LYTIC AGENTS OF THE SPERM OF SOME MARINE ANIMALS

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ABSTRACT

This thesis consists primarily of investigations of lytic agents and related substances that are extracted from sperm of echinoderms, molluscs and mammals.

Sea-urchin fertilizin is found to give a mucin clot reaction with serumprotein to give a stable turbidity. Hyaluronidase, from mammalian sperm, has no dissolving action on sea-urchin egg jelly, nor could a hyaluronidase-like substance, capable of dissolving sea-urchin jelly, acting on purified fertilizin preparations or on hyaluronic acid, be extracted from sea-urchin sperm.

The presence in sea-urchin sperm extracts of egg surface lysin activity is confirmed.

The egg membrane lysin of sperm of the giant keyhole limpet is found to be separable from antifertilizin. Lysin is released from living sperm in sea water at neutral and alkaline pH. Antifertilizin does not appear to be released under these conditions. Release of lysin seems to be independent of metabolic activity. The lysin does not seem to act catalytically. It is inactivated by treatments that denature proteins, and by proteolytic enzymes. The egg membrane is dissolved by SH compounds and is slightly digested by trypsin. It is thought to be of keratin-like nature. It is shown that complete dissolution of the egg membrane is not necessary for fertilization.

An egg membrane lysin is demonstrated in sea-urchin sperm extracts. It dissolves the keyhole limpet egg membrane.

Cross reactions are shown to occur between sperm extracts and egg membranes of the keyhole limpet, abalone and mussel.

TABLE OF CONTENTS

LYTIC AGENTS OF THE SPERM OF SOME MARINE ANIMALS

INTRODUCTION

Interest in problems of fertilization has acquired a fresh impetus in recent years as a result of work which has shown that a number of interacting substances can be extracted from the sperm and eggs of various animals. These are fertilizins of eggs, antifertilizins of eggs and of sperm, and lytic agents of sperm. Of the fertilizins and antifertilizins, fertilizin of echinoderm eggs has been most thoroughly investigated with respect to its action and physico-chemical properties. In the sea-urchin, fertilizin is the chief constituent of the jelly coat of the eggs, and has been characterized as a high molecular weight glycoprotein (cf. Tyler, 1948). Antifertilizin from sperm has been shown to be an acidic protein of relatively low molecular weight (ca. 10,000) and may be localized on the surface of the head of the spermatozoa. Antifertilizin from eggs also appears to be a protein, similar, with respect to those of its properties that have been investigated, to antifertilizin from sperm. It occurs in the interior of the sea-urchin egg. Several different lytic agents from the sperm of various animals have been described. At least one of these (hyaluronidase) has been characterized as an enzyme. Others also appear to be proteins, but their enzymatic nature has not been established. One, the egg surface lysin of sea-urchins and fish (sperm lysin) is described by Runnström as a surface active substance with detergent-like properties.

A promising approach toward resolving some of the problems presented by the reactions of fertilization is that of Tyler and his collaborators, who have correlated many of the reactions involved with those of immunological processes (cf. Tyler, 1948). The first serious attempt to

describe the fertilization process in immunological terms was made by F.R. Lillie (1913, 1914) who introduced the fertilizin terminology. It has not been until recently, however, that adequate experimental evidence has established more than a superficial resemblance.

In a recent review, Tyler (1948) has summarized in considerable detail the evidence correlating fertilization and immunological reactions. Much of this relates to reactions involving fertilizin and antifertilizin. In connection with the present discussion it seems pertinent to present a brief resumé of certain features of this evidence that indicate the possible role of these substances in fertilization.

The agglutination of sperm of various species of animals by egg water is the most striking effect of the reaction between fertilizin in solution and sperm antifertilizin. In a number of species the agglutination reaction normally does not occur, but it has been shown by Metz (1942, 1944, 1945) that after treatment with an adjuvant, such as hen's egg white, sperm of certain of these species are agglutinated by homologous egg water.

Antifertilizin in solution reacts with the intact jelly hull (fertilizin) of the egg in a number of species with the formation of a precipitation membrane on the jelly surface. The eggs may also be agglutinated.

It has been established that fertilizin in solution acts as a barrier to fertilization, but that when present as the jelly hull it serves as an aid to fertilization. Jellyless eggs must be inseminated with significantly higher concentrations of sperm than eggs with the jelly coat present.

In species in which the unfertilized eggs show no distinct gela-

tinous coat the surface of the eggs may contain a fertilizin which reacts with antifertilizin of the sperm. Evidence for the view that a surface layer of fertilizin may be essential for fertilization is provided by the observations of Chambers (1921) and Just (1923) that sea-urchin egg fragments devoid of original surface material are unfertilizable. In this connection it should be noted that in the sea-urchin egg a layer of fertilizin remains intimately bound to the surface even after the jelly hull has been removed, and the presence of this surface layer may account for the fact that jellyless eggs are still fertilizable. Fertilizin, then, as a constituent of the egg surface may be an important factor in specific attachment of sperm to the egg. The presence of antifertilizin in the interior of the egg suggests that it may here be involved in reactions occurring subsequent to sperm entry. There is, however, no experimental evidence concerning this possibility.

Lytic agents of sperm that have been described, include the enzyme hyaluronidase in various species of mammals (cf. Duran-Reynals, 1942), an egg surface lysin (sperm lysin) in sea-urchins and fish (Runnström, and Tiselius, and Lindvall, 1944), and egg membrane lysins in some marine molluscs (Tyler, 1939). There have been occasional reports (Hartmann and Schartau, 1939; Monroy and Ruffo, 1947) in the literature of agents associated with the sperm of echinoderms that dissolve the egg jelly of these animals. The existence of jelly-dissolving lysins in echinoderm sperm, however, is questionable. In general, lytic agents of the sperm act on extraneous membranes or cell layers associated with eggs, dissolving or dispersing these. An exception to this seems to be the egg surface lysin of sea-urchin and fish sperm which acts on the surface

layer of the echinoid egg.

At the present time the best characterized of the lytic agents of sperm is hyaluronidase. Great interest has been shown in this enzyme not only because of its presence in the sperm of a number of mammals, but also because it is found in a variety of substances and organisms that are characterized by the common property of invasiveness. Hyaluronidase has been identified in venoms of reptiles, insects and arachnids, in extracts of leeches and in a number of bacteria. It is of interest that there is no apparent correlation between the presence in bacteria of hyaluronidase and virulence (cf. Duran-Reynals, 1942).

Hyaluronidase hydrolyzes an acidic mucopolysaccharide called hyaluronic acid. This polysaccharide has been demonstrated in a variety of mammalian body fluids and tissues and appears to be one of the chief cementing substances in the subcutaneous tissues. Its presence as a cement substance binding the follicle cells that normally surround the freshly ovulated eggs of most mammals has been inferred since these cells are dispersed in vitro by the action of hyaluronidase. It is this action of the enzyme that makes its participation in the fertilization process in mammals appear likely. Whether hyaluronidase occurs in the sperm of animals other than mammals is an open question at this time. It has been reported to be present in "negligible" quantities in fowl sperm, absent in the sperm of various reptiles and present in echinoderm sperm.

The detergent-like factor extractable from sperm of sea-urchins and fish, and also from sea-urchin eggs, here called "egg surface lysin" (cf. Tyler, 1948), was originally termed "Androgamone III" by Runnström et. al. (1944,1945, etc.) and more recently designated "sperm lysin" by

these workers (Runnström, Monné and Wicklund, 1946). This agent is reported to have a liquefying action on the cortex of sea-urchin eggs. It has been proposed by Runnström and his group that the lysin participates in establishing the block to polyspermy and also in activation of the egg. Because it (or a similar substance) is also obtained from eggs, and because the agent acts on the surface layer of the egg, the name "egg surface lysin" seems more appropriate and less ambiguous than "sperm lysin".

The sperm of several marine molluscs, notably the giant keyhole limpet (Megathura crenulata), abalone (Haliotis cracherodii) and mussel (Mytilus californianus) have been found to contain lytic agents that are able to dissolve the membrane normally present about the unfertilized eggs of these animals. It is believed that the egg membrane lysin of sperm of these animals may serve to facilitate their penetration of the egg membrane, and in this respect, at least, be of importance in the fertilization process.

In considering the possible role in fertilization of lytic agents of sperm it must be borne in mind that the gross effects revealed by action of these substances in vitro or in unphysiological concentration, may not be an adequate basis for drawing conclusions concerning their action in natural fertilization. Illustrations of this point are provided by hyaluronidase and the keyhole limpet egg membrane lysin. These will be discussed in later ~~chapters~~ sections. It should also be noted that lytic agents probably act in conjunction with other sperm and egg substances, fertilizin and antifertilizin, for example. Caution must be employed in attributing an observed phenomenon to a lytic agent when it might as well, or better, be ascribed to interaction of other substances.

This, it should be pointed out, seems of particular importance with respect to action of the sea-urchin egg surface lysin, which is assumed to occur at the egg surface, where fertilizin-antifertilizin reaction also occurs.

The egg membrane lysins of marine molluscs offer several advantages for study. Their primary action appears to be on a clearly visible, distinct membrane surrounding the unfertilized egg. Lysis of the egg membrane can be observed under physiological conditions, equivalent to in vivo observations with higher animals. Their sphere of action may, to some extent at any rate, be differentiated from that of fertilizin and antifertilizin. Among certain disadvantages may be mentioned the difficulty of developing adequate quantitative measures of lytic activity. However, as further study reveals more information concerning the mechanism of the action quantitative techniques can be developed.

The major part of this thesis is devoted to presentation of results obtained in investigations of the egg membrane lysin of the giant keyhole limpet. Studies of lytic agents in sea-urchin sperm have also been pursued and these are reported. Much of the work with sea-urchin sperm was directed toward the possibility of finding a hyaluronidase-like lysin, capable of dissolving the egg jelly. In the course of this work a so-called "mucin clot" reaction of sea-urchin fertilizin was discovered and is included in this thesis in the form of a reprint of a publication. Some experiments with sea-urchin egg surface lysin were also carried out, and the results obtained are reported.

REVIEW OF THE LITERATURE

Hyaluronidase

General survey. No attempt will be made here to review all of the literature concerning hyaluronidase. A brief resumé of pertinent aspects of work which has led to present knowledge of this enzyme will be presented followed by a more detailed review of those papers which relate more directly to the role of hyaluronidase in fertilization. Two exhaustive reviews concerning hyaluronidase are available, covering most of the work prior to 1947 (Duran-Reynals, 1942; Meyer, 1947).

In 1928 Duran-Reynals reported that vaccinial infection of the rabbit is enhanced when the virus is injected into the skin along with an aqueous extract of the testis of one of several animals, including the rabbit, guinea pig and rat. The effect appeared whether the extract was given together with vaccinia or had been injected several days earlier, showing that the action of the extract was exerted upon the host tissues. These observations were duplicated by McClean (1930). Later a variety of substances of different origin were found to possess "spreading power". These were designated "Spreading Factors" or "Diffusing Factors", and were obtained from invasive bacteria (Duran-Reynals, 1933), poisonous insects, snake venoms (Duran-Reynals, 1939), leeches (Claude, 1937) and other sources.

The usual test for spreading power is to inject the substance along with an indicator such as india ink or Evans blue into the skin of a rabbit or guinea pig and compare the amount of spread of the indicator with that of similarly injected dye or ink in saline. Attempts have been made to develop this kind of test into a quantitative assay method (Bacharach, Chance and Middleton, 1940; Humphrey, 1943).

Working along chemical lines Meyer, et al. (1936, 1937, 1938), showed that an enzyme present in autolysates of pneumococcus, tissue hash from rabbit iris, ciliary body and spleen hydrolyzed hyaluronic acid, an acidic mucopolysaccharide that had been isolated by the same group of workers from vitreous humor, synovial fluid, umbilical cord and streptococcus. This finding was duplicated in 1940 by Robertson et al., with preparations from Clostridium welchii acting on synovial fluid. No correlation between all these enzymes and spreading factors was established until 1939 when Chain and Duthie (1939, 1940) identified some of the spreading factors with mucolytic enzymes (hyaluronidase) and described the phenomenon of spreading in animal tissues as an enzymatic effect on hyaluronic acid of connective tissue.

Meyer, et al. (1934, 1936, 1938), worked out the analysis of hyaluronic acid and showed it to be an equimolar combination of glucuronic acid and N-acetylglucosamine. The basic disaccharide (aldobionic) units are polymerized to very large molecules - molecular weights of 200,000 to 500,000 have been reported (Meyer, 1947). The viscosity of the native fluids from which the polysaccharide is obtained is generally much greater than that of the purified hyaluronate, but Meyer has recently (1948) reported the preparation of highly viscous sodium hyaluronate. In tissues and body fluids hyaluronic acid occurs free, as a salt, or it may be bound with protein (Meyer, 1945). In the cornea a sulfuric acid ester of the polysaccharide is found. It is one of the chief components of the intercellular ground substance of connective tissue (Duran-Reynals, 1942).

It has been found (Seastone, 1939; Robertson, Ropes and Bauer, 1940; McClean, 1943; Kass and Seastone, 1944) that hyaluronic acid co-precipitates with protein to give a mucin clot or turbidity, depending upon the

conditions of precipitation. This is a property that is shared with other acidic mucopolysaccharides (nomenclature after Meyer, 1945), e.g., ~~heparin and~~ chondroitinsulfuric acid (~~Cohen, 1942~~ Meyer, 1945). In the case of chondroitinsulfuric acid, the complex formation with protein was found to be stoichiometric (Meyer, Palmer and Smyth, 1937). Depolymerized hyaluronic acid does not form a mucin clot (cf. Meyer, 1947).

The action of hyaluronidase on hyaluronic acid consists of an initial depolymerization of the polysaccharide followed by hydrolysis. Evidence of depolymerization is obtained by measuring reduction of the viscosity. Hydrolysis can be determined by measurement of increase in reducing sugar or of liberated acetylglucosamine.

Assay methods that have been used for testing various preparations for hyaluronidase activity are of four main types:

1. Mucin clot-prevention test (McClean, 1943). This depends upon the failure of depolymerized hyaluronic acid to form a mucin clot. Hyaluronic acid is incubated with various enzyme dilutions and that dilution which is just effective in preventing the formation of a clot gives the titer of the enzyme preparation.

2. Turbidity-reduction test (Kass and Seastone, 1944; Leonard, Perlman and Kurzrok, 1946; Dorfman and Ott, 1948). This test depends upon the formation of a stable colloidal turbidity when dilute solutions of hyaluronic acid are combined with acidified serum or serum protein solutions, and the density of the turbidity can be determined photometrically. After incubation of hyaluronic acid with hyaluronidase, the amount of turbidity produced by reaction with protein is reduced.

3. Viscosity-lowering method. Decrease in viscosity of hyaluronic acid solutions as a result of action of hyaluronidase gives a measure of the activity of different enzyme preparations (Madinaveita and

Quibell, 1940; McClean and Hale, 1941; Robertson, Ropes and Bauer, 1940; Swyer and Emmens, 1947; Sherber, Birnberg and Kurzrok, 1948).

4. Reductometric method. Determination of the products of hydrolysis affords a means of comparing the activity of different enzyme preparations (Meyer, 1946).

Comparison of results obtained with these various assay methods has shown that there are large discrepancies between those from viscosimetric and mucin clot prevention assays (McClean, 1943); turbidimetric and viscosimetric methods have given results showing fair correlation (Kass and Seastone, 1944; Dorfman, 1948); little correlation has been observed in some instances between results obtained with viscosimetric or turbidimetric and reductometric methods - leech enzyme, for example was found by Meyer (1947) to be equal turbidimetrically to the best testes preparations but it showed only 40% hydrolysis.

A number of substances that exhibit spreading power do not hydrolyze hyaluronic acid in vitro. On this basis Duran-Reynals (1942) has proposed a classification of spreading factors. Substances that hydrolyze this polysaccharide and also show spreading power (hyaluronidases) comprise Group A; those which do not hydrolyze hyaluronic acid but do exhibit some spreading power comprise Group B, and among these are a number of simple chemical compounds such as arsenious oxide and glycerol; wintergreen oil, oil of citronella, olive oil, peptones and lecithins are also included in Group B. A third group of substances includes certain compounds that show spreading power and a non-specific viscosity lowering power but no hydrolytic effect. Among these are ascorbic acid, which is effective only in the presence of H_2O_2, and thiolacetic acid, pyrogallol and certain diazo compounds.

The most highly purified hyaluronidase thus far subjected to

electrophoresis proved not to be homogeneous (Hahn, 1943). It showed an isoelectric point at pH 5.7. In distilled water the solution had a pH of 6.2 to 6.9. In general, it may be said that hyaluronidase is a heat-labile protein, inactivated by pepsin and trypsin, ultraviolet irradiation and mild shaking. It is non-dialyzable. Its purification is mainly concerned with the elimination of other proteins present in the crude extract. Among these, at least in material obtained from testicular extracts, may be one or more unrelated enzymes. Thus, a β - acetylglucosaminidase is found associated with crude testicular hyaluronidase but it is not involved in hyaluronidase activity (Meyer, 1947). ~~(Hobby, et al., 1946).~~ Hahn (1945) claims to have separated two enzymes from testicular extracts, one of which hydrolyzes hyaluronic acid to the aldobionic acid stage and the other hydrolyzes the disaccharide to monosaccharides. Hyaluronidases from different sources appear to contain different enzymes. Thus, for example, it has been observed that testicular and leech enzymes give only about half the theoretical yield of reducing sugars, while pneumoccal hyaluronidase gives almost complete hydrolysis and can supplement the action of the testicular and leech materials (Meyer, et al., 1941).

Highly purified hyaluronidase from testicular extracts has been stated to hydrolyze two other mucopolysaccharides beside hyaluronic acid, one the monosulfuric acid ester of hyaluronic acid from cornea (Meyer and Chaffee, 1940), the other the chondroitin sulfate of hyaline cartilage (Meyer, et al., 1941; Madinaveitia and Stacey, 1944). The evidence, on the whole, points to the presence of another enzyme present in the testicular preparations as being responsible for the hydrolysis of chondroitin sulfate (cf. Meyer, 1947). It is of interest, however, that

hyaluronidase activity towards hyaluronate runs practically parallel with the activity towards chondroitin sulfate. Compounds of the general composition of chondroitin sulfate occur beside hyaluronate in mesodermal tissue, like umbilical cord and skin in concentrations about equal to that of hyaluronate.

The action of hyaluronidase can be blocked by means of specific antisera. In contrast to the lack of species-specific action of hyaluronidase, the antisera are highly specific. Thus, antisera against the hyaluronidases of Clostridium welchii and Cl. septicum do not cross-react (McClean, 1943), and in tests of dispersal of follicle cells of rat ova, antisera prepared in rats against bull testis hyaluronidase did not cross-react with the rat testis enzyme (Leonard and Kurzrok, 1946). It may be concluded that the enzymatically active groups of the hyaluronidase are nonantigenic, and neutralization by homologous antibody may be attributed to combination with adjacent antigenic groups on the molecule. Hyaluronic acid, on the other hand, is not antigenic (Meyer, 1947).

