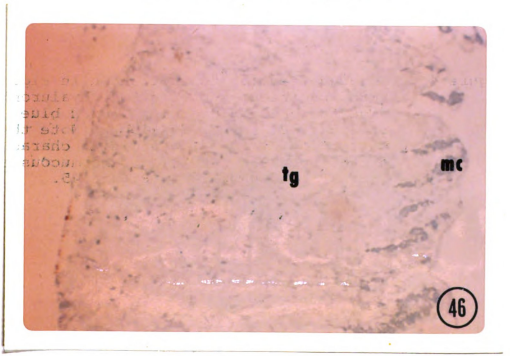


Figure 46. EST_5 MID 3/5/70 FS 100 X
Alc. Blue pH 2.6 Hyal. Digested

Figure 47. Representative section through the middle oviducal region of an untreated R. pipiens male fixed by freeze substitution and stained with the ninhydrin-Schiff procedure (100 X magnification). Tubular glands (tg) stain light pink, while connective tissue in the periphery of the oviduct stains dark pink (see arrow).

Figure 48. Representative section through the upper oviducal region of a non-ovulated, mature R. pipiens female fixed by freeze-substitution and stained with the ninhydrin-Schiff procedure (100 X magnification. Tubular glands (tg) and mucous cells (mc) surrounding the oviducal lumen (lu) stain light pink. No localized staining is observed.

PLATE XXIV

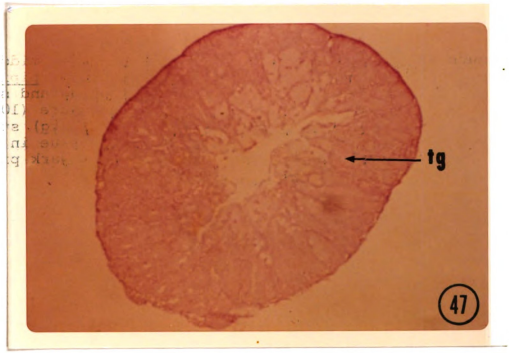


Figure 47. Ninhydrin-Schiff UNT MID 3/5/70 FS 100 X

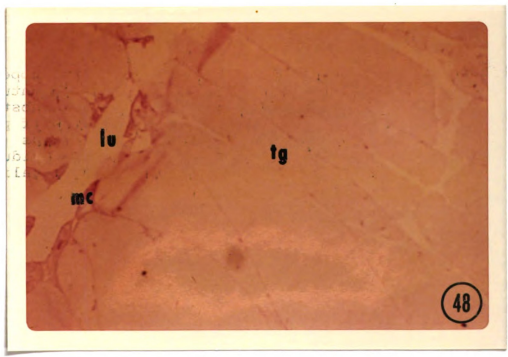


Figure 48. Ninhydrin-Schiff NO UNT UP 3/5/70 FS 100 X

Figure 49. Representative section through the middle oviducal region of an estradiol-treated R. pipiens male fixed by freeze-substitution and stained by the ninhydrin-Schiff procedure (100 X magnification). Tubular glands (tg) stain light pink. No localized staining is observed.

Figure 50. Representative section through the lower oviducal region of an estradiol-treated R. pipiens male fixed by freeze-substitution and stained by the ninhydrin-Schiff procedure (100 X magnification). Tubular glands (tg) stain light pink. No localized staining is observed.

PLATE XXV

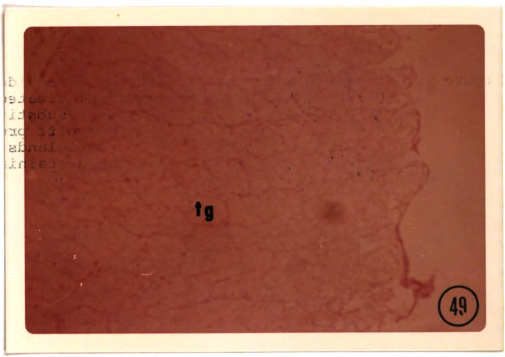


Figure 49. Ninhydrin-Schiff EST_5 MID 3/5/70 FS 100 X

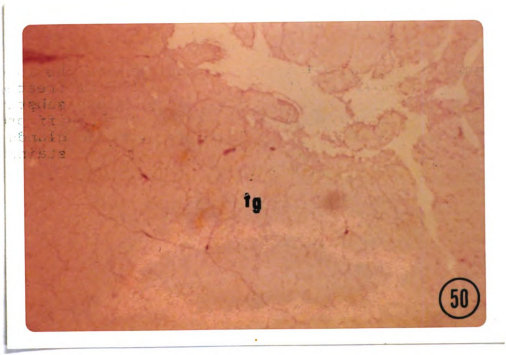


Figure 50. Ninhhdrin-Schiff EST_5 LOW 3/5/70 FS 100 X

DISCUSSION

General Hormonal Effects on Amphibian Oviducts

The studies presented here have shown that estradiol, testosterone and the combination of estradiol and progesterone can induce hypertrophy of the male rudimentary oviduct of R. pipiens. Similar stimulatory effects had been previously reported in several species of amphibians with a variety of sex hormones (Wolf, 1939, 1940; Puckett, 1939; van Oordt and Klomp, 1946= Adams, 1946). These investigators limited their descriptions of this hormone-induced oviducal hypertrophy to indicating that it was due to an accumulation of jelly material within the tubular gland cells. A description of the histochemical nature of these hypertrophic changes and a comparison of the hormone-stimulated male oviducts with those of females have not been previously reported.

Testosterone has been observed to induce hypertrophy of adult male R. pipiens rudimentary oviducts, in these experiments. How an androgenic hormone may exert this feminizing effect could be explained by postulating a conversion of testosterone into estradiol which would act as the

stimulatory hormone. Such a conversion of testosterone to estradiol has been reported in studies of the biosynthesis of steroid hormones in the embryonic gonads of birds (Haffen, 1970). Artificially feminized testes (ovotestis) become able to aromatize testosterone into estrogens. It appears that within the gonads of male and female chicks an enzyme system catalyzing aromatization is present, whose formation or stimulation is induced by exogenous hormones. Such hormone interconversions have been postulated to occur also in the ovaries and testes of adult amphibians, where estrogens, testosterone and progesterone have all been detected (Chieffi, 1967).

Estradiol also proved to have a stimulatory effect on the male rudimentary oviduct of adult R. pipiens. It has been shown that oviducal function is predominantly under ovarian control. Estrogen stimulates the growth of the oviduct by inducing the synthesis of jelly within the tubular gland cells present in amphibian adult females (Christensen, 1931; DeAllende, 1939a; Adams, 1950; Galli-Mainini, 1950a, 1950b; Lee, 1965) and adult males (Puckett, 1939; Wolf, 1928, 1940; van Oordt and Klomp, 1946; Adams, 1946).

Although progesterone has been shown to induce ovulation and jelly secretion in female amphibians: R. pipiens (Lee, 1965), B. bufo (Thornton and Evennett, 1969; Lodge and Smith, 1960), R. temporaria (Lodge and Smith, 1960) and B. arenarum (Houssay, 1950; Galli-Mainini, 1962), in the

experiments reported here no hypertrophic effect (jelly secretion) of the male rudimentary oviduct of R. pipiens was obtained with the 3 dosages of progesterone used. However, when the combination of progesterone and estradiol was used an hypertrophic effect on the male oviduct was observed of the same magnitude as that of estradiol alone (see Text Figure III). This hypertrophic effect of the hormone combination may be accounted for by the action of the estradiol alone. Reports have been made (Penhos and Nallar, 1956; Lee, 1965) that progesterone and estradiol act synergistically in adult female amphibians where the stimulating effect of progesterone on the oviduct is enhanced by administration of estradiol or testosterone.

Similar synergistic effects of estradiol and progesterone have been demonstrated in the chick oviduct (O'Malley et al., 1969) in the induction of ovalbumin and avidin synthesis. Secretion of avidin by the goblet cells of estrogen-stimulated mature chick oviducts does not occur until a single dose of progesterone is administered. Once the tubular glands have differentiated under the influence of estrogen in the chick, progesterone and estrogen seem to work synergistically in the synthesis of ovalbumin by these glands. It appears then, that progesterone can exert its stimulatory effect only in tissues that have been previously exposed to estrogen.

In the case of female adult amphibians, the levels of estrogen appear to be sufficiently high so that progesterone

can exert its effect on the oviduct. In the male, the estrogen levels of the testes seem to be sufficient for maintaining the structure and functioning of the male oviduct at a low level, but these levels do not seem to be high enough to allow progesterone to exert a stimulatory effect.

Major seasonal variations in the female oviduct exist due to the synthesis, accumulation and secretion of jelly by the tubular gland cells. Such variations are predominantly under ovarian hormonal control, although the pituitary has been shown to have some influence also. Slight seasonal variations were found in the male rudimentary oviduct of R. pipiens both untreated and hormone treated (see data of individual experiments (1-5) in Appendix Table I). The maintenance of this highly differentiated organ, must be under control of the low levels of estrogen secreted by the testes, and, perhaps, directly by some pituitary control which may vary seasonally. Lee (1965), however, has observed no variation in the histological appearance of the male rudimentary oviduct of R. pipiens throughout the various seasons of the year.

Hormonal Effects on the Histochemistry of the Male Rudimentary Oviduct

General considerations

Although most of the currently available histochemical techniques do not permit specific identification of individual

carbohydrates, they may be used to identify general classes of complex carbohydrates, such as glycogen, mucopolysaccharides, mucoproteins, glycoproteins, glycolipids. No histochemical distinction can be made between neutral mucopolysaccharides or mucoproteins, or between glycoproteins and mucoproteins (Barka and Anderson, 1963).

Several types of mucopolysaccharides can be distinguished by histochemical methods (Spicer, 1963). This classification, which is based largely on differences in the acid groups and glycol groups of the polymers, recognizes two main groups: neutral and acidic mucopolysaccharides (AMP). Neutral mucopolysaccharides are PAS-reactive, diastase-resistant, and color red in sections stained with the alcian blue-PAS sequential procedure.