Hyaluronidases and hyaluronic acid have been found in many bacteria. In general, the presence of hyaluronidase in bacteria is correlated with invasiveness rather than with virulence (cf. Duran-Reynals, 1942). In some encapsulated forms hyaluronic acid occurs in the capsule, for example in Group A and C hemolytic streptococci. An extensive study of hyaluronidase production in relation to capsule formation in these streptococci showed (Crowley, 1944) that all hyaluronidase positive strains were non-capsulated, although many non-capsulated strains were hyaluronidase negative. Seastone (1939) and Hirst (1941) have presented evidence that the hyaluronate capsule in Group C streptococci is responsible for virulence. Protection against fatal infection in mice was

obtained by the injection of leech and testicular hyaluronidase. According to McClean (1941 a,b) organisms which have hyaluronate appear to produce it defensively in amounts sufficient to offset the available hyaluronidase.

A hyaluronidase inhibitor (antihyaluronidase) is present in normal serum of various animals. This has been reported to be an enzyme or a system of enzymes (Haas, 1946 a,b,c). More recently, evidence has been presented by Dorfman, Ott and Whitney (1948) that inactivation of hyaluronidase in human blood is not enzymatic. These workers suggest that the inhibitory action may be due to a polysaccharide prosthetic group bound to a protein carrier.

Hyaluronidase in fertilization. It was observed by Long (1912) that when unfertilized tubal eggs of rats and mice are inseminated in vitro, the follicle cells, comprising the cumulus oöphorus, are quickly dispersed. According to Long's account, it appears that this was thought to be due to mechanical agitation by the spermatozoa. Later, it was shown by a number of workers (Pincus, 1930; Yamane, 1930, 1935; Pincus and Enzmann, 1932; Gilchrist and Pincus, 1932; Pincus, 1936 - for review of this early work) that dispersal of the follicle cells surrounding unfertilized tubal eggs of the rabbit, rat and mouse is caused, in vitro by sperm suspensions, and also by sperm-free fluid from the vas deferens and from sperm suspensions.

In 1942 McClean and Rowlands found that dispersal of the follicle cells, in vitro, of rat ova is caused by hyaluronidase from various sources. Confirmation of this finding was provided by Fekete and Duran-Reynals (1943) and Leonard and Kurzrok (1945) with eggs of rats and mice, using hyaluronidase from different sources. The active agent was found

roughly to parallel the hyaluronidase in enzymatic and spreading action and in physico-chemical properties.

The complete cell dispersal seen when mammalian ova are denuded by hyaluronidase in vitro led McClean and Rowlands (1942), Fekete and Duran-Reynals (1943) and Leonard and Kurzrok (1945) to suggest that hyaluronidase may participate in the fertilization process by denuding the egg of its follicle cells. This hypothesis would explain the requirement of large numbers of supernumerary sperm commonly present in ejaculates of mammals on the ground that the extra spermatozoa provide sufficient hyaluronidase to denude the ova and thus allow fertilization to be effected.

McClean and Rowlands (1942) suggested that sterility in humans may in some cases be due to deficiency in hyaluronidase production.

In order to test the hypothesis that hyaluronidase may be an important factor in reproductive fertility in mammals, Rowlands (1944) performed a series of seven experiments with rabbits in which heated seminal fluid (presumably containing hyaluronidase) was injected with spermatozoa into superovulated females. It was found that the number of fertilized eggs obtained was increased in the presence of the seminal fluid, the average increase in fertility being such that only one-sixth the normal amount of sperm was needed. Rowlands concluded that this result was due to hyaluronidase in the seminal fluid.

In an experiment with humans, Kurzrok, Leonard and Conrad (1946) reported that fertilization was obtained in six women, with a history of sterile marriage, when bull testis hyaluronidase was added to the semen specimen used for artificial insemination or placed in the genital tract prior to coitus.

In two papers, Swyer (1947 a,b) reported the results of observations

on the hyaluronidase content of semen and the release of hyaluronidase from spermatozoa. He found that there is a close correlation between hyaluronidase content of the semen and sperm density in men, rabbits, bulls and boars. In dogs and fowls no such correlation was found. Swyer also determined that the majority of the total hyaluronidase content of semen is associated with the spermatozoa, and that they do not appear actively to produce the enzyme but merely liberate the preformed substance. In a third paper, Swyer (1947c) reported that hyaluronidase could not be detected in testicular extracts of various reptiles. This is of interest since reptilian ova are devoid of cumuli when ovulated.

Swyer interpreted his findings as lending support to the supposition that hyaluronidase may act in vivo to denude the egg of its follicle cells, for, as it is associated with the spermatozoa and liberated preformed from them, it would presumably be available at the site of fertilization.

Chang (1947) attempted to repeat the experiments of Rowlands (1944), using a larger number of rabbits (thirty-two) and purified testis hyaluronidase, under strictly controlled conditions. It was found that where insemination was performed with a minimal effective number of spermatozoa the fertilizing capacity of the spermatozoa was increased by heated seminal fluid but not by hyaluronidase. In another paper, Chang (1947) reported that the percent of fertilized ova in the rabbit depends upon the ratio of spermatozoa to seminal fluid, significantly higher percentages being obtained when spermatozoa are suspended in a small volume of fluid. The experiments of Chang revealed great variation in the percentage of fertilized ova per doe under strictly controlled conditions and for this reason positive conclusions concerning fertilizability based

upon only a few clinical cases are considered to be of little value.

Direct evidence has been presented by several workers, that shows that, in at least one animal - the rat - no mass removal of the follicle cells prior to fertilization occurs *in vivo*. Leonard, Perlman and Kurzrok (1947) obtained tubal eggs from rats after the animals were bred and found fertilized eggs with the cumulus oöphorus intact. These workers also found that after introduction of hyaluronidase into both uterine horns of a number of rats in heat masses of ova that were recovered 18 to 24 hours later did not contain any denuded eggs. With the use of phase contrast microscopy, Austin (1948), observed male and female pronuclei in eggs of the rat still surrounded by the follicle cells. This author also called attention to the earlier report of Lewis and Wright (1935) who had found fertilized eggs of the rat with the cumulus intact. Blandau and Odor (1949) have made similar observations with fertilized rat eggs. These workers also report that in the rat a very small number of spermatozoa actually arrive at the site of fertilization. This information was obtained by making sperm counts in various parts of the female genital tract at different periods after copulation had occurred. In this manner it was found that 12 hours after insemination an average number of 10 spermatozoa were in that part of the oviduct (ampulla) containing the egg, and within this time a high percentage of the eggs had been fertilized.

At the present time the available evidence indicates that, although hyaluronidase may function in the fertilization process in mammals, it it not essential that the follicle cells be dispersed from about the egg in order that fertilization can occur. It appears that each spermatozoön is equipped with sufficient of the enzyme to enable it to traverse the

follicle cell mass, and thus individual spermatozoa are potentially fitted with respect to hyaluronidase, to effect fertilization without the assistance of others. The observations of Blandau and Odor (1949) reveal that a high percentage of sperm that are introduced into the female genital tract in normal copulation fails to arrive at the site of fertilization, and this indicates that factors other than the amount of hyaluronidase available in the vicinity of the egg are responsible for the success or failure of fertilization.

The possibility that factors other than hyaluronidase may be concerned in sperm penetration through the cumulus oophorus in the rabbit is suggested by the observation of Swyer (1947d) that a tubal factor in this animal disperses the corona radiata - a layer of densely packed follicle cells which frequently remains intact when the cumuli are placed in rabbit sperm suspensions (Pincus, 1930). In a series of experiments Swyer found that the corona radiata was not dispersed in a period of 30 hours in solutions of hyaluronidase that were sufficiently active to cause dispersal of the majority of the cumulus cells in about 25 minutes. In another series of experiments, it was found that eggs treated in hyaluronidase and then inserted into the fimbriated end of the fallopian tube were devoid of the corona radiata when recovered three hours later. Eggs that had not previously been treated with hyaluronidase were recovered intact after being left in the fallopian tube for the same period. The nature of the tubal factor could not be ascertained - tubal extracts were without action on the corona or the cumulus. It was suggested that mechanical factors may be responsible for removal of the corona radiata.

The presence of hyaluronidase in the sperm of animals other than mammals has not been unequivocally demonstrated. As mentioned above it

is absent in reptilian sperm and it is present, if at all, in very small amount in fowl sperm (Swyer, 1947a). Hyaluronidase, or a hyaluronidase-like factor has been reported in the sperm of the Mediterranean species of sea-urchin, Arbacia lixula and Spharechinus granulosa, by Ruffo and Monroy (1946) and Monroy and Ruffo (1947). These authors claim that extracts prepared from sperm of these animals, in the manner employed for extraction of hyaluronidase from bull testis, dissolve the jelly coat of eggs of the same species and also of Psammechinus microtuberculatus and Paracentrotus lividus. They also report that the sea-urchin sperm extracts lower the viscosity of a mucin solution (hyaluronic acid?) and that bull testis hyaluronidase softens, but does not dissolve the jelly coat of the eggs. Egg water is claimed not to inactivate the lytic factor in sea-urchin sperm extracts. Earlier, Runnström, Monné and Broman (1943) had reported that the jelly of sea-urchin eggs is not attacked by hyaluronidase from bull testicle. In various concentrations of the enzyme in sea-water it was found not only that the jelly was not attacked but that fertilization was not disturbed and cleavage occurred normally. In this laboratory, Tyler (unpub.) has been unable to observe any jelly-dissolving effect of sea-urchin sperm extracts, nor has he found such extracts to possess spreading power.

Egg surface lysin (sperm lysin)

A methanol-soluble factor, extractable from sperm of various species of sea-urchin was found by Runnström, and Tiselius, Lindvall (1944) to be active on the surface of the egg. Its activity was demonstrated by subjecting unfertilized eggs, which had been treated with the extract, to hypertonic sea water. As the eggs shrink the surface remains smooth, in contrast to control eggs in which the surface becomes thrown into wrinkles

or folds. Maintenance of a smooth surface during shrinkage of the egg is indicative of a liquefaction of the egg cortex (Runnström, Monné and Broman, 1943). The active factor in the methanol extracts was found to be dialyzable, not attacked by trypsin, slightly inactivated by treatment with N HCl at 100° for 30 minutes, and capable of causing sphering of mammalian erythrocytes. The latter property was shared by a methanol-soluble factor from salmon sperm. In a later communication Runnström, Monné and Wicklund (1944) reported that the methanol-soluble factor from sea-urchin sperm interfered with normal fertilization membrane formation in varying degrees depending on the concentration of the extract, without inhibiting the entrance of the sperm or fusion of pronuclei. Cleavage (but not nuclear division) was more or less inhibited. Further investigations of the action of the egg surface lysin were reported by Runnström, Tiselius and Lindvall in 1945. The presence of this agent was claimed to have the following effects on eggs: (1) it prevents penetration of sperm into the egg without injury to the sperm and the reaction of oöcytes (immature eggs) to insemination is inhibited; (2) it causes unfertilized, mature eggs to shrink with a smooth surface in hypertonic sea water, and a similar effect is exerted on fertilized, uncleaved eggs; (3) it enhances cytolysis of eggs exposed to hypertonic sea water and favors the formation of surface blisters of lipid nature; (4) it makes eggs more sensitive to cytolysis by hypotonic sea water; (5) it causes eggs to fragment more readily upon centrifugation; (6) it inhibits the usual change in form of oöcytes immersed in hypertonic sea water; (7) it prevents gastrulation more or less completely, but promotes the animalization of isolated animal halves. Two detergents, Duponol and Dupont QB, and honey bee venom in appropriate concentrations, were found

to imitate the action of the sperm factor. An extract with similar effects was obtained from sea-urchin eggs. It was suggested that the changes conferred upon the egg surface by the lysin indicate that it is in some manner involved in the natural mechanism which prevents polyspermy. Support for this hypothesis was claimed on the basis that effects similar to those produced by the methanol extracts were obtained with seminal fluid obtained by centrifugation of concentrated sperm suspensions and that an analogous factor is extractable from the eggs.

Another report published by Runnström and Monne (1945) describes in considerable detail the influence of various conditions on the rate of smoothing of mature unfertilized sea-urchin eggs, and a theoretical discussion of the possible role of the egg surface lysin in fertilization is presented. With respect to factors influencing smoothing of the egg surface in hypertonic medium, it was found that this reaction is favored by elevated pH, sheep serum, KCN, sodium azide, absence of both Ca and Mg (but not of either alone), and the egg surface lysin. Additionally, it was claimed that activation of mature unfertilized eggs is accompanied by the same kind of cortical reaction that is observed as a result of treatment with the egg surface lysin. As mentioned above, this reaction is interpreted by these workers as a liquefaction of the cortex. They suggest that surface liquefaction may correspond to a first step in activation that involves the release of a surface-liquefying egg-factor (egg surface lysin of the egg). The egg factor is considered to affect primarily the vitelline membrane, and complete activation may involve a spreading of the factor to the underlying plasma membrane. In a later paper (1946) Runnström and Lindvall present evidence to show that the methanol-soluble egg surface lysin inhibits sperm agglutination by egg

water. On the basis of this, and the earlier evidence cited above - principally the promotion of smooth shrinkage in lysin-treated eggs subsequently exposed to hypertonic medium, and inhibition of fertilization by treatment of eggs with the lysin - these authors suggest that the mechanism of the block to polyspermy may consist of cortical liquefaction induced by release of egg surface lysin by attachment of the (first) spermatozoon. Other spermatozoa are thereby prevented from becoming attached to the egg surface. This hypothesis has been criticized by Tyler (1948), who points out that supernumerary spermatozoa do attach to the egg surface and remain attached (?) after fertilization.

Some inferences concerning the mechanism of the cortical liquefaction induced by egg surface lysin, bee venom and detergents, have been made by Runnström and his co-workers. The surface of the egg was shown by Runnström (1923, 1924, 1928) to contain a lipid layer. Danielli and Harvey (1938) proposed that the plasma membrane consists of a film of lipid molecules with protein molecules adsorbed at the interfaces. Ohman (1944) suggests that in the lipo-protein film (plasma membrane) the peptide chains of the protein constituents are oriented parallel to the egg surface, with polar side chains directed toward the water and lipophilic side chains directed inward and imbedded among the hydrocarbon chains of the lipid molecules. Unpublished experiments of Cheesman (cited by Runnström and Lindvall, 1946) showed that the egg surface lysin penetrates lipo-protein films, causing an expulsion of part of the protein molecules and in this process liquefying the film. Bee venom, owing to its content of lecithinase attacks the phosphatides of the egg surface (cf. Runnström, Monne and Wicklund, 1946; Ohman, 1944) causing separation of lipids from the cell structure, which leave the egg in the form of

hyaline blisters. Egg surface lysin, according to Runnström, Monné and Wicklund (1946) acts in a manner essentially similar to that of bee venom. The action of detergents on the egg surface is likewise attributed to their ability to penetrate lipo-protein films and cause disruption of the lipo-protein complex. It is not clear from the published statements of Runnström and his co-workers whether they consider the action of the egg surface lysin to be due to the presence in the material of a lecithinase-like, or of a detergent-like substance. According to the work of Cheesman, cited above, comparison of liquid-expanded films of the lysin formed at an air-water interface and films formed by it at an oil-water interface suggested that at least two, and sometimes three, surface-active substances were present. These had, respectively, the surface properties of a phosphatide, a long-chain detergent and a fatty acid.

It is of interest that Runnström has recently (1947) reported that in order to obtain sufficient quantities of the egg surface lysin it has been necessary to resort to fish spermatozoa. Mackerel sperm was used as the source material. The lysin extracted from fish sperm is considered identical with the methanol-soluble factor obtained from sea-urchin sperm and eggs. It appears, therefore, that this agent is not species-specific.

Egg membrane lysin of marine molluscs

In 1939, Tyler reported that extracts of sperm of the giant keyhole limpet, Megathura crenulata, contain a lytic agent which dissolves the membrane that is normally present about the unfertilized egg of this animal. Abalone (Haliotis cracherodii) sperm was also found to yield a lysin which acts on the egg membrane of this species. Cross-lysis between limpet and abalone was not observed. Von Medem (1942) confirmed the presence of an egg membrane lysin in sperm extracts of other species

of keyhole limpet and abalone. Berg (1949) has reported that sperm of the mussel, Mytilus californianus, contain a lytic agent that dissolves the egg membrane and, additionally, may dissolve material that normally holds together the blastomeres of the cleaved egg. It was noted by Berg that if fertilized eggs are allowed to remain in a solution of the lysin the blastomeres round up and become almost separated. This effect always parallels the egg membrane lysis. No other reports concerning the occurrence of egg membrane lysins in molluscs have come to the attention of the writer. A brief summary of observations concerning the action and properties of the keyhole limpet egg membrane as reported by Tyler (1939) will conclude this literature review.

The eggs of the giant keyhole limpet, when they are shed, are somewhat irregular in shape and have a rather thick membrane closely adherent to the surface. Outside of the membrane is a jelly layer which slowly swells in sea water to attain a diameter somewhat greater than the diameter of the egg. As the unfertilized eggs stand in sea water the membrane separates from the surface and after about 20 to 30 minutes in sea water it is fully raised leaving a space equal to about one-tenth of the egg diameter between it and the surface of the egg (see figure 11a).

When the eggs are placed in a concentrated sperm suspension the membrane is observed to disappear (figure 30). The spermatozoa penetrate the jelly rapidly and adhere to the membrane. When a sufficient number have accumulated the membrane begins to get thinner and lifts off further from the surface of the egg. It then buckles in, particularly in the region where there is the greatest accumulation of sperm. Presently is vanishes in that region while it continues to expand and thin out elsewhere. Finally it disappears completely.

Extracts containing an active lytic agent were obtained by freezing and thawing a suspension of the sperm in sea water. Membrane dissolution by an extract prepared in this way proceeds in essentially the same manner as when living spermatozoa are employed (figure 11).

Time required for complete dissolution of the egg membrane provides the basis for assay of lytic activity of extracts. The rate of dissolution was found to bear an approximate linear relation to dilution of an extract. The rate of dissolution was also influenced by the jelly coat of the eggs. In the absence of jelly it was found that membrane dissolution may occur as much as $7\frac{1}{2}$ times faster. The presence of the jelly layer apparently hinders diffusion of the agent.