In general, AMP show strong γ-metachromasia when stained with toluidine blue. Sulfated and non-sulfated AMP can be distinguished by histochemical means. Sulfomucins, give a strong positive reaction with alcian blue at pH 0.5, show strong alcohol-resistant metachromasia and stain either blue or purple with the alcian blue-periodic acid-Schiff (AB-PAS) sequential procedure. Non-sulfated acidic mucopolysaccharides give a strong positive reaction with alcian blue at pH 2.6. Three kinds of non-sulfated acidic mucopolysaccharides can be differentiated histochemically: 1. Sialomucins are mucopolysaccharides rich in sialic acid residues and show a decrease in their staining affinity for basic dyes upon

sialidase digestion. The action of sialidase is clearly seen in the change from blue- or purple to red staining with the alcian blue-PAS procedure. 2. Hyaluronic acid, which upon hyaluronidase digestion, also shows a decrease in its staining affinity for basic dyes (i.e., a decrease in basophilia). 3. Those mucopolysaccharides with unidentified acid groups, supposedly rich in carboxyl groups.

Although histochemical techniques can only identify general classes of carbohydrates, they can detect differences in the chemical nature of tissue constituents, if appropriate controls are used. Histochemical procedures have been quite successfully used in detecting differences in the composition of jelly secretions present in the oviducts of R. pipiens females (see Introduction, section on the histochemistry of the female oviduct).

The PAS reaction is used for demonstration of complex carbohydrates rich in neighboring hydroxyl groups or equivalent amino-substitutions (hydroxy-amino substitutions) (Mowry, 1963). Appropriate controls such as protein digestion, lipid extraction and diastase digestion help in determining the nature of the PAS-reactive compounds with greater accuracy (Barka and Anderson, 1963).

Alcian blue at pH 2.6 stains complex carbohydrates rich in free acidic groups, i.e., polycarboxylates. Some sulfate groups, however, may also contribute to this staining at pH 2.6 (Mowry, 1963). Mowry (1968) has used alcian blue

at low pH (0.5-1.0) for selective coloration of sulfated mucopolysaccharides. At this pH binding of alcian blue is apparently confined to polysulfates, polycarboxylates being undissociated. The specificity of the alcian blue reaction can be increased by methylation with acidic methanol. Such treatment prevents alcian blue stainability by blocking reactive groups.

The alcian blue (pH 2.6)-PAS sequential procedure has been used for the combined coloration of both free acidic and neighboring hydroxyl groups of complex carbohydrates (Mowry, 1963). The red coloration of the PAS reaction predominates in neutral mucopolysaccharides, while a blue or purple coloration predominates in acidic ones.

The presence of specific residues in complex carbohydrates, such as hyaluronic acid and sialic acid, can be ascertained indirectly by digesting tissues with hyaluronidase or sialidase, and then testing for a decrease in staining affinity for basic dyes.

The presence of hyaluronic acid was not detected in any of the oviducal tissues tested. It appears that the hyaluronic acid of these tissues is present in such low concentrations that it is undetectable by the histochemical procedure used. A concentration of at least 0.5% to 1% hyaluronic acid is necessary for detecting hyaluronic acid by this method (Barka and Anderson, 1963).

Complex carbohydrates present in the oviducal secretions of the tubular glands and mucous cells appear to be

in the form of mucopolysaccharides. A strong positive PAS reaction accompanied by a very weak protein reaction was obtained, evenly distributed in both tubular gland cells and mucous cells. No localized staining was obtained with either reaction.

Histochemistry of non-ovulated, mature female tubular glands

The histochemical tests performed on female oviducal materials for comparison purposes revealed that the tubular glands present in the upper oviducal regions of non-ovulated, mature females contain mucopolysaccharides rich in both neutral and acidic groups. A strong positive PAS reaction, as well as a strong metachromasia with toluidine blue and moderate alcianophilia at pH 2.6 was obtained in oviducal sections from this region. Furthermore, upper oviducal sections stained purple with the AB-PAS sequential procedure, another indication of the presence of both neutral and acidic mucopolysaccharides in this region. The AMP of this upper region may be in the form of sialomucins (i.e., mucopolysaccharides high in sialic acid residues) since a change in color, from purple to red, was observed after sialidase digestion.

Oviducal jelly secretions in the middle and lower regions of non-ovulated, mature females appear to contain a preponderance of mucopolysaccharides rich in neutral groups.

Histochemical determinations of sulfomucins by the alcian blue reaction at pH 0.5 were not done with female oviducts. The three oviducal regions of female oviducts, however, showed intense metachromasia with toluidine blue, which disappeared upon alcoholic dehydration. This last observation tends to support the idea that no sulfomucins are present in any of the three oviducal levels of females. Other histochemical studies (Pereda, 1970a; Shivers and James, 1970; Steinke and Benson, 1970; Kelly et al., 1970) have demonstrated either directly, by radioactive sulfate incorporation or indirectly, by histochemical tests, the presence of sulfomucins in female oviducts. Sulfate is present in higher concentrations in the upper oviducal regions of females, than in the other oviducal regions (middle and lower) of R. pipiens females. The inability to detect sulfomucins in female oviducal sections may be due to the fact that only small pieces (approximately 3 mm) of oviduct from any of the three oviducal regions were taken for histochemical analyses, and these did not contain those areas previously reported by other authors to be rich in sulfated mucopolysaccharides.