Preliminary attempts to characterize the egg membrane lysin showed that it is non-dialyzable and can be completely precipitated from the crude extract with ammonium sulfate. Material thus precipitated and dialyzed was tested by the usual protein tests and, except for the Hopkins-Cole, all were positive. Incubation with crystalline trypsin and chymotrypsin resulted in inactivation of the lysin. It was also found that alcohol precipitates and at the same time inactivates the lytic agent, and that in distilled water its activity is soon lost. The egg membrane lysin was found to be heat-labile - 2 minutes at 60° sufficed to inactivate it.

Associated with the lytic agent in the extracts of frozen-thawed sperm was antifertilizin, which was precipitated with the lysin upon addition of ammonium sulfate. Unlike the lysin, however, the antifertilizin is relatively heat-stable, so that heating an extract to 60° for 2 minutes was without apparent effect on it. On the basis of this observation, it was suggested that both antifertilizin and the heat-labile

agent may be concerned in the lytic action, one (heat-labile component) acting in the manner of complement.

The lytic action of sperm extracts appeared to be confined to the egg membrane, since no particular effect was noted on the development of fertilized eggs whose membranes had been dissolved (see figure 3), and freshly shed unfertilized eggs whose membranes had been lysed fertilized as readily as untreated eggs. It was suggested, somewhat indirectly, that complete dissolution of the membrane is not a necessary prerequisite to fertilization. It was concluded that the lytic agent appears simply to be concerned in the penetration part of the fertilization process.

EXPERIMENTS WITH HYALURONIDASE AND SEA-URCHIN FERTILIZIN

1. A mucin clot reaction with sea-urchin fertilizin

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Introduction

Recent work on the fertilizins (the sperm-agglutinating constituents of egg water) of the eggs of sea-urchins and other animals has shown them to be of the nature of mucoproteins. Tyler and Fox (1939, 1940) showed that the fertilizins of Strongylocentrotus and of Megathura possess protein characteristics, but are of low nitrogen content. Similar evidence has been obtained with Arbacia fertilizin by Kuhn and Wallenfels (1940) and with Psammechinus fertilizin by Runnström, Tiselius, and Vasseur (1942). The latter workers also obtained a positive carbohydrate test. Tyler (1948) reported the presence of reducing sugars to the extent of about 15 per cent in hydrolyzed, purified preparations of Strongylocentrotus fertilizin and identified galactose as one of the constituents. As will be shown later in this paper, the present author has found hexosamine to be present in amounts equivalent to about 2 per cent of the original material. According to Runnström, Tiselius, and Vasseur (1942) and Tyler (1946) the sea-urchin fertilizins are of pronounced acidic character.

Acidic mucopolysaccharides are known (cf. Meyer, 1945, and Stacey, 1946) to co-precipitate with proteins upon acidification of the native fluid or of the neutral extracts in the form of "mucin clots," stringy or granular precipitates, depending upon the conditions of precipitation. This reaction is given, for example, by hyaluronic acid (Meyer and Palmer, 1936) and has been used in the assay of the enzyme hyaluronidase (McClean, 1943). It was of interest to determine whether or not fertilizin preparations would give the mucin clot reaction. As the work reported here shows, fertilizin preparations do give such a mucin clot reaction. A titration method, based upon this, was developed for these preparations, and comparisons made with their sperm-agglutinating activity in untreated condition, after dialysis, and after exposure to heat and to ultra-violet irradiation.

Materials and Methods

The sea-urchins *Lytechinus pictus, Strongylocentrotus purpuratus* and *S. franciscanus* were used in these experiments. Most of the work was done with *S. purpuratus.*

Two kinds of fertilizin preparations were employed. One, which will be termed "crude fertilizin," was prepared by acidifying a 20 per cent suspension of washed eggs to pH 3–3.5, removing the supernatant fluid after five or ten minutes and readjusting the pH of this solution to 7–7.5. The other, which will be termed "purified fertilizin," was further subjected to alkali precipitation, dialysis against 3.3 per cent acid saline (pH 3.5–4) and alcohol precipitation according to the method described by Tyler (1948). Material prepared in this manner has been

found to be electrophoretically homogeneous (Tyler, unpub.). For the various tests the solutions were made up in 3.3 per cent NaCl at a pH of about 7.

Sperm-agglutinating titer of the fertilizin preparations was determined using the drop method of preparing two-fold serial dilutions with sea water in Syracuse watch glasses. To two drops of each dilution of fertilizin solution one drop of a uniform sperm suspension, usually 1 per cent (calculated as 1 cc. dry sperm per 100 cc. sea water suspension), was added. The highest dilution in which agglutination is observable under the microscope gives the titer of the preparation.

Tests for univalent fertilizin were made according to the method described by Tyler (1941) and Metz (1942). Essentially, this method consists of first treating sperm with the solution containing univalent fertilizin, which does not agglutinate the sperm, and then adding an equal volume of strong normal fertilizin solution to the suspension of sperm. Failure of the sperm to be agglutinated by the normal fertilizin presumably indicates that the combining groups on the sperm surface have been occupied by univalent fertilizin groups and are no longer available to unite with the normal fertilizin. Univalent fertilizin was obtained by heating and by irradiation with ultra-violet light of normal fertilizin preparations (cf. Tyler, 1941, and Metz, 1942).

Bovine serum albumin prepared by the Armour laboratories was used in 1 per cent solution for the co-precipitation tests.

Hyaluronic acid was obtained from human umbilical cords according to the method described by McClean (1943), whereby the distilled water extract of acetone-dried, ground cords, extracted with 90 per cent acetic acid according to the method of Meyer and Palmer (1936), was precipitated with 1.25 volumes of cold, potassium acetate-saturated 95 per cent alcohol. The precipitate was washed with alcohol, acetone, and ether and dried over P_2O_5. The dry product was dissolved in distilled water as required; a solution of 0.1–0.2 per cent of the dry material was clear, viscous and did not form a precipitate upon the addition of acetic acid, but co-precipitated with serum albumin in the presence of acetic acid, forming a stringy clot. In higher dilutions the mixture of serum albumin, acetic acid and hyaluronic acid solution resulted in the formation of a fine precipitate or turbidity. The highest dilution in two-fold serial dilutions in which turbidity was perceptible by visual inspection was taken as the titer of the hyaluronic acid solution.[1]

Experiments and Observations

Co-precipitation of fertilizin with serum albumin in acid solution

A viscous solution of crude fertilizin of *Lytechinus pictus* was prepared as described above and combined with a 1 per cent solution of bovine serum albumin in 0.9 per cent NaCl and 2 N acetic acid according to the method described by McClean (1943) in the mucin clot test. With this solution a very large clot was formed similar in character and appearance to the clot formed by hyaluronic acid. Purified fertilizin preparations of *Strongylocentrotus purpuratus, S. franciscanus,* and *L. pictus* were tested in the same manner and in each case a clot or precipi-

[1] A quantity of pure potassium hyaluronate was later supplied to me by the Schering Corporation, through the courtesy of Dr. W. Alan Wright and Dr. Erwin Schwenk.

tate formed, depending upon the concentration of the solution. In Figure 1 a series of photographs of the mucin clot reaction of *S. purpuratus* fertilizin is shown, with the reaction given by hyaluronic acid for comparison.

By taking advantage of the biological activity of fertilizin, i.e., its sperm-agglutinating activity, a simple test was performed which provided definitive evidence that it is the fertilizin which is co-precipitated with serum albumin and not some hitherto undetected component of the material.

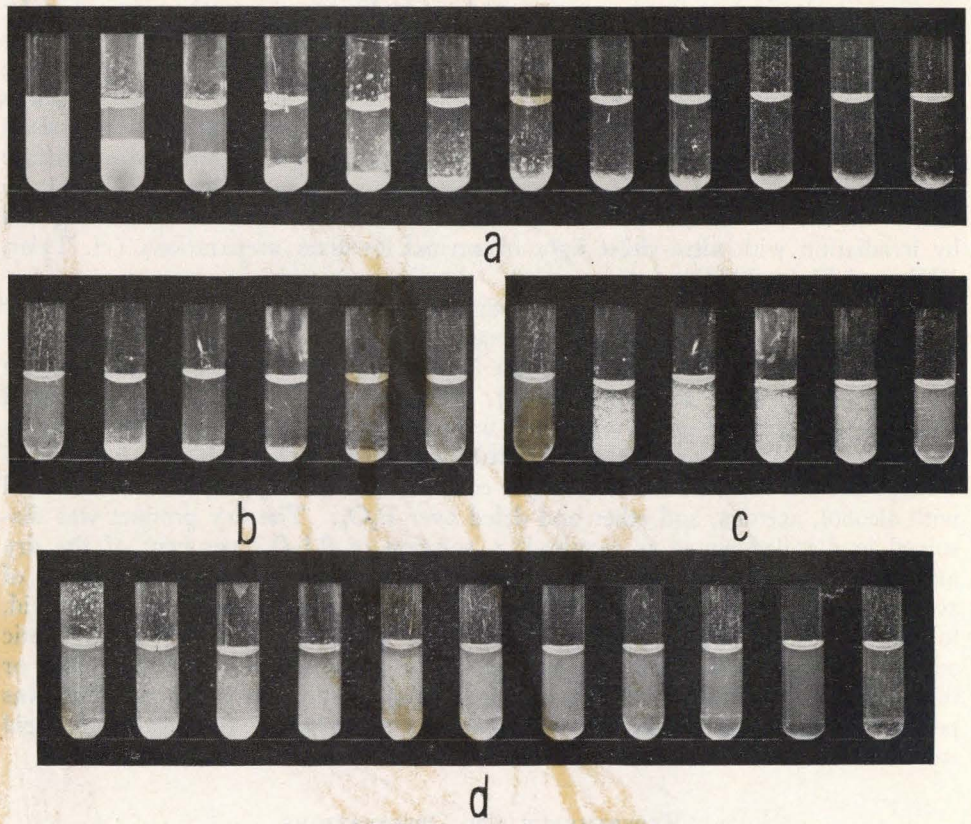

FIGURE 1. Mucin clot reactions of *Strongylocentrotus purpuratus* fertilizin and of hyaluronic acid in two-fold dilutions. **a.** Purified *S. purpuratus* fertilizin. The first tube contains fertilizin solution and serum albumin, but distilled water instead of acetic acid. The opacity of the first tube is due to the opalescence of the mixture. **b.** Crude *S. purpuratus* fertilizin. The first tube contains fertilizin solution and acetic acid but 0.9 per cent saline instead of serum albumin. **c.** The same as **b** with the tubes shaken prior to being photographed. **d.** Hyaluronic acid.

A solution of purified fertilizin in 3.3 per cent saline was mixed with serum albumin and the pH brought to about 3.5 with 2 N acetic acid. The resulting precipitate was thrown down by centrifugation, the supernatant withdrawn and its pH adjusted to 7. As shown in Table I, the supernatant exhibited no sperm-agglutinating activity.

TABLE I

Sperm-agglutinating activity of the supernatant and of the precipitate recovered separately after the addition of bovine serum albumin to an acidified solution of purified fertilizin, and results of control tests

	Reaction mixture	Per cent of sperm-agglutinating activity*
0.5 ml. fertilizin in 3.3% saline	0.05 ml. distilled H_2O 0.2 ml. 0.9% saline	100
	0.05 ml. 2 N acetic acid 0.2 ml. 1% bovine serum albumin in 0.9% saline	Supernatant: 0 Dissolved precipitate: 100
	0.05 ml. distilled H_2O 0.2 ml. 1% bovine serum albumin in 0.9% saline	100
	0.05 ml. 2 N acetic acid 0.2 ml. 0.9% saline	100
	0.05 ml. distilled H_2O 0.2 ml. 0.9% saline, acidified and neutralized	100
0.5 ml. 3.3% saline	0.05 ml. 2 N acetic acid 0.2 ml. 1% bovine serum albumin in 0.9% saline	0

* Sperm-agglutinating activity of fertilizin, distilled water and 0.9% saline mixture taken as 100%.

It had previously been found that the precipitate formed by the addition of serum albumin to a fertilizin solution acidified to a pH of about 3.5 dissolves completely at a pH of 5.6 or higher. After centrifugation and withdrawal of the supernatant of the material being tested, the precipitate was resuspended in 3.3 per cent NaCl solution, its pH brought to about 7, and the volume made equal to that of the original mixture. Upon testing this solution it was found, as is shown in the table, that the sperm-agglutinating activity was equal to that of the original fertilizin solution, showing that the activity was recovered quantitatively from the precipitate.

A number of control tests were made, the results of which are summarized in Table I. These showed that (1) co-precipitation of fertilizin and serum albumin does not occur in the absence of acid; (2) it does not occur in acidified solution in the absence of added protein; (3) the presence of albumin does not affect the sperm-agglutinating activity of the fertilizin; (4) the sperm-agglutinating activity is not affected by acidification and subsequent neutralization of the solution, and (5) sperm agglutination does not occur in saline solution in the absence of fertilizin, nor is a precipitate formed when albumin and acid are added to saline.

Quantitative recovery of the sperm-agglutinating activity from co-precipitated fertilizin and serum albumin, which is achieved by the simple expedient of raising the pH to 5.6 or higher, shows that the specific combining groups of the fertilizin are not irreversibly altered by its reaction with the albumin. The significance of this phenomenon cannot be evaluated at the present time, however, since essentially nothing is as yet known of the structure of the fertilizin molecule.

Titration by the mucin clot method

The method employed for titration was as follows: Two-fold serial dilutions of fertilizin in 3.3 per cent NaCl at about pH 7 are made in 10 × 75 mm. test tubes in 0.5 ml. quantities. To each tube 0.05 ml. of 2 N acetic acid is added and the contents mixed. The tubes are inclined and 0.2 ml. of 1 per cent bovine serum albumin in 0.9 per cent saline is slowly pipetted down the side of each tube; after this the tubes are carefully returned to a vertical position and in most cases a ring of precipitate forms immediately at the zone of contact between the albumin solution and the acidified fertilizin solution. The rings are easily detected at high dilutions in which a diffuse turbidity is difficult to detect and score visually. The reactions are read at once in good natural light, since the rings tend to disperse rapidly as the albumin diffuses through the mixture. The highest dilution at which a ring is observed is taken as the mucin clot titer of the preparation.

Effect of pH, albumin concentration and salt concentration

Numerous titrations with the same stock samples of purified fertilizin using the method described above have given consistently identical mucin clot titers in the course of routine testing. It was thought advisable, however, to carry out some controlled tests to determine in a more definitive manner the amount of variability in titer to be expected within a rather limited range of pH, albumin concentration, and salt concentration.

A sample of a purified fertilizin solution from eggs of *S. purpuratus* was centrifuged at 3000 r.p.m. for five minutes. A slight sediment was thrown down, the clarified supernatant was drawn off, and the pH adjusted to 7.0 with the glass electrode. To assure maximum uniformity of different samples of the supernatant, it was thoroughly mixed before removing an aliquot. Two-fold dilutions were made in 0.5 ml. quantities with 3.3 per cent NaCl solution. Serum albumin was made up in 1 per cent solutions, and the same stock solution of 2 N acetic acid was used throughout. Various amounts of the latter two solutions were added to different sets of tubes of the fertilizin dilutions. The total albumin and salt concentration in the different sets was thus altered, as noted below. The pH was measured with a Beckman glass electrode pH meter. After each titration the pH of the final mixtures was measured in the first tube, in the tube giving the end-point, and in an intermediate tube. The maximum difference in pH observed within any set was 0.26 unit. The results obtained in a series of nine titrations are given in Table II. Each of the pH values listed represents the mean of three determinations in each set. The total salt concentration is expressed as per cent NaCl. The mucin clot titers (last column of table) are the end points of visible precipitation as determined by the ring method described above.

As the data in the table show, no marked difference in mucin clot titer occurs, for the most part, as a result of the differences in pH, total salt and albumin concentrations employed. In titration 5, the observable end-point would probably have been at least one dilution higher had rings formed. The failure of rings to form is attributable to the relatively large quantity of serum albumin solution used; as noted above, in high dilutions a diffuse turbidity such as was produced in this case is more difficult to detect visually than a ring at the same dilution. It has

been found that 0.5 ml. of albumin solution is about the largest quantity practicable for obtaining consistent ring formation. Since there is always a tendency to pipette too rapidly when a large number of tests is being performed, it has proven more convenient to use 0.2 ml. of albumin solution.

The evidence presented in Table II indicates that pH is not a critical factor with respect to observed titer within the range tested. The albumin concentration is not critical, nor is the total salt concentration of the system in the range from 2.0 per cent to 3.1 per cent. Below 2.0 per cent the salt concentration may be a more important factor, as shown by the higher titer obtained in titration 6. Although the total salt concentration in titration 5 is the same as that in number 6 (1.6 per cent), the two sets are not comparable for the reason mentioned above.

TABLE II

Mean pH, total albumin concentration, total salt concentration and mucin clot titers in nine titrations using uniform samples of a homogeneous purified fertilizin preparation of S. purpuratus

Titration no.	Ml. 2 N acetic acid	Ml. 1% albumin solution	NaCl concentration of albumin solution, per cent	Mean pH	Total albumin concentration, per cent	Total NaCl concentration, per cent	Mucin clot titer
1	0.05	0.1	0.9	3.09	0.15	2.7	64
2	0.05	0.2	0.9	3.30	0.27	2.4	64
3	0.05	0.3	0.9	3.33	0.35	2.3	64
4	0.05	0.5	0.9	3.54	0.47	2.0	64
5	0.05	1.0	0.9	3.74	0.65	1.6	32*
6	0.05	0.5	0.0	3.58	0.47	1.6	128
7	0.05	0.5	3.3	3.58	0.47	3.1	64
8	0.01	0.2	0.9	3.88	0.28	2.6	64
9	0.02	0.2	0.9	3.78	0.28	2.5	64

* Scored as turbidity, not as ring.

In the next section it will be shown that the mucin clot and sperm-agglutinating titers of a fertilizin solution are both reduced after dialysis of the preparation against distilled water with consequent removal of salt (NaCl). The data to be presented indicate that the physical state of the fertilizin is reversibly modified in the absence of electrolytes, at least under certain conditions of temperature. It seems likely, therefore, that there may exist some optimum concentration of electrolytes between zero concentration and that represented by 2.0 per cent NaCl (equivalent to an ionic strength of 0.34) at which co-precipitation of fertilizin and serum albumin attains a maximum. Further analysis of the effect of salt concentration on the co-precipitation of fertilizin and serum albumin is under way and will be reported in a later communication.

In the system as employed in the routine titration procedure in the present investigation, however, the mean pH, total albumin and total salt concentration fall well within the range for each of these factors in which identical titers are obtained, using uniform samples of a homogeneous fertilizin preparation.