Histochemistry of the tubular glands of untreated male rudimentary oviducts

The jelly secretions present in the upper, middle and lower oviducal regions of untreated males appear to contain a preponderance of mucopolysaccharides rich in neutral groups

(i.e., neighboring hydroxyl or hydroxyamino groups). Very little acid mucopolysaccharide seems to be present in the jelly secretions of these untreated males, i.e., mucopolysaccharides with very few acidic groups. These secretions exhibit a very strong positive PAS-reaction, even after protein digestion and lipid extraction, and are diastase resistant. They color red in sections stained with the AB-PAS procedure, give a very weak reaction with alcian blue at pH 2.6 and show very weak metachromasia with toluidine blue. No sulfomucins (negative reaction with alcian blue at pH 0.5), nor sialomucins (sialidase resistant) are present in the secretions of these tubular glands.

Estradiol-treated male tubular glands

Tubular glands present in the upper oviducal region of estradiol-treated male rudimentary oviducts appear to contain a preponderance of mucopolysaccharides rich in acidic groups. Tubular glands from this region gave an intense alcian blue reaction at pH 2.6, and a positive PAS reaction.

The acidic mucopolysaccharides present in the tubular glands of the upper region of estradiol-treated male rudimentary oviducts appear to be of three kinds. 1) First, are those AMP rich in sulfate groups. An intense positive reaction was obtained with alcian blue at pH 0.5 and, when staining with the AB-PAS procedure, blue staining predominated in the vast majority of the tubular glands present

in this region; 2) Second, are those AMP rich in sialic acid groups. Furthermore, the sialic acid residues present in these mucopolysaccharides appear to be in the acetylated form (N-acetyl-O-diacetyl) as indicated by the fact that sialidase digestion was ineffective in altering the AB-PAS pattern of staining until the sections were deacetylated prior to digestion. Pereda (1970b) has shown that sialomucins present in the jelly secretions of R. pipiens female oviducts appear also to have sialic acid moieties in the acetylated form. 3) Third, are those mucopolysaccharides, which appear to be non-sulfated, but are rich in carboxyl groups, as indicated by the fact that some blue staining from the AB-PAS procedure remained in the sections even after sialidase digestion.

The tubular glands present in the middle and lower oviducal regions of estradiol-treated male rudimentary oviducts appear to be rich in neutral mucopolysaccharides, with very little AMP. The tubular glands present in these regions show a very strong-positive PAS reaction, weak alcianophilia at pH 2.6, and in the combined coloration of AB-PAS only the red coloration characteristic of neutral mucopolysaccharides was apparent. No sulfomucins nor sialomucins were detected histochemically in these two regions.

Testosterone-treated male tubular glands

The jelly secretions of the tubular glands present in the upper and middle regions of testosterone-treated male rudimentary oviducts appear to be composed mainly of mucopolysaccharides rich in neutral groups, with very small amounts of AMP. A strong positive PAS reaction was obtained in the tubular glands of these two regions, as well as a very weak or moderate alcianophilia at pH 2.6. A moderate metachromatic reaction with toluidine blue, which is alcohol-fast, is an indication of non-sulfation in the acid mucopolysaccharides present here. After the sequential AB-PAS procedure, the red coloration of the PAS reaction predominates in these two oviducal regions. A negative staining reaction with alcian blue at pH 0.5 was obtained, suggesting that no sulfomucins are present in these two regions of testosterone-treated male oviducts. No sialomucins were detected histochemically in either of these two regions.

The tubular glands present in the lower oviducal regions of testosterone-treated male tissues appear to contain mucopolysaccharides rich in both neutral and acidic groups. The jelly secretion present in the tubular gland cells of these oviducal regions are rich both in neutral and acid mucopolysaccharides, as indicated by the strong positive PAS reaction, moderate reaction with alcian blue at pH 2.6, and moderate metachromasia with toluidine blue. Furthermore, the tubular glands of this region stained purple when stained with the AB-PAS procedure.

Sulfomucins may be present in the lower oviducal region, as indicated by the alcianphilia at pH 0.5 exhibited by the tubular glands of this region. However, one may suggest that these sulfomucins are not present in high concentrations in the lower oviducal regions, since only a moderate reaction with alcian blue at pH 0.5 was obtained, and the metachromasia exhibited by the tubular glands of this region was alcohol-fast.

The presence of sialomucins was not determined in testosterone-treated male rudimentary oviducts, since no more sialidase was available to conduct the digestion.

As mentioned previously, upper oviducal regions of estradiol-treated male oviducts appear to contain high concentrations of acid mucopolysaccharides (i.e., sulfomucins, sialomucins) while in the corresponding region of testosterone-treated male oviducts a high concentration of neutral mucopolysaccharides was observed. This discrepancy between the kinds of mucopolysaccharides induced by different hormones in corresponding male oviducal regions may only be apparent. It may well be that corresponding segments from the different upper oviducal regions were not taken. In any case, induction of synthesis of new polysaccharides, not present in the untreated male oviduct is obtained after hormonal stimulation.

Histochemistry of the mucous cells

The secretions characteristic of the mucous cells of untreated male rudimentary oviducts appear to be rich in both acidic and neutral mucopolysaccharides. They show strong positive PAS-reaction, strong reaction with alcian blue at pH 2.6, and stain purple with the AB-PAS sequential procedure. Furthermore, the AMP of these jelly secretions may be sulfated. A moderate staining reaction was obtained with alcian blue at pH 0.5 (although toluidine blue metachromasia was not alcohol-resistant).

No sialic acid residues were detected histochemically (by sialidase digestion) in the mucous cells present in any of the three oviducal regions of untreated male rudimentary oviducts.

The histochemical composition of the secretions of the mucous cells in any of the three oviducal regions of hormone-treated male oviducts remained histochemically identical with or similar to that found in the mucous cells of untreated male rudimentary oviducts. The secretion present in the mucous cells of the three oviducal levels of non-ovulated, mature females is histochemically identical or similar to that of untreated male rudimentary oviducts and hormone-treated male rudimentary oviducts. In other words, no major changes in the histochemical composition of the mucous cells of male rudimentary oviducts appear to be induced upon hormonal stimulation.

Conclusions

By the histochemical data, various kinds of mucopolysaccharides and their distribution in the male rudimentary oviduct before and after hormonal stimulation were compared with those present in non-ovulated, mature female oviducts. Neutral mucopolysaccharides predominate in the three oviducal regions of untreated male rudimentary oviducts. Very little acid mucopolysaccharides is present. No sulfomucins nor sialomucins were detected histochemically in the untreated male oviducts. However, upon hormonal stimulation, a preponderance of acidic mucopolysaccharides, mainly sulfomucins and sialomucins, is detected in the upper regions of the estradiol-treated oviducts. The secretions in the middle and lower regions of the estradiol-treated oviducts remain identical to that found in untreated male oviducts, consisting mainly of neutral mucopolysaccharides with very little AMP.

The only histochemical difference detected between testosterone-treated and untreated male oviducts is the presence of acidic mucopolysaccharides (sulfomucins) in the lower oviducal region, which was mostly composed of neutral mucopolysaccharides, but after hormonal stimulation, is rich in both neutral and acidic mucopolysaccharides. The jelly secretions present in the upper and middle oviducal regions of testosterone-treated male oviducts remain identical or similar to that present in the untreated male oviducts,

namely, a preponderance of neutral mucopolysaccharides over acidic ones.

On hormonal stimulation, at least using estradiol, the male oviduct has the same kinds of mucopolysaccharides as non-ovulated, mature female oviducts. The fact that histochemical differences between testosterone-treated and untreated male oviducts are less pronounced than those observed with estradiol-treated ones could be due to the possibility that all of the injected testosterone was not converted into estradiol.

Although hormone-treated male rudimentary oviducts and non-ovulated, mature female oviducts show quite large differences in their respective lengths, by dividing each of these two kinds of oviducts into three approximately equal regions, one may get some idea as to the distribution of mucopolysaccharides along the lengths of these two oviducts. Approximately the same mucopolysaccharides are present in corresponding oviducal regions of female oviducts and male hormone-treated oviducts. For example, the same kinds of mucopolysaccharides are present in the upper oviducal region of estradiol-treated male rudimentary oviducts as in the upper oviducal region of non-ovulated, mature females, namely, sulfomucins, sialomucins, non-sulfated, mucopolysaccharide rich in carboxyl groups, and very little neutral mucopolysaccharide.

Changes in Male Frog Oviducal Secretion As Detected by Immunological Techniques

Untreated R. pipiens male rudimentary oviducts show at least eight different antigens as indicated by the number of immunoprecipitation lines obtained in double-diffusion tests. Six of these antigens are common to both untreated male rudimentary oviducts and non-ovulated, mature female ones. The other two antigens are unique to the untreated male oviduct. These appear to be male-specific antigens.

One new antigen appears in male oviducts after either estradiol or testosterone stimulation. This new antigen appears to be identical to one of the antigens found in non-ovulated, mature female oviducts. A total of seven lines of identity are evident between testosterone- and estradiol-treated rudimentary oviducts and non-ovulated, mature female oviducts.

A correlation could be made between the different types of mucopolysaccharides identified by histochemical procedures and the "new antigen" line identified by double-diffusion test in male rudimentary oviducts after hormonal stimulation.

In estradiol-treated male oviducts sulfomucins and sialomucins appear to be induced in the upper oviducal region after hormonal stimulation, as detected by histochemical techniques. In the case of testosterone-treated oviducts only sulfomucins appear to be induced in the lower oviducal region. One new immunoprecipitation line is evident

in male oviducts, treated with either hormone, which was not present in the untreated male oviduct. One may suggest that either sulfomucins or sialomucins could be the new antigen detectable by double-diffusion tests.

The identification of the new antigens appearing in the hormone-stimulated male oviducts with the new histochemically identifiable molecules is only speculative at this time. Further tests to determine such an identity could be made, employing isolated antigen-antibody complexes to produce specific antibodies (Shivers and James, 1967) to the "new" molecular configurations. The antibodies could then be used in immunofluorescent staining tests to determine an identity.

Implications of This Research

Attempts to isolate and identify the individual antigens (mucopolysaccharides) making up amphibian egg jellies (Shivers, unpublished results; Freeman, 1968; Oliphant *et al.*, 1970) with the purpose of demonstrating their possible role(s) in fertilization have not been entirely successful to date due to the high degree of complexity of these jellies. At least 9-10 different antigens are present in *R. pipiens* egg jelly, as determined in double-diffusion plates (Shivers and James, 1970). Jelly of *X. laevis* eggs dissolved by disulfide bond reduction showed five distinct macromolecular species. Each of the 3 jelly layers of *X. laevis* eggs had

a distinct macromolecular composition (Oliphant et al., 1970).