Mucin clot and sperm-agglutinating titrations of fertilizin in salt-free solution

A sample (I) of a stock preparation of purified fertilizin in 3.3 per cent saline was made salt-free by dialyzing against distilled water at 1° C. until it no longer formed a precipitate with $AgNO_3$. It was found that a white, flocculent material was present in the dialyzed solution, whereas no such flocculence was evident in the control in 3.3 per cent NaCl solution which was kept at 1° for the same period. A considerable reduction in both mucin clot and sperm-agglutinating titer of the salt-free sample as compared with the control was observed. In the mucin clot titration, the character of the precipitate formed in the salt-free preparation upon the addition of serum albumin in the presence of acid differed from that in the control in being of larger particle size and somewhat stringy. Difficulty was encountered in making sperm-agglutinating titrations (where the salt content of the preparation was adjusted just prior to titration to 3.3 per cent NaCl by adding an equal volume either of 6.6 per cent NaCl or of double sea water to the salt-free solution) in cases where suspended material was present. The spermatozoa clumped about the particulate matter, and it was impossible to score the dilutions satisfactorily. Where tests were made with samples of the dialyzed solution in which the amount of suspended material was visibly less than was originally present, both the mucin clot precipitation and sperm agglutination occurred in the manner characteristic of the control solutions; in these cases the two titers were also somewhat higher, although they did not necessarily equal the values obtained for the control solution.

A second sample (II) of the same stock preparation of purified fertilizin was dialyzed against distilled water until salt-free. This time the first five changes of the water used for dialysis, totaling 3 liters, were saved, combined, and lyophilized to dryness. The residue was taken up in about 10 cc. of distilled water and the resulting solution was approximately isotonic with sea water, as shown by the fact that sperm of *S. purpuratus* remained active when placed in it. This "dialysate-concentrate" was 300 times more concentrated than the original dialysate, but proved to be negative for both mucin clot formation and sperm-agglutinating activity. Sample II behaved in all respects like sample I. The results of tests with the two samples are summarized in Table III.

The evidence obtained from the present experiments indicates that at temperatures near the freezing point (1° C.) the physical state of fertilizin can be reversibly modified by the removal of electrolytes. Macroscopic aggregates may appear in a fertilizin preparation under these conditions, and there is a correlated decrease in the mucin clot and sperm-agglutinating titers. Under the influence of added salt and elevated temperature (up to 21.5° C.), either separately or combined, there occurs a correlated decrease in the amount of visible macroscopic material and increase in mucin clot and sperm-agglutinating titers to values approaching those obtained with control solutions. The negative results of tests with the "dialysate-concentrate" show that there was no actual loss of fertilizin during dialysis.

Analysis of a purified fertilizin preparation

The co-precipitation of fertilizin with protein in acidic solution in a manner analogous to the behavior of acid mucopolysaccharides suggests affinity of fertilizin with this class of substances. It has recently been claimed, moreover, that a preparation

TABLE III

Results of titrations of salt-free fertilizin preparations of S. purpuratus after various treatments

Test no.	Fertilizin preparation	Treatment after removal from dialysis bath at 1° C.	Sperm-agglutinating titration		Mucin clot titration	
			pH	Titer	pH	Titer
1	I	None			5.2	8
2	I	Salt content adjusted to 3.3% NaCl. pH adjusted	7.2	64		
3	I	Salt content adjusted to 3.3% NaCl			5.2	16
4	I	2.5 hrs. at 21.5° C.			5.2	16
5	I	2.5 hrs. at 21.5° C. pH adjusted			7.2	16
6	I	Dialyzed vs. 3.3% NaCl at 1° C. 2 hrs. at 21.5° C. pH adjusted	7.0	64	7.0	16
7	I	Dialyzed vs. 3.3% NaCl at 1° C. 24 hrs. at 8° C. pH adjusted	7.45	1024	7.45	32
8	I	Control. Not dialyzed vs. distilled water. pH adjusted	7.4	512	7.4	64
9	II	1 hr. at 21.5° C. pH adjusted			6.9	32
10	II	Salt content adjusted to 3.3% NaCl. 3 hrs. at 21.5° C. pH adjusted	6.9	512		
11	II	Salt content adjusted to 3.3% NaCl. 1 hr. at 21.5° C. pH adjusted			6.9	64
12	II	Control. Not dialyzed against distilled water. pH adjusted	7.3	1024	7.3	128

from bull testes, presumably containing the enzyme hyaluronidase, is capable of causing the jelly of intact sea-urchin eggs to swell (Ruffo and Monroy, 1946; Monroy and Ruffo, 1947). It was of interest, then, to attempt to determine the extent to which fertilizin may be chemically similar to hyaluronic acid. The few available data, which have been reviewed briefly in the introductory section, indicate that fertilizin is by no means identical with hyaluronic acid. The present investigation has shown that fertilizin differs from hyaluronic acid to a marked degree with respect to the two chief constituents of the latter substance, hexosamine and glucuronic acid.

A homogeneous sample of a purified fertilizin preparation in 3.3 per cent NaCl was prepared as described above. In the present instance the supernatant fluid was filtered through hardened filter paper. The pH of the filtrate was adjusted to 7 and

TABLE IV

Results of chemical analysis of a purified fertilizin preparation of S. purpuratus

Filtrate					Hydrolysate	
Sperm-agglutinating titer	Mucin clot titer	Dry weight, mg./ml.	Total nitrogen, per cent	Glucuronic acid	Hexosamine, per cent	α-amino acids
2048	512	8.95	4.1	none	1.6	positive Ninhydrin

mucin clot and sperm-agglutinating titers were obtained. Aliquots of the filtrate were taken for the various analyses, which included determinations of dry weight, total nitrogen, hexosamine, α-amino acids and glucuronic acid. The results of the analyses are presented in Table IV. Dry weight per ml. was calculated from the weight of material precipitated from an aliquot of the filtrate with 1.25 volumes of cold 95 per cent alcohol. According to Tyler (1948), precipitation of fertilizin is complete under these conditions. The precipitate was washed with alcohol and dried in an oven at 55° C. to constant weight. Total nitrogen was determined for duplicate samples of the dried precipitate by the micro-Kjeldahl method. Another portion of the dried material was hydrolyzed by boiling in a sealed tube with 4N HCl for eight hours. Hexosamine was determined in an aliquot of the hydrolysate by the method of Palmer, Smyth and Meyer (1937). Another portion of the hydrolysate was treated with Ninhydrin reagent for the determination of α-amino acids. For the determination of glucuronic acid the colorimetric method recently described by Dische (1947b) was employed, using a sample of the original filtrate,

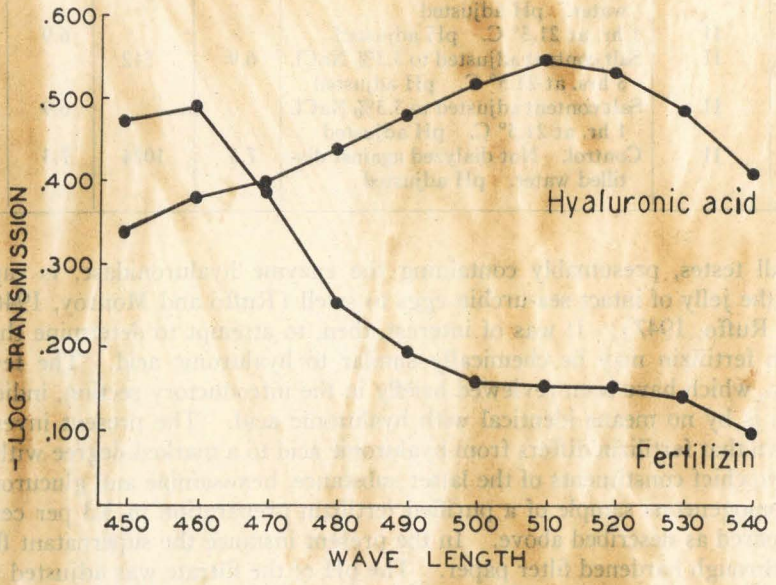

FIGURE 2. Absorption curves of reaction mixtures of mannose-thioglycolic acid-fertilizin and mannose-thioglycolic acid-hyaluronic acid. Wave lengths in mμ. Fertilizin $\Delta E_{(510-480)} = -0.097$; hyaluronic acid $\Delta E_{(510-480)} = +0.115$.

and for comparative purposes, a solution of pure potassium hyaluronate (Schering) was tested at the same time by the same method. According to Dische, the reaction of carbohydrates with -SH compounds in H_2SO_4, which differentiates between various classes of carbohydrates and individual hexoses and hexuronic acids, is highly characteristic for glucuronic acid when mannose is employed. This reaction is the basis of the test. The reaction mixture with glucuronic acid gives a typical absorption curve in the range 450–540 mμ, and it was found by Dische that the curve for hyaluronic acid is almost identical with that of glucuronic acid. In practice, accord-

ing to Dische, it is only necessary to measure the intensity of the mannose reaction at 510 and 480 mμ and subtract the second value from the first. This difference is positive for glucuronic acid and polyglucuronides, and negative for the other hexuronic acids. Figure 2 shows the absorption curves for fertilizin and for hyaluronic acid. The difference between the intensity of the mannose reaction with fertilizin at 510 and that at 480 mμ is negative, and hence it may be concluded that fertilizin does not contain glucuronic acid. This result is in agreement with previous results obtained by Tyler (unpub.) using an earlier method of Dische's (1947a). As shown in Table IV, fertilizin does contain hexosamine, but in small amount, which is in agreement with earlier results obtained by the present author using Kunitz's (1939) method. The Ninhydrin reaction was very weak but probably positive. Total nitrogen (4.1 per cent) of this material is somewhat lower than has been reported previously for *S. purpuratus* fertilizin by Tyler and Fox (1940), who found an average total nitrogen content of 5.2 per cent with crude preparations.

These data show that fertilizin differs markedly from hyaluronic acid in its chemical constitution. It is obvious, therefore, that the ability to give the mucin clot reaction does not by any means indicate close similarity between fertilizin and hyaluronic acid, even though it may be evidence that the former is related to the group of acidic mucopolysaccharides.

At the suggestion of Dr. Albert Tyler, the mucin clot titration procedure was used in conjunction with sperm-agglutinating titrations to investigate the effects of various kinds of treatment on fertilizin. In the following sections the results of parallel titrations of preparations subjected to heat and to ultra-violet irradiation are presented.

Parallel titrations with heat-treated fertilizin solutions

According to Tyler and Fox (1940), the sperm-agglutinating activity of *Strongylocentrotus purpuratus* fertilizin is rapidly destroyed at 100° C. The rate of inactivation, according to these authors, is a function of the pH, the fertilizin being most stable in the range from 4 to 7. Their data show that at pH 7.3 the agglutinin is 95–100 per cent inactivated in 20–30 minutes at 100°. In the present experiments in which *S. purpuratus* purified fertilizin solutions were used, the preparations have proven to be considerably more heat-stable than the material used by Tyler and Fox. Since the solutions employed by Tyler and Fox corresponded to crude fertilizin as defined in this paper, it may well be that the relatively purer condition of the fertilizin in the present preparations accounts for its greater stability.

Initial loss of agglutinating activity does not appear to involve complete destruction of fertilizin. At first the agglutinating fertilizin is converted into a "univalent," non-agglutinating form (cf. Tyler, 1941, Metz, 1942). It was of interest, accordingly, to test samples of heat-treated fertilizin for their univalence (inhibition) titer as well as for their sperm-agglutinating and mucin clot titers. The method employed for detecting univalent fertilizin has been briefly described in an earlier section of this paper; determination of inhibition titer consists in determining the greatest dilution in which no agglutination occurs upon the addition to the test dilutions of equal amounts of normal (untreated) fertilizin solution (Metz, 1942).

In the first experiments 1.5 ml. samples of the stock purified fertilizin solutions were placed in 13 × 100 mm. test tubes and immersed in a boiling water bath. The

exposed portions of the tubes were cooled by means of a stream of air so that heating could be continued for long periods without appreciable loss of fluid. Since sperm are quickly inactivated in even slightly hypertonic medium, the fact that the sperm remained active in the solutions that had been heated was assumed to indicate that evaporation of water from the tubes during heating was insignificant. A thermometer placed in the water bath with the tubes showed that the temperature of the bath fluctuated between 96° and 98° C.

In later experiments a quantity of fertilizin solution large enough to permit the withdrawal of a number of 1.5 ml. samples was placed in a flask with a reflux condenser attached by means of a ground glass joint. The solution was refluxed and loss of water was thus kept to a minimum. The temperature of the boiling fluid in the flask could be assumed to be about 100° C. A considerable excess of solution was used so that its concentration would not be significantly affected by the slight amount of water that failed to run back down. Before withdrawing a sample, the neck of the flask and the lower part of the condenser were cooled with cold water from a wash bottle. In all of the experiments the pH of the fertilizin solutions was adjusted with the glass electrode just before heating was begun; a control sample was allowed to stand at room temperature throughout the total time of heating. As each sample was removed from the water bath or from the reflux flask,

TABLE V

Results of parallel titrations of heat-treated, purified fertilizin preparations of S. purpuratus

Fertilizin preparation	Initial pH	Sample	Temperature, degrees Cent.	Time in hours	Final pH	Titer		
						Sperm agglutination	Mucin clot	Inhibition
I*	7.1	a	96–98	0.5	—**	256	64	—**
		b	96–98	1.0	—	64	64	—
		Control	room	1.0	—	512	64	—
II*	7.85	a	96–98	4.5	—	16	64	—
		Control	room	4.5	—	256	64	—
III*	7.4	a	96–98	3.5	—	128	256	0
		b	96–98	5.5	—	64	64	32
		Control	room	5.5	—	1024	256	—
IV***	7.5	a	100	2.0	7.5	4096	512	—
		b	100	3.0	7.2	4096	512	—
		c	100	5.0	7.1	2048	256	—
		d	100	6.0	7.1	2048	256	0
		e	100	7.5	7.1	1024–2048****	256	0
		Control	room	7.5	6.8	4096	512	—

* Individual 1.5 ml. samples heated in water bath.

** Dashes indicate not tested. Zero inhibition titer indicates tested but inhibition not detected.

*** Samples (1.5 ml.) withdrawn from refluxed solution.

**** A trace reaction probably present in the higher dilution.

it was placed immediately in the freezer. In the later experiments the pH of each heated sample was recorded before it was frozen. The titrations were performed as soon thereafter as possible. The results of the experiments are presented in Table V.

As may be seen from the table, reduction of sperm-agglutinating titer by heating is not necessarily accompanied by parallel reduction in mucin clot titer. Thus, for example, samples IIa and IIIa, heated for 4.5 and 3.5 hours respectively, showed no significant reduction in mucin clot titer although the sperm-agglutinating titer of the former was reduced to about 6 per cent and that of the latter to about 12 per cent of the original values. Sample IIIb was heated for 5.5 hours with a reduction of sperm-agglutinating titer to approximately 6 per cent of its original value. In this case the mucin clot titer was reduced to 25 per cent of the original value. Samples IVa–IVe show a more nearly parallel reduction of sperm-agglutinating and mucin clot titers than any of the others. Preparation IV was refluxed. After boiling for 7.5 hours the sperm-agglutinating titer was reduced to 25–50 per cent of the original value and the mucin clot titer was reduced to about the same per cent of the original value. Sample IIIb was the only one which gave an inhibition (univalence) titer. In the samples which were tested for inhibition but in which none was detected (IIIa, IVd, IVe), it is probable that insufficient univalent fertilizin was present in the high dilutions to permit detection. The inhibition test is unambiguous only in dilutions containing sufficient univalent fertilizin to react with most of the added sperm. In the high dilutions enough sperm remain uncombined to be agglutinated upon the addition of normal fertilizin and thus obscure the slight amount of inhibition that may be present. In the present experiments, sample IIIb was the only one in which sufficient univalent fertilizin was produced in the lower dilutions to give clear-cut evidence of inhibition. Since the inhibition titer of IIIb was 32, while the mucin clot titer was 64, it appears that the mucin clot reaction of fertilizin does not depend upon maintenance of the multivalent condition. Stronger evidence to support this view was afforded by experiments in which fertilizin was irradiated with ultra-violet light.

Parallel titrations with ultra-violet irradiated purified fertilizin preparations

Metz (1942) showed that univalent fertilizin is produced by irradiation of normal (multivalent) fertilizin by ultra-violet rays. In the present experiments ultra-violet irradiation was carried out in an apparatus consisting of glass tubing, 150 × 35 mm., fitted on the mid-section of a 15 watt General Electric "Germicidal" lamp, the diameter of which is 25 mm. The major part of the output of this lamp is concentrated in the 2537 Å wave-length band. The space between the outer wall of the lamp and the inner wall of the tubing is the irradiation chamber. The chamber and lamp assembly is mounted on a motor-driven rocker. An opening in the top of the chamber, which can be closed with a rubber stopper, permits the introduction and withdrawal of fluid. The chamber is cooled by means of a small electric fan mounted on the rocker platform; when the fan is in operation the temperature of fluid inside the chamber does not rise above 35° C. during irradiation.

In the first experiment, a purified fertilizin preparation of *S. purpuratus,* the pH of which was first adjusted to 7, was irradiated for a total of 2.5 hours. It was found, as shown in Table VI, that the sperm-agglutinating titer was reduced to

TABLE VI

Results of ultra-violet irradiation of purified fertilizin

Fertilizin preparation	Sample no.	Initial pH	Time of irradiation in hours	Final pH	Sperm agglutination titer	Mucin clot titer	Inhibition titer
I	Control	7.0	—	—	256	64	—
	1	7.0	$2\frac{1}{2}$	—	4	32	—*
II	Control	7.67	—	6.60	1024	1024	—
	1	7.67	$4\frac{1}{3}$	5.52	0	1024	128
	2	7.67	6	5.49	0	1024	128
	3	7.67	$7\frac{3}{4}$	5.49	0	512	4
	4	7.67	9	5.49	0	256	4

* Univalence present in this sample by inhibition test but titer not obtained.

about 2 per cent of the original value and the mucin clot titer was decreased to 50 per cent of the original. Tested by the inhibition method, the irradiated preparation was found to contain univalent fertilizin. The inhibition titer of this sample was not obtained. In a second experiment, a quantity of the fertilizin preparation which was found to be very heat-stable with respect to its sperm-agglutinating activity (preparation IV of the preceding section) was irradiated. The pH of the solution was first adjusted to 7.7. Small portions (1.5 ml.) were withdrawn at intervals up to nine hours; the first sample was removed after 4.3 hours of irradiation. A control sample was allowed to stand in natural light (filtered through window glass) at room temperature throughout the entire period of irradiation. Immediately upon the removal of each sample from the irradiation chamber, its pH was measured with the glass electrode, and then it was placed in the freezer. All of the samples, including the control, were stored in the freezer until the titrations could be performed. As shown in Table VI, all of the irradiated samples showed complete loss of agglutinating activity. Tested by the inhibition method, all of them were found to contain univalent fertilizin. The inhibition titers showed a progressive decrease as time of irradiation was increased. The mucin clot titers also showed a progressive decrease with increased time of irradiation. After nine hours the mucin clot titer was reduced to 25 per cent of its original value, and the inhibition titer was reduced to about 6 per cent of the value found after 4.3 hours of irradiation.