In the studies presented here, it has been shown by means of histochemical and immunological techniques, that there is a great degree of similarity between the antigens (mucopolysaccharides) present in hormone-treated male rudimentary oviducts and those present in non-ovulated, mature female oviducts. Furthermore, at least one new antigen is produced after hormonal stimulation of the male rudimentary oviduct and 2 new kinds of AMP (sulfomucins and/or sialomucins) appear only after hormonal stimulation of male rudimentary oviducts, as detected by histochemical tests.

Therefore, it might be possible to use hormone-treated male rudimentary oviducts as a source of material for isolating these 2 new kinds of mucopolysaccharides produced after hormonal stimulation. An indirect way for isolating these antigens could be by absorption procedures. One could absorb hormone-treated male rudimentary oviducts with antisera prepared against untreated male oviducts. Such an absorption would remove common components between the 2 types of oviducts and leave in solution the new antigen(s) that is(are) produced under hormonal stimulation.

In turn, such isolated antigen could be injected into rabbits for the production of specific antibodies. Such antibodies against the new antigen(s) produced under hormonal stimulation, can then be tested for their role(s) in fertilization by inhibition of fertilization tests on R. pipiens eggs.

That indeed new antigens (mucopolysaccharides) are produced under hormonal stimulation needs more direct evidence. Studies on the incorporation of radioactive sulfate ($^{32}SO_4$) by male oviducal tubular glands after hormonal stimulation might provide such an evidence.

It has been suggested by Humphries (1966), Soupart and Noyes (1964), Pereda (1970b) that sialomucins and sulfomucins (Pereda, 1970a) may be involved in sperm penetration. Sulfomucins may be involved in the cortical granule breakdown reaction and establishment of the block to polyspermy (Kelly et al., 1970).

The histochemical data reported here has provided some insight as to the chemical nature of the mucopolysaccharides present in male oviducal jelly secretions. This information could prove to be useful when attempting to chemically isolate the individual mucopolysaccharides.

The untreated male rudimentary oviduct of R. pipiens may prove to be a good model for studying the effect of hormones on the synthesis of specific mucopolysaccharides at either the transcriptional or translational levels of the genome as has been described in the chick oviduct by O'Malley et al. (1969). These investigators have demonstrated that estrogens induced changes in nuclear transcription such as nuclear RNA synthesis (McGuire and O'Malley, 1968) and chromatin template activity (O'Malley et al., 1969); and new species of hybridizable RNA (O'Malley and

McGuire, 1968; O'Malley et al., 1968, 1969) precede the appearance of cell specific proteins (i.e., ovalbumin, lysozyme and avidin).

Similarly, since in the frog male rudimentary oviduct the production of at least 2 new mucopolysaccharides is induced under hormonal stimulation, it could be used as a model for the study of estrogen-mediated functional differentiation.

SUMMARY

A variety of sex hormones was tested on adult male Rana pipiens for their effectiveness in inducing hypertrophy of the rudimentary oviducts. Only two hormones, estradiol and testosterone, were effective. An increase in the size, weight, and degree of convolution of these oviducts, as well as an induction of jelly secretion was observed after hormonal stimulation.

Both histochemical and immunological techniques were used to detect the kinds of mucopolysaccharides present in the oviducal jelly secretion before and after hormonal stimulation and their distribution along the length of the oviduct. Furthermore, to get some idea as to this distribution of mucopolysaccharides, oviducts were divided into approximately three equal regions, namely, upper, middle and lower. Non-ovulated, mature female oviducts were used for comparison (as controls).

Neutral mucopolysaccharides predominate in the three oviducal regions of untreated male rudimentary oviducts. Very little acid mucopolysaccharide is present. No sulfomucins nor sialomucins were detected histochemically in these oviducts.

Upon hormonal stimulation, a preponderance of acid mucopolysaccharide, mainly sulfomucins and sialomucins, was detected in the upper regions of the extradiol-treated male oviducts. The jelly secretions in the middle and lower regions of the estradiol-treated oviducts remain identical to that found in untreated male oviducts, consisting mainly of neutral mucopolysaccharides with very little acid mucopolysaccharides.

The presence of acid mucopolysaccharides (sulfomucins) was detected in the lower oviducal region of testosterone-treated male oviducts, thus making this region rich in both neutral and acidic mucopolysaccharides. The jelly secretions present in the upper and middle oviducal regions of these oviducts remained identical or similar to those present in untreated male oviducts, namely, a preponderance of neutral mucopolysaccharides over acidic ones.

On hormonal stimulation, at least using estradiol, the male rudimentary oviduct has the same kinds of mucopolysaccharides as non-ovulated, mature female oviducts. Also approximately the same mucopolysaccharides are present in corresponding oviducal regions of female oviducts and hormone-treated male oviducts.

Untreated male rudimentary oviducts show at least 8 different antigens as indicated by the number of immunoprecipitation lines obtained in double-diffusion tests. Six of these antigens are common to both untreated male

rudimentary oviducts and non-ovulated, mature female ones. The other two antigens are unique to untreated male oviducts.

One new antigen appears in male rudimentary oviducts after either estradiol or testosterone stimulation. This "new antigen" appears to be identical to one of the antigens found in non-ovulated, mature female oviducts. It is suggested that the "new antigen" line identified in these hormone-treated oviducts by the double-diffusion tests may be either the sulfomucins or sialomucins detected histochemically.

A total of 7 lines of identity are evident between testosterone- and estradiol-treated rudimentary oviducts and non-ovulated, mature female oviducts.

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APPENDIX

APPENDIX TABLE I

HORMONAL TREATMENTS

Date of Experiment	$\frac{\text{Oviducal wt}}{\text{final body wt}}$ x 1000								
	Prog A	Prog B	Prog C	Est	Prog+Est	Test A	Test B	UNT	Oil
May 15/69	2.8	1.3	---	---	---	2.5	1.6	---	0.7
	1.1	1.1	---	---	---	2.8	1.8	---	0.6
	---	0.5	---	---	---	2.3	---	---	1.1
June 23/69	0.3	0.8	---	---	---	5.1	5.6	0.8	0.1
	0.6	1.1	---	---	---	2.4	---	0.6	0.6
	---	---	---	---	---	---	---	0.4	1.0
July 23/69	1.5	1.1	---	10.9	2.9	1.9	2.0	0.8	1.0
	2.5	1.3	---	4.4	5.0	2.5	3.8	0.5	0.4
	3.0	1.2	---	---	5.2	1.8	8.0	---	0.7
	1.5	0.9	---	---	4.8	---	9.7	---	0.9
	---	---	---	---	3.6	---	---	---	---
Oct. 27/69	---	1.2	3.4	5.4	2.3	---	---	1.5	2.1
		1.9	0.7	8.9	4.1	---	---	2.7	1.9
	---	1.2	2.4	5.6	3.7	---	---	1.3	1.3
	---	3.5	3.4	4.5	4.1	---	---	3.1	0.7
	---	1.7	1.2	4.0	7.8	---	---	2.7	2.1
	---	1.1	2.1	6.2	0.8	---	---	1.1	0.7
	---	1.9	---	2.6	5.2	---	---	---	---

Feb. 19/70	---	---	---	2.1	---	3.3	---	1.0	0.6
	---	---	---	1.5	---	2.6	---	0.7	0.3
	---	---	---	3.8	---	2.3	---	1.0	1.5
	---	---	---	1.8	---	3.0	---	0.3	1.0
	---	---	---	2.9	---	3.1	---	0.4	0.7
	---	---	---	2.6	---	3.0	---	0.6	0.3
	---	---	---	1.7	---	2.8	---	---	0.6
	$\bar{y}$ = 1.7	1.3	2.1	4.6	4.0	2.8	4.6	1.2	0.9
	n = 8	17.0	7.0	17.0	13.0	15.0	7.0	17.0	24.0
	s = 1.0	0.7	1.1	2.8	1.8	0.8	3.2	0.9	0.5

APPENDIX TABLE II

EXPERIMENT NO. 1 (May 15-May 29/69)

Frog No. and Treatment		Initial Body Wt. (gms)	Final Body Wt. (gms)	Oviducal Wt. (gms)	$\frac{\text{Oviducal wt}}{\text{Final Body wt}} \times 1000$
Prog. A	3	39.0	28.0	0.079	2.82
	4	45.5	40.0	0.042	1.05
Prog. B	1	37.0	30.6	0.040	1.33
	2	42.0	32.0	0.036	1.13
	4	39.0	32.0	0.017	0.53
Test A	2	37.5	32.0	0.079	2.47
	3	38.0	33.0	0.092	2.79
	4	45.0	35.0	0.081	2.31
Test B	1	42.0	35.0	0.055	1.57
	3	38.0	29.5	0.053	1.80
UNT			all died		
OIL	1	410	36.0	0.022	0.61
	2	43.0	38.0	0.042	1.11
	3	41.0	36.0	0.026	0.72

APPENDIX TABLE III

EXPERIMENT NO. II (June 23-July 7/69)

Frog No. and Treatment		Initial Body Wt. (gms)	Final Body Wt. (gms)	Oviducal Wt. (gms)	$\frac{\text{Oviducal Wt.}}{\text{Final Body Wt}} \times 1000$
Prog. A	1	28.0	22.0	0.000	0.3
	2	38.0	27.0	0.017	0.6
Prog. B	1	30.0	25.0	0.022	0.8
	2	38.0	27.0	0.030	1.1
Test A	1	41.0	35.-	0.179	5.1
	2	36.0	29.0	0.071	2.4
Test B	2	35.5	32.0	0.182	5.6
UNT	1	36.0	31.0	0.025	0.8
	2	30.0	27.0	0.017	0.6
	3	42.0	36.0	0.015	0.4
OIL	1	40.0	34.5	0.005	0.1
	2	33.5	29.0	0.019	0.6
	3	34.0	28.0	0.029	1.0

APPENDIX TABLE IV

EXPERIMENT NO. III (July 23-August 7/69)