The results of these experiments demonstrate conclusively that the mucin clot reaction of fertilizin does not depend upon maintenance of the multivalent condition. They also show that ultra-violet irradiation is a more effective agent than heat in converting multivalent, purified fertilizin to the univalent condition. The progressive decrease in inhibition titer found in the second experiment indicates that degradation of the fertilizin by ultra-violet light proceeds beyond the stage in which it exhibits univalence.

DISCUSSION

In general it may be said that the mucin clot titer of untreated fertilizin preparations parallels their sperm-agglutinating activity. Sperm agglutination is usually detectable in higher dilutions than is the mucin clot reaction where the latter is observed by the ring method used in the present experiments.

Destruction of the sperm-agglutinating activity of fertilizin is not necessarily accompanied by a reduction of mucin clot titer. Conversely, however, it is clear that fertilizin which has been subjected to treatment that causes a reduction in mucin clot titer, for example heating or irradiation by ultra-violet light for extended periods, will invariably show at least a parallel decrease in sperm-agglutinating activity. It has been shown in the experiments with ultra-violet irradiation that the capacity of the fertilizin to agglutinate sperm may be completely destroyed with but little, if any, loss of its ability to give the mucin clot reaction. The evidence shows that when the agglutinating (multivalent) form is degraded to the non-agglutinating (univalent) form, the latter continues to co-precipitate with protein in the mucin clot reaction. If a preparation in which all of the fertilizin has been made univalent is subjected to continued irradiation by ultra-violet light, a progressive decrease in both mucin clot and inhibition titers occurs.

The phenomenon of sperm agglutination by fertlizin has been interpreted by Tyler (1941, 1942, 1947, 1948) as an antigen-antibody type of reaction in which complementary combining groups of a substance (antifertilizin) on the surface of the sperm cells unite in "lock and key" fashion with the combining groups of fertilizin. Where a number of such combining groups are available on the surface of the fertilizin molecule, agglutination occurs as the result of the building up of a lattice, as postulated for analogous immunological reactions by Heidelberger (1938) and Marrack (1938). The formation of univalent fertilizin is brought about by various agents—e.g. heat, ultra-violet light, x-rays—which, according to Tyler (1941), split the molecule into fragments, each of which contains a single combining group. These fragments are still of large size, since they are non-dialyzable (Tyler, 1941). They are also capable of co-precipitating with protein in the presence of acid, giving the mucin clot reaction.

The ability to give the mucin clot reaction is, at least in the case of hyaluronic acid, presumably a function of the degree of polymerization of the molecule (Meyer, 1947). Depolymerized molecules are incapable of giving the reaction. Although fertilizin has been shown to be very different from hyaluronic acid in its chemical composition, the fact that it co-precipitates with protein in acid solution in an analogous manner suggests that it may be similar in its physical structure. Thus, fertilizin may normally exist in a polymerized condition. Sperm-agglutinating activity may, then, accompany a range of polymer size, and the univalent condition may represent a state of polymerization with which but a single combining element is associated. Degradation of multivalent fertilizin to the univalent form would then entail a progressive splitting off of relatively stable univalent units. The evidence from the experiments with ultra-violet irradiation indicates that the univalent form is in fact the more stable, since complete conversion to univalence was observed after 4.3 hours of irradiation, whereas even after nine hours both the inhibition and mucin clot titers retained significant values.

Summary

1. Preparations of fertilizin of three species of sea-urchin have been found to give a mucin clot reaction similar to that given by hyaluronic acid. Upon the addition of bovine serum albumin to an acidified solution of fertilizin, a precipitate forms which dissolves at a pH of 5.6 or higher. All of the sperm-agglutinating activity accom-

panies the precipitate and it is recovered quantitatively when the precipitate is dissolved.

2. A method for the determination of mucin clot titer of fertilizin is described.

3. At temperatures near the freezing point (1° C.) the physical state of fertilizin can be reversibly modified by the removal of electrolytes by dialysis. Macroscopic aggregates appear, accompanied by a parallel decrease in mucin clot and sperm-agglutinating titers. Disappearance of the aggregates is accompanied by an increase in both titers.

4. Chemical analysis of fertilizin shows that it contains no glucuronic acid, about 2 per cent hexosamine and amino acids. Fertilizin, therefore, differs greatly from hyaluronic acid, but its ability to give the mucin clot reaction suggests an affinity with the class of mucopolysaccharides.

5. In general, mucin clot titer parallels sperm-agglutinating titer of the same untreated fertilizin preparation, although sperm agglutination is detectable in higher dilutions than is the mucin clot reaction where the latter is observed by the ring method used in the present experiments.

6. Parallel mucin clot and sperm-agglutinating titrations were made with fertilizin preparations in untreated condition and after exposure to heat and to ultra-violet irradiation. The purified preparations used in these experiments proved to be exceptionally heat-stable; irradiation by ultra-violet light was found to be a more effective treatment in degrading the material.

7. Destruction of the sperm-agglutinating activity of fertilizin by heat and by ultra-violet irradiation does not necessarily cause a parallel decrease in mucin clot titer. The mucin clot reaction continues to be given by preparations in which all of the fertilizin has been converted from the normal, agglutinating condition to the non-agglutinating, "univalent" form. Continued irradiation of the univalent fertilizin is accompanied by a progressive decrease in both inhibition and mucin clot titer.

8. It is suggested that fertilizin may normally exist in a polymerized condition and that the non-agglutinating, "univalent" condition may represent a relatively more stable lower polymer of the native, agglutinating form.

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2. Effect of sea-urchin sperm extracts and hyaluronidase on the mucin clot reaction of fertilizin

After it was found that fertilizin gives a mucin clot reaction, it was of interest to determine whether this could be prevented by prior incubation of fertilizin with sea-urchin sperm extracts or hyaluronidase. Several sperm extracts were prepared and their effect on the mucin clot reaction tested. An active hyaluronidase extract was prepared from bull testes and this was tested for its effect on the fertilizin mucin clot reaction. The various extracts were also tested with the preparation of hyaluronic acid, described in the first section.

Preparation of sea-urchin sperm extracts

Sperm of two species of sea-urchin, Strongylocentrotus purpuratus and Lytechinus pictus were obtained dry by allowing the excised testes to shed through silk bolting cloth into a dry vessel.

Three methods were used for the preparation of the extracts. Each of these is effective in the extraction of active hyaluronidase from mammalian testis or sperm.

The first of these procedures was that of Claude and Duran-Reynals (1937). Dry sperm was combined with an equal volume of M/10 acetic acid and allowed to stand in the refrigerator overnight. In the morning the suspension was centrifuged and cold acetone added to the supernatant. The precipitate was treated with a small quantity of distilled water. The resulting extract, after removal of the undissolved residue, was used in the tests.

The second procedure was that described by Hahn (1943). Following this method, an acetic acid extract of dry sperm was subjected to fractionation with ammonium sulfate. The final preparation corresponded to Hahn's Solution I.

The third method used in this investigation was a modification of the procedure described by Perlman, Leonard and Kurzrok (1948) for the extraction of hyaluronidase from rat sperm. The main feature of this method was that extraction was accomplished by freezing and thawing the sperm in a hypotonic medium (sperm suspension diluted with equal volume of distilled water). The extract so obtained was dialized with distilled water until it was salt-free, and then lyophilized. The resulting dried powder was treated with sea water overnight and the extract from this material, after removal of undissolved residue, was used in the tests.

Bull testis extract was prepared from fresh bull testes, obtained from the Cudahy packing plant, by the method of Hahn (1943) and corresponded to his Solution I. This preparation contained active hyaluronidase as determined by a mucin clot prevention test with hyaluronic acid. It was also effective in dispersing follicle cells from mouse ova in vitro.

In addition to the sea-urchin sperm extracts whose preparation is outlined above, a number of antifertilizin containing extracts that had been prepared by Dr. Tyler were tested for mucin clot preventing action. These extracts had been variously obtained by freeze-thawing and by acid extraction of sperm suspensions (cf. Tyler, 1939; Tyler and O'Melveney, 1941).

In order to have a check on the possibility that a hyaluronidase-like agent may be present in the sperm or seminal fluid of sea-urchins, but inactivated by the various extraction procedures, tests were made with suspensions of live sperm and with seminal fluid from concentrated sperm suspensions.

Finally, an extract of whole testes of S. purpuratus prepared by the method of Claude and Duran-Reynals was tested.

Mucin clot prevention tests

For the tests with fertilizin, 0.5 ml of a purified fertilizin solution, 3.3 percent in NaCl at a pH of 7 was combined with an equal volume of extract, the pH of which had been adjusted to 7. The mixture was allowed to stand 30 minutes to 1 hour at room temperature. In some cases the mixtures were placed at 37° for 30 minutes. At the end of the incubation period 0.5 ml of a 1 percent bovine serum albumin solution in 0.9 percent saline solution was added and then 0.2 ml 2N acetic acid for a final pH of 3 to 3.5. The reaction was scored after 30 minutes. In these tests no attempt was made to get ring formation such as was done in the experiments described in section 1. For the tests with hyaluronic acid, a 0.1 to 0.2 percent solution of hyaluronic acid in distilled water was used.

Results. All of the extracts of sea-urchin sperm, as well as of sea-urchin testis and seminal fluid, and bull testis hyaluronidase, were found to be ineffective in preventing the mucin clot reaction of the fertilizin. In none of these tests was it possible to observe any diminution of the clot. As a matter of fact, in many cases, including the tests with seminal fluid and whole sperm, the clot was enhanced rather than diminished. Similarly, when tested for prevention of the mucin clot reaction of hyaluronic acid, the sea-urchin sperm extracts again proved ineffective, and here too, the reaction was often enhanced. Only in the case of hyaluronic acid and bull testis preparation was clot formation prevented.

Since other evidence was at hand (cf. section 5) which strongly suggested that none of the sea-urchin sperm extracts that were tested for mucin clot-preventing action were capable of dissolving the intact jelly hull of sea-urchin eggs, it did not appear profitable to continue this phase of the investigation.

It did seem important, however, to attempt to obtain more definitive results with hyaluronidase. For this purpose a purified preparation of bull testis hyaluronidase was obtained from the Schering Corp. Instead of using this in further mucin clot prevention tests, however, it was decided to attempt to develop a test analogous with the turbidity reducing test used in assay of hyaluronidase activity with hyaluronic acid as substrate. A quantitative basis for this test is provided by photometric measurement of the stable colloidal turbidity resulting from reaction of dilute or purified hyaluronate with acidified protein solution.

From observations made in connection with the mucin clot reaction of fertilizin it seemed probable that the turbidity produced by reaction of dilute purified fertilizin and acidified serum albumin solutions was sufficiently stable to enable reproducible determinations of optical density to be made photometrically. As will be shown in the next section, this was found to be the case.

3. Turbidity reaction with sea-urchin fertilizin

Preliminary tests with dilute solutions of fertilizin mixed with acidified bovine serum albumin indicated that reproducible results could be obtained by photometric measurement of the turbidity using a Beckman spectrophotometer at 580 mu. An attempt was therefore made to determine the optimum conditions for turbidity development.

In hyaluronic acid systems, Dorfman and Ott (1948) found that maximum turbidity of a solution containing 40 micrograms of hyaluronate per ml was produced at a pH of 3.8 in the presence of an excess of albumin and with the ionic strength at a minimum consistent with adequate buffering capacity.

In a series of tests with fertilizin the optimum conditions of pH, total salt content and albumin concentration were determined for mixtures

containing up to 40 micrograms of fertilizin per ml. It was found that with these quantities of fertilizin there is a linear relation between fertilizin concentration and density of the turbidity.

The methods used and the results obtained are described in the following sections.

Optimum pH for the turbidity reaction

A stock solution, 3.3 percent in NaCl, containing 1.75 mg purified fertilizin per ml was used for all of the tests. For the pH determinations, a portion of this was diluted with 3.3 percent NaCl solution to a concentration of 0.2 mg per ml.

For the tests, 1 ml aliquots of the fertilizin solution were pipetted into each of a number of 15 X 125 mm test tubes. To each tube was then added 4 ml of 1 percent bovine serum albumin in 0.2M acetate buffer, making a total volume of 5 ml, containing approximately 40 micrograms of fertilizin per ml, and 8 mg of serum albumin per ml. The pH of the buffer in each of the tubes had been individually adjusted so that pH increments of 0.2 to 0.4 unit were obtained over the range from 3.6 to 5.2 The final pH of each mixture was checked with the glass electrode. After the mixtures had stood for 30 minutes at room temperature, readings were made with the Beckman spectrophotometer at 580 mu. At least three readings were made with each mixture. Fertilizin (40 γ per ml) in 1 percent albumin solution 0.2M in sodium acetate at pH 7 was used as a blank.

As shown in curve I in figure 3, maximum turbidity is attained at pH 4.4 to 4.5 when the system contains 25 to 40 micrograms of fertilizin per ml.

Interesting results were obtained when systems containing smaller amounts of fertilizin, but otherwise identical with that containing 40 micrograms per ml, were examined. As shown by curves #II, III, IV and V

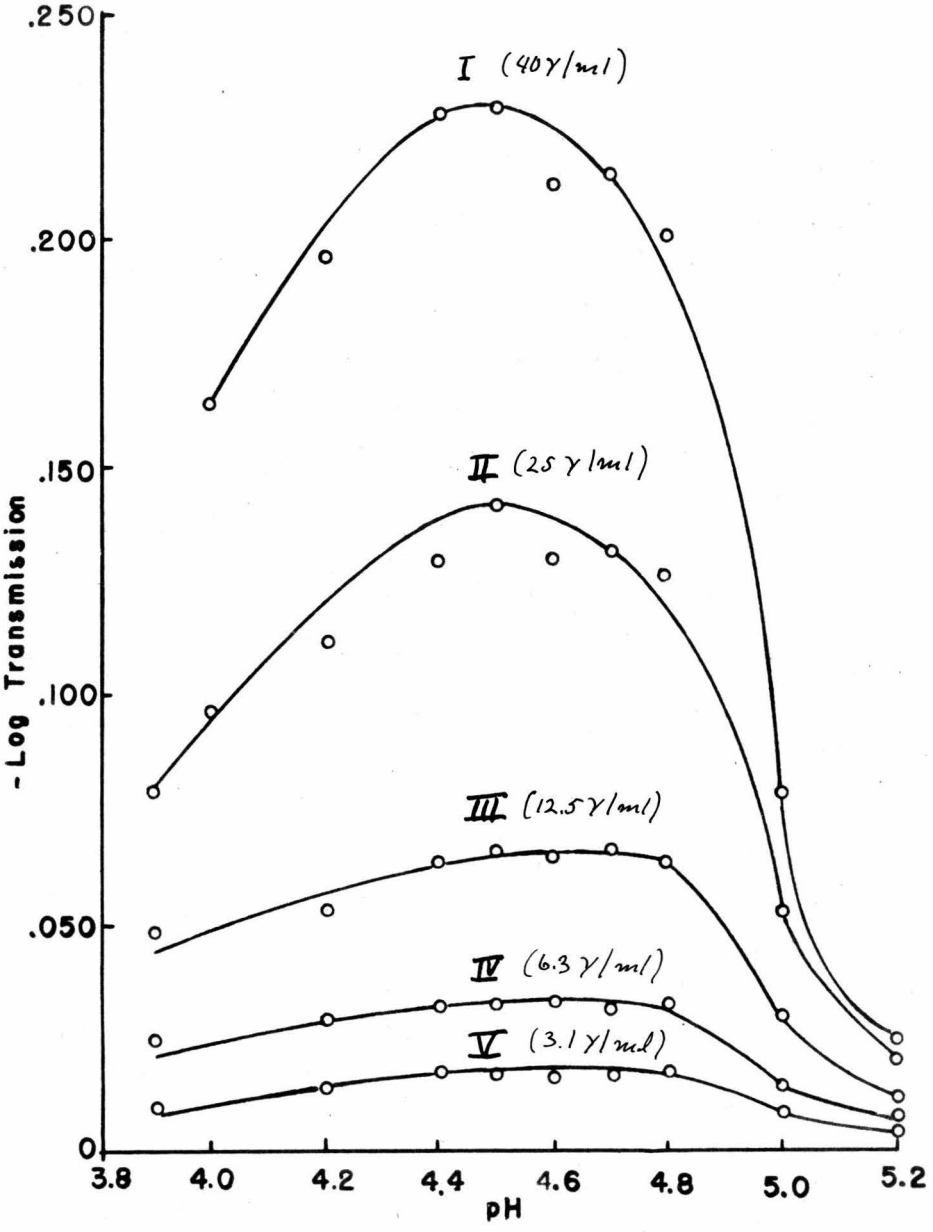


FIGURE 3. EFFECT OF pH ON TURBIDITY REACTION OF SEA URCHIN FERTILIZIN.

in figure 3, with lower concentrations of fertilizin there is a corresponding broadening of the pH range over which maximum turbidity is developed. With about 12.5 micrograms per ml, and less, practically uniform turbidities are obtained from approximately pH 4.4 to 4.8, the turbidity decreasing above and below these values.

Another interesting feature of the results of these tests is that the reaction between fertilizin and serum albumin occurs not only on the acid side of the isoelectric point of serum albumin (pH 4.8 for bovine serum albumin, according to Cohn, 1941) but on the alkaline side as well. In this respect the reaction resembles that in which inorganic and organic anions are bound by serum albumin on both the alkaline and acid sides of the isoelectric point of the protein (cf. Brand and Edsall, 1947).

Evidence from electrophoresis experiments (Runnström, Tiselius and Vasseur, 1942; Tyler, 1948) shows that fertilizin is of pronounced acidic character and Tyler found that it migrates to the anode with practically constant velocity from pH 8.6 to 2. Within the pH range investigated in the present study then, fertilizin behaves as an anion. In this respect, it is comparable with other organic ions which react with serum albumin in the manner described.

According to a number of authors (cf. Brand and Edsall, 1947; Teresi and Luck, 1948, for references), the combination of protein with organic ions apparently depends upon both electrostatic and Van der Waal's forces. On the acid side of the protein isoelectric point positively charged groups in the albumin molecule, belonging, for example, to lysine or arginine, or any free α-amino groups are especially important. On the alkaline side of the isoelectric point Van der Waal's forces predominate until a pH is reached at which repulsion between the protein and anion

overcomes these forces (Teresi and Luck, 1948). In the case of the fertilizin-serum albumin complex, it has been found that dissociation is complete at about pH 5.6 (cf. section 1). The turbidity curves shown in figure 3 fall off more or less rapidly above pH 4.8.

The decrease in turbidity observed below about pH 4.4, which is especially marked in systems containing 25 micrograms of fertilizin per ml and more (to 40 micrograms per ml) is not so readily interpretable. A possible explanation is that the net negative charge of the fertilizin molecule may decrease at lower pH. This assumption is not in harmony with the results of electrophoresis experiments (Tyler, 1948b), but Tyler has expressed the view (personal communication) that the mobility data from these experiments may be open to revision. The presence in fertilizin of amino acid residues (Tyler, 1948b) suggests that these may influence the reaction with protein.

The data (figure 3) show that development of maximum turbidity at a more or less sharply defined pH depends upon the concentration of fertilizin, where albumin is present in excess. Thus, for example, with from about 3 to 6 micrograms of fertilizin per ml, essentially identical turbidity values were observed from about pH 4.3 to 4.8, decreasing outside of this range. This is of interest since it shows that the ratio of the concentrations of fertilizin and protein must be taken into account in assessing the significance of the pH effect on the reaction. Further investigation of this phenomenon was not undertaken.

Optimum electrolyte concentration for the turbidity reaction

Two sets of determinations of the effect of electrolyte concentration of the fertilizin-serum albumin system on turbidity development were made. In each of these, the pH was maintained at 4.5 by the use of acetate buffer. For this reason, the minimum salt content was established

by the minimum concentration of buffer. In each case this was 0.1 molar. In one set, the salt concentration of the different mixtures was varied by adding NaCl in appropriate amount; in the other by using various concentrations of acetate buffer. The albumin concentration was the same in all the mixtures - about 8 mg per ml. The fertilizin concentration in each mixture was approximately 25 micrograms per ml.

For the preparation of the fertilizin solution, a portion of the stock solution (1.75 mg per ml in 3.3 percent NaCl) was diluted to 0.5 mg per ml with 0.1M acetate buffer, pH 4.5. The solution was dialyzed against a large volume of buffer, and made to 4 times the original volume with buffer. For the tests, 1 ml aliquots, each containing approximately 0.125 mg fertilizin, were mixed in each of several 15 X 125 mm test tubes with 4 ml quantities of 1 percent serum albumin in pH 4.5 acetate buffer.

For the first series of determinations, aliquot portions of a 1 percent solution of serum albumin in 0.1M acetate buffer at pH 4.5 were combined separately with weighed quantities of NaCl in each of several 5 ml volumetric flasks. After making to volume, 4 ml of each of these solutions were combined with the 1 ml fertilizin aliquots. The amount of NaCl in the various albumin solutions was calculated to give the following total salt concentration in the final mixtures (expressed as ionic strenth): 0.1 (no NaCl), 0.16, 0.22, 0.26, 0.32, 0.38.

For the second series of determinations, the salt content of the mixtures was varied by employing 4 ml of 1 percent albumin solution made up in concentrations of pH 4.5 acetate buffer calculated to give final concentrations in the different systems of 0.1M, 0.2M, 0.3M, 0.4M, 0.5M.

Readings were made as before, with the Beckman spectrophotometer at 580 mu, after the reaction in each tube had been allowed to develop for 30 minutes at room temperature.

The two curves shown in figure 4 illustrate the results of these tests. It is seen from these curves that maximum turbidity of the fertilizin system is developed at the minimum electrolyte concentration, and that essentially similar results are obtained whether the salt content is varied by Cl^- of Ac^-. This indicates that the effect is not due to a specific anion. In this respect, then, the turbidity reaction of fertilizin is similar with that of hyaluronic acid.

Optimum albumin concentration for the turbidity reaction

In this determination, aliquot portions of the fertilizin solution in 0.1M acetate buffer at pH 4.5 used in the preceding tests were used. Serum albumin solutions of varying concentration were made up in 0.1M acetate buffer, pH 4.5. The mixtures were prepared as before, allowed to stand 30 minutes at room temperature and readings made with the spectrophotometer. The concentration of serum albumin in the different mixtures was (mg per ml): 0.05, 0.15, 0.30, 0.50, 0.75, 1.0.

The results plotted in figure 5 show that maximum precipitation occurs at a concentration of about 0.15 mg per ml of serum albumin, and remains relatively constant as the concentration of albumin is increased. This agrees with the observations of Dorfman and Ott on hyaluronic acid systems.

It was of interest to determine the ratio of albumin to fertilizin at which maximum turbidity is developed. For this purpose, several sets of tests were made using the same procedure as for the initial determinations, except that in the different sets, various fertilizin concentrations were employed. It was found that the critical serum albumin - fertilizin ratio lies somewhere between about 7 and 12. Taking the higher figure, in lieu of more precise determinations, it can be said that for the development of maximum turbidity in the fertilizin reaction

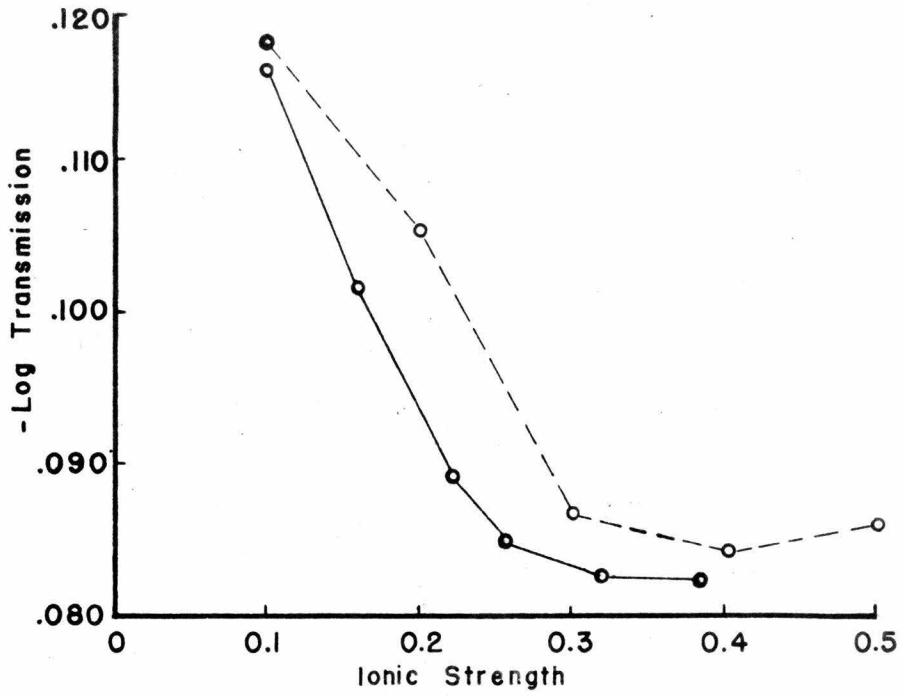


FIGURE 4. EFFECT OF SALT CONCENTRATION ON TURBIDITY REACTION OF FERTILIZIN. SOLID LINE = SALT CONTENT VARIED WITH Cl^-. BROKEN LINE = SALT CONTENT VARIED WITH Ac^-.

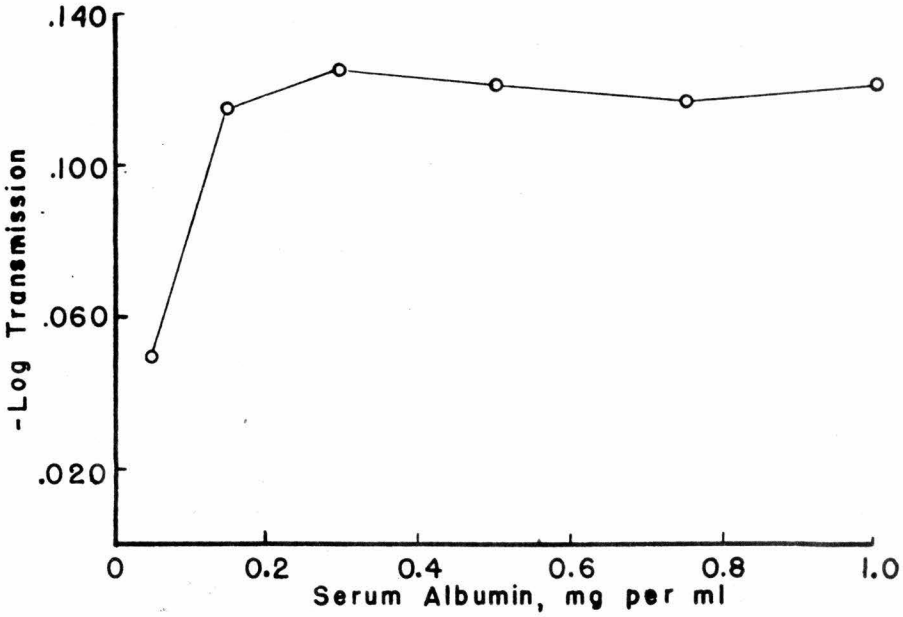


FIGURE 5. EFFECT OF ALBUMIN CONCENTRATION ON TURBIDITY REACTION OF FERTILIZIN. CONCENTRATION OF FERTILIZIN = 25 MICROGRAMS PER ML.

with serum albumin, the ratio of albumin to fertilizin in the system should not be less than 12. A large excess of albumin does not seem to influence the reaction.

Stability of the turbidity in fertilizin-albumin mixtures

On several occasions fertilizin-albumin mixtures containing up to about 40 micrograms fertilizin per ml in acetate buffer at pH 4.5 were left overnight in the refrigerator. Turbidity values obtained after overnight storage (about 20 hours) were compared with those obtained 30 minutes after the mixtures had been prepared. Essentially identical readings were obtained.

Relation between fertilizin concentration and turbidity

Turbidity values were obtained for a number of mixtures differing only in the concentration of fertilizin. Where the optical density (negative log of the transmission) is plotted against fertilizin concentration a linear relation is observed up to 40 micrograms fertilizin per ml, as shown in figure 6. Each mixture was buffered at pH 4.5 with 0.2M acetate and contained 8 mg serum albumin per ml.

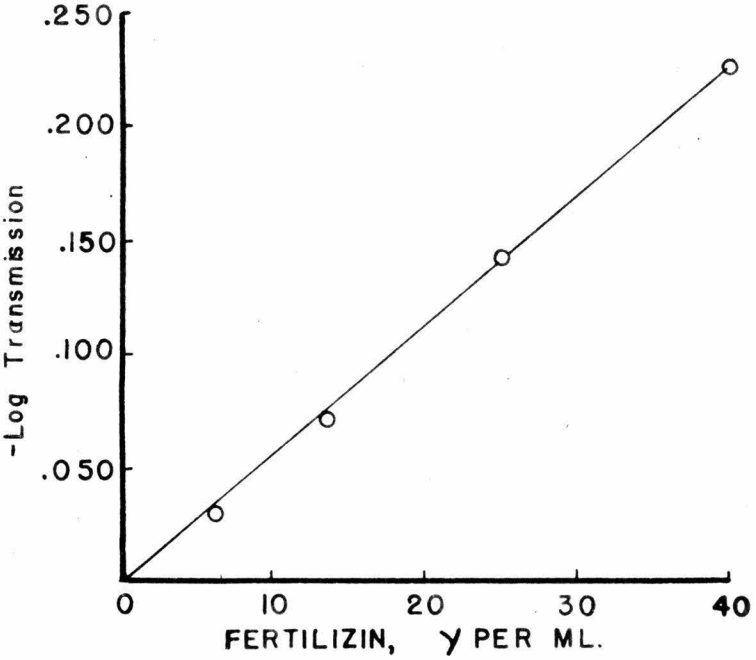


FIGURE 6. RELATION OF FERTILIZIN CONCENTRATION TO OPTICAL DENSITY AT 580 mμ.

4. Effect of hyaluronidase on the fertilizin turbidity reaction

The turbidity method may be applied to the estimation of hyaluronidase in two ways. The first of these consists in determination of the reduction in the rate at which turbidity develops. The second consists of determining the amount of turbidity which remains after some specific time. The second procedure was used here, since the initial aim was to attempt to determine if fertilizin can serve as a substrate for hyaluronidase.

Methods and materials

The general procedure described by Leonard, et al. (1946) for the turbidimetric determination of hyaluronidase in mammalian semen and tissue extracts was used in the present study. Appropriate modifications were introduced in order to achieve as nearly as possible the optimum conditions for turbidity development in the fertilizin system. These have been described in the preceding section. Another modification was suggested by the report of Dorfman and Ott (1948) that pH 5.5 is optimum for hyaluronidase activity. Leonard et al. buffered their hyaluronidase solutions at pH 6. According to Meyer (1947) NaCl appears to be necessary for hyaluronidase activity. For the present tests, hyaluronidase, fertilizin and hyaluronic acid solutions were made up containing NaCl.

For the tests, a solution containing ca. 0.4 mg purified fertilizin per ml in 0.1M acetate buffer at pH 5.5, 0.3M in NaCl was prepared from the same stock solution of fertilizin that had been used for the earlier studies. The method used in making up the fertilizin solution was the same as previously described except that the stock solution was diluted to contain 1 mg fertilizin per ml prior to dialysis with buffer. After dialysis, the solutions were further diluted to make the concentration approximately 0.4 mg per ml.

A 1 percent solution of Schering hyaluronidase, made fresh for each experiment, in 0.1M acetate buffer at pH 5.5, 0.3M in NaCl, was used as a stock solution from which desired dilutions were made.

Two serum albumin solutions, each 1 percent, were prepared in 0.2M acetate buffer. One of these, at pH 4.4 was used in the experiments with fertilizin; the final pH of the system was about 4.5. The other albumin solution, pH 3.8, was used with hyaluronic acid, and the final pH in this system was about 4.

Hyaluronic acid solution was prepared by dissolving hyaluronic acid (Schering) in 0.1M acetate buffer at pH 5.5, 0.3M in NaCl. The solution was made to contain 0.4 mg hyaluronic acid per ml.

In the experiments, 1 ml aliquots of each of a number of dilutions of the stock hyaluronidase solution were mixed with 1 ml portions of the fertilizin (or hyaluronic acid) solutions in each of a number of 3 ml test tubes. The mixtures were incubated 30 minutes in a water bath at 37°, then chilled 5 minutes in ice water. From each tube, 1 ml, containing approximately 0.2 mg substrate was pipetted and mixed with 4 ml of the appropriate serum albumin solution in each of several 15 X 125 mm test tubes. In the final systems the concentration of substrate was approximately 40 micrograms per ml, and the total salt content equivalent to an ionic strength of about 0.2. After the mixtures had stood 30 minutes at room temperature, readings were made with the Beckman spectrophotometer at 580 mu.

In order to provide reference points by which to estimate hyaluronidase activity in terms of turbidity reducing units, it was necessary to provide two standards. One of these contained 40 micrograms substrate per ml, and the other 20 micrograms per ml, and both were prepared in the same way as the other mixtures with the exception that hyaluronidase was

omitted in the incubation mixtures. A single turbidity-reducing unit is that amount of hyaluronidase required to reduce the turbidity of the system from that produced by 40 micrograms substrate per ml (0.2 mg) to that produced by 20 micrograms per ml (0.1 mg) upon 30 minutes incubation.

Results of the tests. The results showed hyaluronidase to have no measurable effect on fertilizin as tested by this method. Data from one experiment are shown in figure 7. It is seen that with concentrations of hyaluronidase in the incubation mixtures of about 0.3 mg per ml to 1.25 mg per ml, the final turbidities were not significantly different from that of the 40 microgram per ml standard. On the other hand, with hyaluronic acid as substrate, hyaluronidase concentrations of 0.03 mg per ml to 0.004 mg per ml were effective in reducing the turbidity - essentially to zero in the higher concentration and well below that of the 20 microgram per ml standard in the lowest concentration (figure 8).

Discussion. The evidence presented here shows that purified fertilizin is not degraded by hyaluronidase - at least not in a manner or to an extent detectable by the turbidimetric method.

In the next section, the results of direct observations of the action of hyaluronidase, and also of sea-urchin sperm extracts, on the intact jelly hull of sea-urchin eggs are presented.

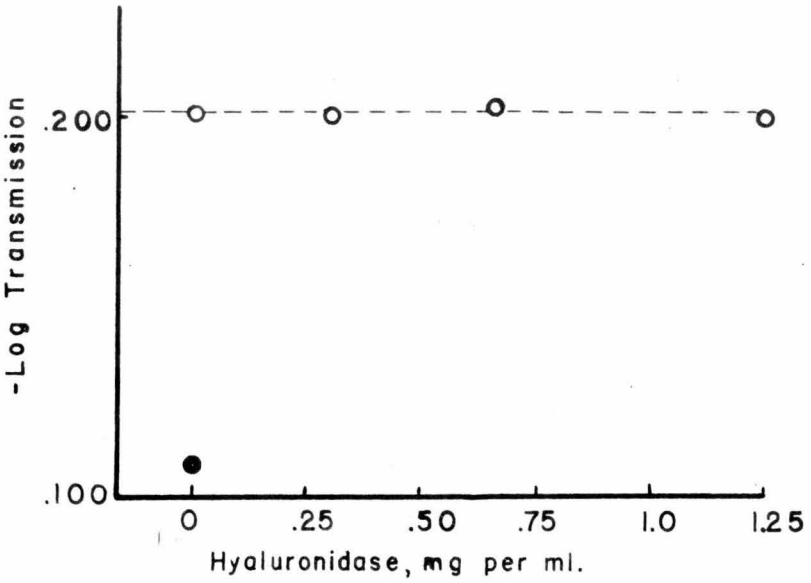


FIGURE 7. TURBIDITY OF FERTILIZIN TURBIDIMETRIC SYSTEMS AFTER INCUBATION OF FERTILIZIN WITH HYALURONIDASE. OPEN CIRCLES = 40γ FERTILIZIN PER ML. CLOSED CIRCLE = 20γ PER ML.

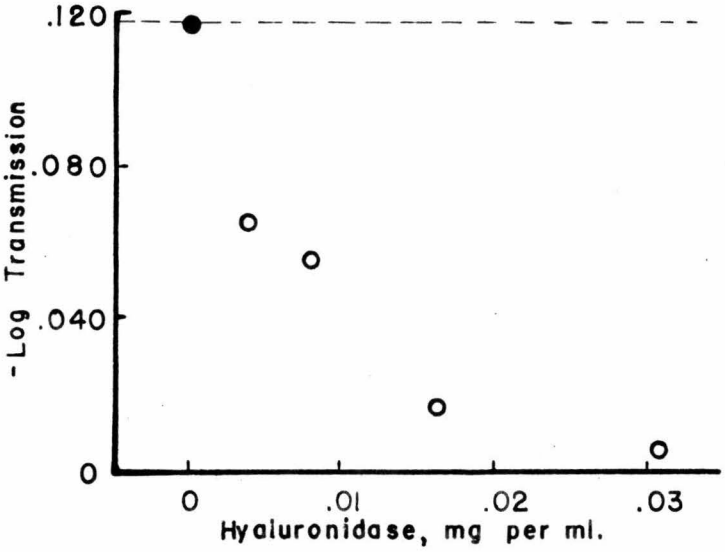


FIGURE 8. TURBIDITY OF HYALURONIC ACID TURBIDIMETRIC SYSTEMS AFTER INCUBATION OF HYALURONIC ACID WITH HYALURONIDASE. OPEN CIRCLES= 40γ HYALURONIC ACID PER ML. CLOSED CIRCLE = 20γ PER ML.