Frog No. and Treatment		Initial Body Wt. (gms)	Final Body Wt. (gms)	Oviducal Wt. (gms)	$\frac{\text{Oviducal Wt.}}{\text{Final Body Wt.}} \times 1000$
Prog. A	1	24.0	20.0	0.030	1.5
	2	23.5	19.0	0.048	2.5
	3	20.0	±4.5	0.044	3.0
	4	24.5	22.0	0.033	1.5
Prog. B	1	24.5	21.0	0.023	1.1
	2	30.0	24.0	0.032	1.3
	3	23.5	19.0	0.022	1.2
	4	23.5	20.5	0.019	0.9
EST	1	22.0	20.0	0.217	10.9
	2	23.0	20.0	0.087	4.4
Prog B + EST	1	27.0	22.0	0.063	2.9
	2	24.0	21.0	0.104	5.0
	3	27.5	25.0	0.129	5.2
	4	28.0	23.0	0.109	4.8
Test A	2	27.0	26.0	0.049	1.9
	3	24.0	20.0	0.050	2.5
	4	24.0	25.0	0.044	1.8

Test A	2	27.0	23.0	0.109	4.8
	4	24.0	20.0	0.049	1.9
Test B	1	22.0	22.0	0.044	2.0
	2	24.5	19.0	0.073	3.8
	3	25.5	22.5	0.180	8.0
	4	24.0	20.0	0.194	9.7
UNT	2	28.5	26.0	0.020	0,8
	4	22.0	22.0	0,010	0,5
OIL	1	24.0	24.0	0.025	1.0
	2	25.5	25.0	0.010	0.4
	3	26.5	25.5	0.017	0.7
	4	29.0	27.0	0.025	0.9

APPENDIX TABLE V

EXPERIMENT NO. IV (October 27-November 10/69)

Frog No. and Treatment		Initial Body Wt. (gms)	Final Body Wt. (gms)	Oviducal Wt. (gms)	$\frac{\text{Oviducal Wt.}}{\text{Final Body Wt.}}$ x 1000
Prog. B	1	46.0	40.0	0.050	1.2
	2	48.0	43.0	0.083	1.9
	3	47.0	44.0	0.057	1.2
	4	40.0	39.0	0.137	3.5
	5	47.0	44.0	0.076	1.7
	6	45.0	43.0	0.050	1.1
	7	45.0	44.0	0.048	1.0
	8	52.0	47.0	0.090	1.9
Prog. C	2	37.0	35.0	0.120	3.4
	3	48.0	46.0	0.035	0.7
	4	44.0	40.0	0.098	2.4
	5	45.0	44.0	0.151	3.4
	6	41.0	37.0	0.045	1.2
	7	53.0	49.0	0.104	2.1
	8	38.0	33.0	0.042	1.2
EST	1	43.0	35.0	0.192	5.4
	2	41.0	37.0	0.331	8.9
	3	43.0	38.0	0.215	5.6
	4	42.0	37.0	0.168	4.5
	5	46.0	42.0	0.170	4.0
	6	46.0	41.0	0.256	6.2
	7	40.0	35.0	0.311	8.8
	8	42.5	36.0	0.095	2.6

Prog. + EST	1	48.0	42.0	0.100	2.3
	2	46.0	42.0	0.174	4.1
	3	42.5	37.0	0.139	3.7
	4	43.0	41.0	0.172	4.1
	5	46.5	40.0	0.313	7.8
	6	51.0	45.0	0.036	0.8
	7	41.0	33.0	0.073	2.2
	8	50.0	41.0	0.216	5.2
UNT	1	45.0	42.0	0.065	1.5
	2	40.0	38.0	0.105	2.7
	3	50.0	45.0	0.061	1.3
	6	50.0	46.0	0.144	3.1
	7	43.0	38.0	0.104	2.7
	8	36.0	32.0	0.037	1.1
OIL	1	41.5	39.0	0.084	2.1
	2	48.0	46.0	0.090	1.9
	3	48.0	48.0	0.065	1.3
	5	51.0	46.0	0.033	0.7
	6	40.0	37.0	0.080	2.1
	7	40.0	38.0	0.027	0.7
	8	43.0	39.0	0.048	1.2

APPENDIX TABLE VI

EXPERIMENT NO. V (February 19-March 5/70)

Frog No. and Treatment		Initial Body Wt. (gms)	Final Body Wt. (gms)	Oviducal Wt. (gms)	$\frac{\text{Oviducal Wt.}}{\text{Final Body Wt.}} \times 1000$
EST	1	35.0	28.0	0.060	2.1
	2	38.0	32.0	0.050	1.5
	3	30.0	23.5	0.090	3.8
	4	37.5	32.0	0.060	1.8
	5	37.5	30.5	0.090	2.9
	6	29.0	22.5	0.060	2.6
	8	37.5	33.5	0.060	1.7
Test A	1	34.0	27.0	0.090	3.3
	2	35.0	30.0	0.080	2.6
	3	34.5	30.0	0.070	2.3
	4	35.5	32.5	0.100	3.0
	5	35.5	32.0	0.100	3.1
	7	38.0	33.0	0.100	3.0
	8	37.0	32.0	0.090	2.8
UNT	1	34.0	29.0	0.030	1.0
	2	33.0	27.0	0.020	0.7
	4	35.0	30.0	0.030	1.0
	6	35.5	30.0	0.010	0.3
	7	36.0	30.5	0.015	0.4
	8	36.5	30.5	0.020	0.6
OIL	1	37.5	31.0	0.020	0.6
	2	36.0	31.0	0.010	0.3
	3	35.0	30.0	0.045	1.5
	5	36.0	32.0	0.035	1.0
	6	34.0	27.0	0.020	0.7
	7	35.0	30.0	0.010	0.3
	8	39.5	33.0	0.020	0.6