5. Effect of sea-urchin sperm extracts and hyaluronidase on the jelly hull of sea-urchin eggs.

The experiments to be reported in this chapter were done in collaboration with Dr. Tyler. Most of the tests were made at the Marine Laboratory at Corona del Mar.

Materials and methods. The sea-urchin sperm extracts whose preparation has previously been described (section 2) were used in these tests. In addition to these, both crude and purified hyaluronidase preparations were tested. For the tests, all extracts were made isotonic with sea water, either by dialysis with 3.3 percent NaCl solution, or by addition of an equal volume of double sea water to the solution, and the pH was, in every case, adjusted to between 7 and 8.

Eggs of the sea-urchin Lytechinus pictus were used for the most part. Some tests were made with eggs of S. purpuratus, but no distinction is made in this report. The eggs were obtained by the usual method in which the entire ovaries are removed from the animal and placed in sea water, into which the eggs are released. From this suspension, a quantity of eggs was removed, washed twice in fresh sea water and transferred to a small flask with sufficient sea water to make about a 1 percent suspension. From this suspension of eggs, approximately uniform quantities could be removed for testing. Care was observed in handling the eggs in order to avoid shaking the jelly from them.

A standard procedure was employed in all tests. Using the drop method, two-fold dilutions in sea water were made with each extract to be tested. Salt cellars were found to be the most favorable type of dish. One drop of eggs from the stock suspension was placed in each dish containing 2 drops of the extract. Controls were placed in sea

water. After 10 minutes, the eggs in each dish were examined in a manner described below.

To determine the effect on eggs treated in the various extracts, they were examined with a compound microscope under low power. Presence or absence of the jelly layer was readily determined by noting whether the eggs came into direct contact when the dish was gently swirled. If the eggs could not be caused in this manner to touch each other, it was either because they were surrounded by the jelly or else had become stuck to the bottom of the dish. The absence of a jelly layer as ascertained by this test, however, did not mean that the jelly had been dissolved. The basis for this statement will be made clear in the next section.

Some observations of treated eggs in Chinese india ink suspensions supplemented the usual examinations. The ink particles render the jelly coat visible, where this is present. In all cases the two methods of observation gave identical results with respect to showing the presence or absence of the jelly.

Results of the tests. The effects of the various extracts on the jelly coat of sea-urchin eggs are summarized in Table VII. In the table the data refer to effects noted in full-strength extracts, except in those cases in which the extract was initially salt-free and required dilutions with double sea water prior to testing. In the case of the controls, the data represent the range of variation in results recorded for all of the control tests made with sea water. It is seen from the table that many of the extracts were effective in causing disappearance of the jelly hull, but in most of these cases, a parallel phenomenon was observed. This was the formation of a precipitation membrane on the jelly surface, which often contracted to the surface of the egg with concomittant disappearance of the jelly. The two or three instances in

TABLE VII

Precipitation membranes, percentage of eggs stuck to the dish and percentage of jellyless eggs observed in suspensions of unfertilized sea-urchin eggs treated with various sperm extracts. Extracts 1-8, anti-fertilizin preparations; 9, sea-urchin sperm extract, method of Claude and Duran-Reynals; 10-11, sea-urchin sperm extracts, method of Hahn; 12, sea-urchin sperm extract, method of Perlman, et al.; 13, seminal fluid of sea-urchin sperm, combined with washings; 14-15, bull testis hyaluronidase.

Extract	Precipitation membrane	Eggs stuck to dish (percent)	Jellyless eggs (percent)
1	+	100	100
2	+	0	0
3	+	100	100
4	+	100	100
5	+	100	100
6	+	10	10
7	+	100	100
8	+	100	100
9	0	0	5
10	0	0	20
11	0	0	0
12	+	10	0
13	+	5	0
14	0	0	0
15	0	0	0
Controls	0	0	0-70

which jellyless eggs were found, but in which precipitation membranes were not observed are not significant in view of the variability shown by the control eggs. The data also show that in most instances in which precipitation membranes were observed, the eggs were also stuck to the bottom of the dish. There is evidently a parallelism between the presence of precipitation membranes and sticking of the eggs. It should also be mentioned that agglutination of the eggs usually accompanied, to a greater or lesser degree, the appearance of precipitation membranes.

Of particular interest is the fact that only one of the sea-urchin sperm extracts prepared according to methods for hyaluronidase extraction from mammalian tissues (No. 12 in the table) was effective in producing precipitation membranes on the jelly. Extract No. 13, which also produced precipitation membranes, was sea-urchin seminal fluid combined with two sea-water washings of the sperm. In neither of these extracts, however, were jellyless eggs found in the dishes. The precipitation membranes were light, did not contract to the egg surface, and only 5 to 10 percent of the eggs stuck to the dish. Some jellyless eggs were observed in extracts 9 and 10, but the percentage in each case can hardly be considered significant. Neither of the bull testis preparations had any observable effect on the jelly.

In contrast to the slight effects obtained with extracts prepared by methods for hyaluronidase extraction were those shown by the series of preparations obtained from sea-urchin sperm by methods with which antifertilizin is extracted. Of eight antifertilizin preparations tested (No. 1 to 8 in Table VII), 100 percent of the eggs treated in each of six of these were found to be "jellyless" and stuck to the dishes. Strong precipitation membranes were seen on these eggs, and in most cases were observed to contract to the egg surface. With the other two anti-

fertilizin preparations, 0 and 10 percent "jellyless" eggs were found and corresponding percentages of eggs stuck to the dishes. Light precipitation membranes, or none at all, were seen in these.

Of interest in connection with these experiments is the fact that when isolated jelly hulls are treated in active antifertilizin solutions, they shrink to a small size, but do not disappear. In two photomicrographs shown in figure 9 (a,b), an isolated jelly hull is shown in successive stages of shrinkage. For comparison, a jelly-coated egg with precipitation membrane forming (c, figure 9) and fully contracted to the egg surface (d, figure 9) is shown.

The presence on the egg surface of a contracted precipitation membrane can be demonstrated by subjecting eggs, treated first in antifertilizin solution, to high-speed centrifugation. Distortion, or fragmentation of the eggs often reveals the presence of a distinct membrane, as shown in figure 10a. No such membrane is seen on non-treated centrifuged eggs (figure 10b).

Discussion. The formation of a precipitation membrane on the surface of the jelly hull of sea-urchin eggs is one of the manifestations that characterizes the reaction between antifertilizin and fertilizin, where the latter is present as the jelly hull of the egg (Tyler and O'Melveney, 1941). Substances other than antifertilizin are known to produce precipitation membranes on the jelly of sea-urchin eggs, for example, basic proteins (Metz, 1942, 1949; Hultin, 1947 a,b; Borei, 1948). It is reasonably certain, however, that antifertilizin is not a basic protein (Metz, 1949). A significant difference between the action of antifertilizin and basic proteins is that the former is apparently specific for fertilizin, while the latter is not specifically directed against fertilizin.

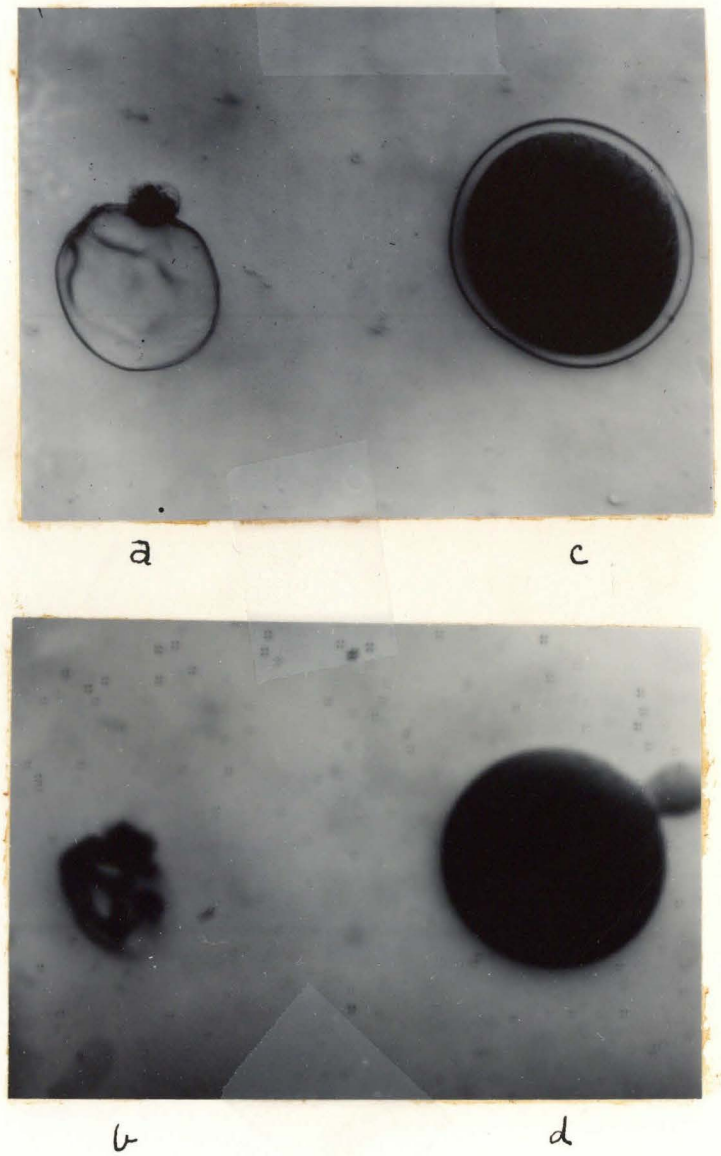
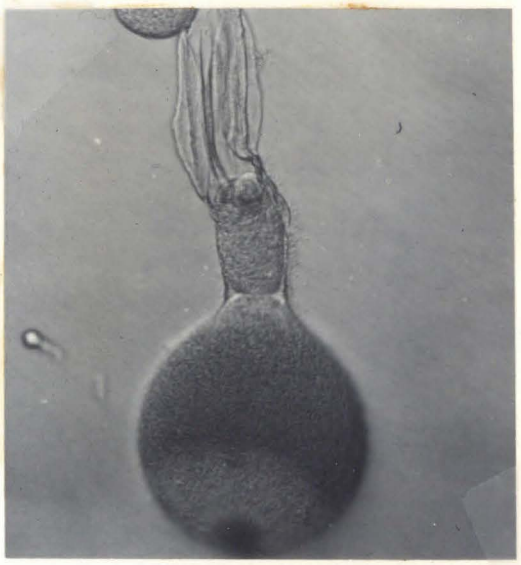
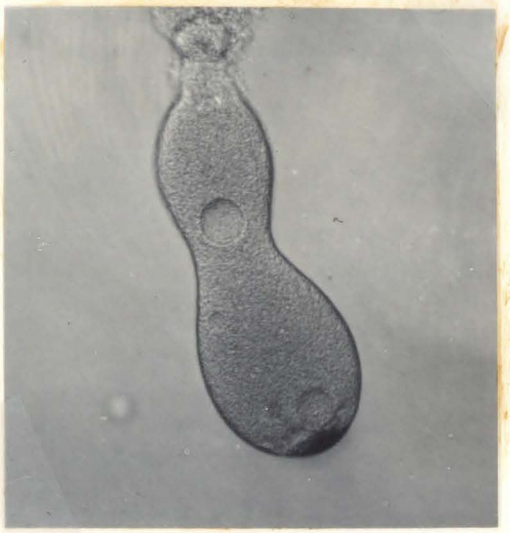


Figure 9. Action of antifertilizin on an isolated jelly hull of Lytechinus pictus. Two stages of shrinkage shown in a and b; precipitation membrane developing in c, and contracted to the surface in d.



a



b

Figure 10. Eggs subjected to high-speed centrifugation. Egg treated in antifertilizin shown in a, with precipitation membrane; egg not treated in antifertilizin shown in b, with no precipitation membrane.

The evidence presented here lends no support to the thesis that sea-urchin sperm extracts are capable of dissolving the egg jelly. On the contrary, it is clear from the present experiments, as well as from earlier observations (Tyler and O'Melveney, 1941), that the apparent dissolution of the jelly of sea-urchin eggs in the presence of sperm extracts is attributable to the presence of antifertilizin in these extracts, and that this results from the formation of a precipitation membrane and incorporation of the jelly in it with consequent contraction of the membrane to the egg surface. The failure of hyaluronidase from bull testis to effect jelly dissolution, or to have any observable effect of the jelly, is in agreement with the observations of Runnström, Monné and Broman (1943) and contrary to the results of Hartmann, Schartau and Wallenfels (1940), Ruffo and Monroy (1946) and Monroy and Ruffo (1947). Consistently negative results obtained in fertilizin mucin clot prevention and turbidity reducing tests with sperm extracts and bull testis hyaluronidase provide additional evidence that fertilizin is not degraded by these preparations.

EXPERIMENTS WITH EGG SURFACE LYSIN FROM SEA-URCHIN SPERM

1. Extraction of egg surface lysin. Effects of extracts on eggs. Methanol extracts of sea-urchin sperm

Materials and methods. Sperm of the purple urchin, S. purpuratus, was used for the preparation of most of the extracts. In some cases, sperm of Lytechinus pictus was used. Dry sperm was obtained either by injection of KCl into the animals, which causes them to shed, or by collecting sperm shed from excised whole testes. The first method was used where it was desired to obtain small quantities relatively free of body fluid. The second method was used for the collection of larger quantities of sperm.

For extraction of the egg surface lysin, the method described by Runnström and Lindvall (1946) was followed. Dry sperm was lyophilized and the powder was extracted with 5 to 10 times its volume of hot, absolute, redistilled methanol in a Soxhlet continuous extraction apparatus. Methanol extraction was continued for 8 to 12 hours. At the end of this time, the clear, yellow extract was evaporated to dryness in vacuo. The residue was extracted 5 times with 10 ml portions of chloroform, or petroleum ether, and the combined extracts passed through a column of $ZnCO_3$ in a chromatograph tube. The column was washed with about 100 cc of absolute alcohol and the alcohol eluent evaporated in vacuo. The residue was then taken up in a small volume of sea water. That part of the residue which failed to go into solution after shaking the suspension at room temperature at intervals over a period of 2 or 3 hours, was removed by filtration. The pH of the filtrate was adjusted to about 8, if this was necessary, and it was then tested with eggs for surface lysin activity.

For the tests, unfertilized eggs of Lytechinus pictus or S. purpur-

atus were used. As a rule, jellyless eggs were used, the jelly having been removed by brief treatment in acid sea water.

Two kinds of test were used to detect surface lysin activity. These were the hypertonicity test and the hypotonicity test (Runnström et al., 1945). In both tests, treatment of eggs in the various extracts was done in the same way. A drop of egg suspension containing 50 to 100 eggs was placed in 2 ml of the extract and left for varying intervals of time, usually 30 to 50 minutes. For the hypertonicity test, 0.5 ml of 2.5N NaCl was then added to the suspension and the condition of the eggs determined at intervals starting about 4 minutes after addition of the NaCl. For the hypotonicity test distilled water was added to the suspension and the time required for 50 percent of the eggs to show cytolysis was compared with the time required for 50 percent of the control eggs to show cytolysis in equally diluted sea water. In both tests, eggs in 2 ml of sea water served as controls.

Results of the tests. On the whole, the results of tests with the methanol extracts were similar to those reported by Runnström et al., insofar as eggs treated in these extracts showed smooth shrinkage in hypertonic sea water, and in hypotonic sea water cytolysis of treated eggs was accelerated as compared with untreated eggs. Furthermore, in confirmation of the Swedish workers, treated eggs in hypertonic sea water tended to show varying degrees of surface "blistering" and cytolysis of the dark type. It should be noted, however, that positive results were not obtained with all extracts, or even consistently with one extract. No attempt was made to analyse the factors which may have been responsible for occasional erratic results, but these may have been related to the condition of the eggs used in testing, or to the influence of pH, temperature, etc., on the activity of the extracts. With respect

to pH, there was some indication that lytic activity was enhanced at a slightly acid pH (ca. pH 6.7) but this was not investigated systematically. Data obtained from tests of four different extracts are shown in Table VIII. In the table, the data refer to results with undiluted extracts.

Tests of seminal fluid

The hypothesis that the egg surface lysin is of physiological significance would be considerably strengthened if it can be demonstrated in seminal fluid, as has been reported by Runnström, et al. (1945). In the present study, several attempts were made to demonstrate surface lysin activity in sea-urchin seminal fluid by means of the hypertonicity test. In only one test were unequivocally positive results obtained. This is described below.

Experiments. A concentrated (25%) suspension of Lytechinus sperm was centrifuged 30 minutes at 3500 rpm. The supernatant was diluted with an equal volume of sea water (cf. Runnström et al., 1945) and frozen overnight. After thawing, portions of the solutions were diluted 1:1 and 1:3 respectively with sea water and these were tested with Lytechinus eggs. In each dilution, the eggs were agglutinated to some extent. When examined 4 minutes after addition of NaCl the treated eggs were smooth, while control eggs in sea water were strongly wrinkled. The treated eggs also appeared somewhat larger than the control eggs. No difference between eggs treated in 1:1 and 1:3 solutions was apparent.

The results of this experiment cannot be taken as providing complete confirmation of the results reported by Runnström, et al., since in the present case, antifertilizin was present in the sperm supernatant, as shown by agglutination of the eggs. The Swedish workers reported that

TABLE VIII

Hypertonicity and hypotonicity tests of 4 methanol extracts of sea-urchin sperm. All extracts adjusted to pH 7-8.

	Hypertonicity test		Hypotonicity test	
Solution	Time of treatment	Condition of eggs 5 min. after adding NaCl	Time of treatment	Time for cytolysis of 50 % of eggs
Methanol extract #1	45 min.	smooth; 10% dark cytolysis		
Sea water		wrinkled		
Methanol extract #2	50 min.	smooth		
Sea water		wrinkled		
Methanol extract #3	35 min.	100% cytolysis, shrinkage not observed	40 min.	Less than 1 min. (100%)
Sea water		No cytolysis, wrinkled		7 min.
Methanol extract #4	40 min.	"Angular" shrinkage with smooth surface	40 min.	1½ min.
Sea water		Usual shrinkage, wrinkled		7 min.

their solutions obtained in a similar manner did not agglutinate the eggs, but this does not mean that antifertilizin may not have been present in their preparations, particularly since in a recent publication (Runnström, Monné and Wicklund, 1946) they have reported that antifertilizin preparations show an effect on the egg cortex in dilutions much greater than those causing appearance of precipitation membranes on the jelly. Frozen-thawed extracts of sperm were reported by Runnström, et al. (1945) to show both surface lysin and antifertilizin activity.

Experiments to be reported in a later part of this paper show that a lytic agent can indeed be demonstrated in frozen-thawed extracts of sea-urchin sperm, which may well be distinct from antifertilizin. This agent, however, is inactivated by brief heating at 60° and accordingly is not identical with the heat-stable methanol-soluble egg surface lysin. On the whole, the available evidence concerning the presence in sea-urchin seminal fluid of egg surface lysin (i.e., as a distinct agent) is not very convincing.

One experiment was performed in which the effect of Lytechinus seminal fluid on the fertilizability of homologous and heterologous eggs was tested. Dry sperm of Lytechinus was centrifuged at 4500 rpm for 50 minutes. The supernatant was diluted with an equal volume of sea water and frozen. After thawing, the solution was used without further dilution to treat eggs of Lytechinus and Dendraster excentricus. After 50 minutes treatment the eggs were inseminated with homologous sperm in the test solutions. It should be mentioned that the jelly hull was present on the eggs, and the amount of sperm used for insemination was in excess of that required for 100% fertilization of samples of untreated eggs of the same lots used in the experiment. Of the treated Lytechinus eggs, about 10 percent produced fertilization membranes, and of the Dendraster

eggs, 100 percent of the treated eggs produced fertilization membranes.

Discussion. The results of this experiment are of interest insofar as they indicate the possibility of a species-specific effect. The egg surface lysin is apparently not species-specific, since its effects are duplicated by extracts of fish sperm, by detergents and by bee venom. If, in the experiment cited, the failure of 90 percent of the treated Lytechinus eggs to produce fertilization membranes is to be attributed to the action on the egg surface of a detergent-like material of low molecular weight, it is not readily apparent why the Dendraster eggs were not also affected.

It is, of course, not surprising that a complex chemical system such as a sperm cell may contain constituents that, upon extraction, may exhibit detergent-like properties. With respect to the egg surface lysin, Runnström (1947) has stated that the substance is probably present within intact cells in the form of a precursor. What the nature of this precursor may be is not indicated. Presumably it may be a lipo-protein complex.

It appeared to be of some interest to attempt to extract material from sea-urchin sperm, that would exhibit some of the properties of the egg surface lysin, by methods other than those employed by Runnström, et al. Two different procedures were utilized in this study. The methods used and the results obtained are described in the following section.

Total lipid extracts of sperm

Methods. A total lipid extract of S. purpuratus sperm was prepared, with some modifications, according to the method of Bloor (1943). Lyophilized sperm was extracted with a mixture of 3 parts methanol and 1 part ether in a Soxhlet apparatus. The extract was evaporated nearly

to dryness in vacuo at 60°. Evaporation to dryness was completed in a stream of N_2, which was passed over hot copper to remove oxygen. The dried residue was then extracted with several 5 ml portions of hot petroleum ether, which were decanted into a 15 ml centrifuge tube and clarified by centrifuging. About 3 ml of the clear petroleum ether extract was saved as the "total lipid" fraction. The remainder of the supernatant was evaporated to about 2 ml. and phospholipid precipitated by the addition of 12-13 ml acetone with 2-4 drops of a 5 percent alcoholic solution of magnesium chloride. The precipitated phospholipid was recovered by centrifugation and dissolved in petroleum ether. The supernatant was evaporated to dryness, washed with water and finally dissolved in petroleum ether. This portion was assumed to contain the bulk of the fatty acids as well as any sterols present.

Each of the three fractions, total lipid, phospholipid and fatty acid, was passed through a column of $ZnCO_3$ in a chromatograph tube. A petroleum ether blank was also run through a column of $ZnCO_3$. The columns were eluted with absolute alcohol, the eluents evaporated to dryness and each residue taken up in a small volume of sea water. The sea water suspensions were shaken and allowed to stand for several hours at room temperature, after which they were filtered. The sea water filtrates were tested with eggs for surface lysin activity. These comprise the Extract I fractions of Table IX.

Another total lipid extract of S. purpuratus sperm was prepared from washed, dry sperm according to the method of Zamecnik, et al. (1945). About 100 cc of dry sperm were combined with an equal volume of distilled water, frozen and thawed. The thawed mass was combined with approximately 9 volumes of a mixture of 1 part ethanol and 1 part ether and allowed to stand overnight at room temperature. The suspension was then reduced

to a smaller volume by evaporation over a steam bath and dialyzed against several changes of distilled water for 4 days at 1° C. The dialyzed material was lyophilized. The residue, consisting of about 450 mg of material, was taken up in 50 ml of sea water. This suspension was blended on a Waring blender for 30 seconds and then centrifuged at 3500 rpm for 10 minutes. Insoluble material was discarded. The supernatant was saved for testing. This is Extract II of Table IX.

The various fractions of Extract I were tested by means of the hypertonicity test, and they were also tested for their effect, combined with hypertonic treatment, on fertilizability of eggs. Extract II was tested by the hypertonicity and hypotonicity tests. In general, it appears that the hypotonicity test is more suited for quantitative determinations of surface effects, where these accelerate the rate of cytolysis in diluted sea water. Cytolysis of eggs in hypotonic medium is an all or none phenomenon that occurs with sufficient rapidity so that there is never any serious question as to whether an egg is cytolyzed. In the hypertonicity test, on the other hand, there may be various degrees of wrinkling of eggs, so that a clear-cut distinction between affected and unaffected eggs is sometimes difficult to make.

Results. The results of the tests are summarized in Table IX. It is seen from the table that, of the various fractions of Extract I, the most active, as judged by the two kinds of tests employed, was the total lipid fraction. The phospholipid fraction showed somewhat less activity than the total lipid fraction and the fatty acid fraction showed less effect than the phospholipid fraction. In the case of Extract II, it is of interest that cytolysis of most of the treated eggs occurred very rapidly in hypertonic medium. In two hypotonicity tests

TABLE IX

Hypertonicity and hypotonicity tests of 2 lipid extracts of sea-urchin sperm. All extracts adjusted to pH 7-8.

		Hypertonicity test		Hypotonicity test	
Extract	Fraction	Time of treatment	Condition of eggs 5 min. after adding NaCl	Time of treatment	Time for cytolysis of 50% of eggs
I	Total lipid	40 min.	70-80% dark cytolysis; surface blistering without wrinkling	Not tested	
	Phospholipid	"	50% dark cytolysis; surface blistering without wrinkling	"	
	Fatty acid	"	10-20% dark cytolysis; surface blistering with possible wrinkling	"	
	Blank	"	5% dark cytolysis; wrinkled	"	
	Sea water control		5-10% dark cytolysis; wrinkled		
II	Total lipid	30-40 min.	100% cytolyzed. Shrinkage not observed.	40 min.	Less than 1 min. (100%)
	Sea water		wrinkled		7 min.

of this extract, a marked acceleration of cytolysis of the treated eggs was observed.

Discussion. In attempting to assess the significance of these results at least three points seem clear. The first of these is that total lipid extracts of sea-urchin sperm yield material that is soluble in sea water which shows, when tested with eggs, certain effects that are similar to those ascribed to the egg surface lysin. The second point is that more of the surface active material may be associated with one component of the total lipid extract than with others. It is impossible to say, on the basis of this experiment, that a significant fraction of activity resembling that of the egg surface lysin is concentrated in the phospholipid fraction. It does, however, seem reasonable to suggest that by using standard procedures for the fractionation of lipid extracts, it might be possible to identify by chemical means, the kind of substance, or substances that show significant surface effects on eggs. Finally, activity resembling that of the egg surface lysin has been demonstrated in association with non-dialyzable material. This probably indicates that the active substance is combined with water-insoluble material rather than that it is non-dialyzable. In the Runnström procedure adsorption on $ZnCO_3$ and alcohol elution may effect the splitting off of the alcohol-water-soluble constituent(s). The point of interest here is that extraction of lyophilized total lipid material with sea water yields a solution that has marked effects on eggs. It appears from this that the active constituent(s) may be loosely bound to water-insoluble components.

With respect to the general question of the physiological significance of egg surface lysin in fertilization of sea-urchin eggs, it seems premature to assign this a definitive role. There is no doubt that material can be extracted from sea-urchin sperm which may affect the egg surface

in various ways. The effects may even all be attributable to one general kind of action - liquefaction of the cortex, as Runnström claims. At least two points require clarification: (1) the availability of sperm egg surface lysin under physiological conditions; (2) the relation of this substance to the various processes of fertilization.

EGG MEMBRANE LYSIN OF THE GIANT KEYHOLE LIMPET (Megathura crenulata)

1. Materials and basic methods. Estimation of lytic and antifertilizin activities in sperm extracts

Materials and basic methods

Keyhole limpets were collected from the breakwaters at the entrance to Newport Bay. Some were obtained from the Santa Monica breakwater*.

Some of the experiments were performed at the Marine Laboratory at Corona del Mar, but most of the experiments and processing of materials were done in Pasadena. For the latter purpose, either live animals were transported, or the eggs and sperm were removed at the Marine Laboratory and brought to Pasadena in iced containers. Routine methods employed in securing sperm and eggs and in their subsequent preliminary processing are briefly described below.

Removal of sperm and eggs. Animals are opened by cutting through the mantle and body wall with a knife, starting at the radula and continuing around the body just under the shell. The cut portions of the body are separated, exposing the body cavity. The gonads are freed from the gut and body wall with sharp-pointed forceps, and removed in toto. Testes are placed in a dry vessel and ovaries into sea water.

Preliminary processing of sperm. The purpose of preliminary processing of sperm is to free it from foreign material, body fluid and testis tissue. This is best accomplished by first mincing the whole testes with scissors and washing out as much sperm as possible with sea water, straining the suspension through silk bolting cloth in order to separate testis fragments. Sperm obtained in this manner are then washed several

*Animals provided through the courtesy of Dr. Theodore Bullock of U.C.L.A.

times by centrifuging with sea water in a refrigerated centrifuge. In the final washing, the sperm are packed by hard centrifuging, the supernatant poured off and the volume of packed sperm estimated.

Preliminary processing of eggs. Eggs are released from ovaries after these have been put into sea water. The eggs are strained through bolting cloth. To remove the jelly, a suspension of eggs is acidified to pH 3 to 3.5 and centrifuged. It may be necessary to repeat the acid treatment two or three times. This is followed by two or three washings with normal sea water. To preserve eggs with the membrane intact, formalin is added to a suspension in sea water to make a 1 or 2 percent solution.

For experiments in which it is desired to leave the jelly hull intact, eggs are washed in several changes of normal sea water.

Estimation of lytic activity of sperm extracts

The egg and its membrane. When shed, the keyhole limpet eggs are irregular in shape, and have a thick membrane closely adherent to the surface. As the unfertilized eggs stand in sea water, they gradually round out and the membrane lifts from the surface. The average diameter of many eggs measured is 165 u, and that of the fully elevated membrane is 185 u. The jelly layer surrounds the membrane.

The egg lies eccentrically within the membrane. There is a micropyle in the membrane, as shown in figure 34. Other points concerning the egg and its membrane will be presented later.

Standard techniques for assay of sperm extracts. A stock supply of jellyless eggs preserved in 2 percent formalin in sea water was kept in the refrigerator. For each day's tests, a small number of eggs was removed from the stock supply, washed 3 times in sea water and placed in fresh sea water in a Syracuse dish.

For assays, eggs are transferred individually with a fine dropping

pipet, each to a separate salt cellar containing an aliquot of the solution being tested. Each egg is pipetted into the test solution in a single drop. The same pipet is used for all transfers so that the size of the drop is constant. A uniform quantity of test solution is used. Approximately 0.5 ml of solution has been employed, measured, in most cases from a dropping pipet which delivers approximately 0.05 ml per drop. For greater precision, the test solution is measured from a measuring or volumetric pipet.

The effect of a test solution on the egg membrane is scored after 5 minutes exposure. To time the tests, a stop watch is started at the instant the egg is released into the solution. Each dish is gently agitated at intervals until it is placed on the stage of a compound microscope about 4½ minutes after start of the test. The membrane is examined at a magnification of 100X. In most cases, it is possible to roll an egg about sufficiently by gentle agitation of the dish so that enough of the membrane surface can be brought into view in the course of a few seconds to permit an evaluation of its condition. With practice, it is possible to complete the observations within ± 10 seconds of the five minute period.

Criteria of lytic action

Tyler (1939) described a method for the assay of egg membrane lysin (EML) which depends upon the time required for the complete dissolution of the membrane. With very active preparations the accuracy of this method is reported to be about ± 5 percent for the different eggs in any test.

In the experience of the writer, this method has proved impractical for routine assay of moderately active or weak preparations. With very active preparations in which the membrane is dissolved within one or two minutes, it is not difficult to determine the end-point - i.e., the instant

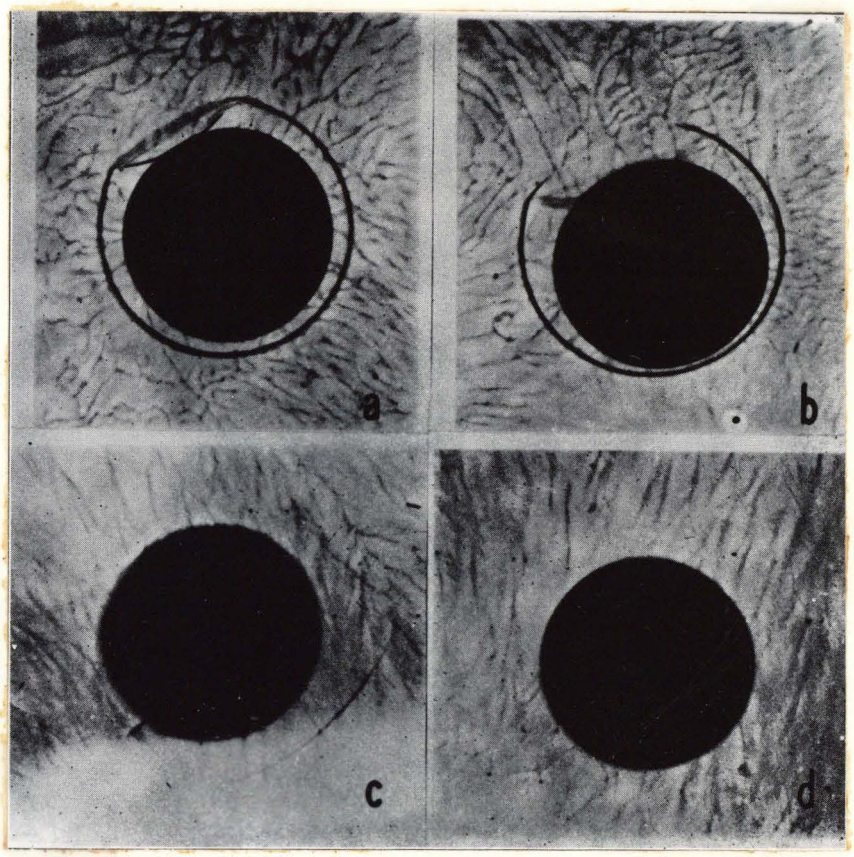


Figure 11. Action of sperm extract on egg membrane of Megathura crenulata. Successive stages of dissolution are shown. From Tyler, 1939.

at which the last trace of the membrane disappears. The process of membrane dissolution is illustrated in figure 11. In less active solutions, however, in which the rate of dissolution is slower than this, the instant at which the membrane can be said to have completely disappeared is very difficult to determine. Depending upon the activity of the solution, traces of the membrane in the different eggs of a test may be seen to persist for many minutes or even hours. An example of results obtained in attempting to determine the dissolution-time with a moderately active preparation is presented in Table X.

The data shown in the table were obtained by observing the time required for apparently complete dissolution of the membrane of each of 10 eggs at room temperature in an extract of frozen-thawed Megathura sperm diluted 8-fold with sea water buffered at pH 8.1 with 0.02M barbiturate. It is seen from the table that the average deviation from the mean dissolution time is ±29 percent. Since assays of preparations containing but a fraction of this activity were required, some other method of estimating their activity was necessary.

The assay method that was finally adopted depends upon the appearance in the membrane of indentations when eggs are treated with lytic preparations (see figure 11a). Indenting of the membrane may be the first indication of lytic action. This is followed, or accompanied by thinning and swelling. Non-lytic agents may effect indenting of the membrane, but this is not accompanied by dissolution or swelling of the membrane. The highest dilution of a lytic preparation which causes the appearance of a single indentation in the membrane of at least two out of three eggs in a period of five minutes is defined as the apparent titer. The concentration of lysin represented by the apparent titer is defined as one lysin unit.

TABLE X

Time required for complete dissolution of the membrane of each egg in a set of 10 treated in aliquots of a moderately active EML solution.

	Egg											
	1	2	3	4	5	6	7	8	9	10	Mean DT	St'd dev
DT*	4.5	6.8	6.0	8.8	16	5.0	8.5	7.6	7.8	12.5	8.6	29%

*DT = dissolution time in minutes.

Arbitrary limits of the dimensions of an indentation that would be scored as "one" indentation had to be established and adhered to. In optical section, the membrane appears as a circle, hence the size of an indentation in it can be expressed in terms of degrees of arc. To be scored as one indentation, not less than 30° or more than 90° of arc must be involved. The diagrams shown in figure 12 illustrate this. In practise, it was not difficult to observe these limitations.

Because indenting of the membrane is not a specific effect the indentation assay method could be used only after lytic action of a substance was established. For this, the definitive criterion is membrane dissolution, or obvious swelling and thinning of the membrane. Degree of membrane swelling could be estimated by measuring increase in the diameter with an ocular micrometer. This method could not be used for assay, since increase in membrane diameter is usually accompanied by sufficient distortion to render the membrane no longer spherical. Estimates of percentage increase in membrane diameter, however, were often used in conjunction with the indentation method.

Validity of the indentation assay method

The basic assumption with any assay method for the estimation of biological activity of a substance is that there is a relation between the phenomenon measured and the amount or concentration of active material. In the present case, data have been obtained which indicate that a direct relation exists between the number of indentations produced in an egg membrane in five minutes and the concentration of lytic substance in the test solution.

Relation between number of indentations and dilution. A number of experiments was performed in which eggs were treated in each of several dilutions of different Megathura sperm extracts for a period of five

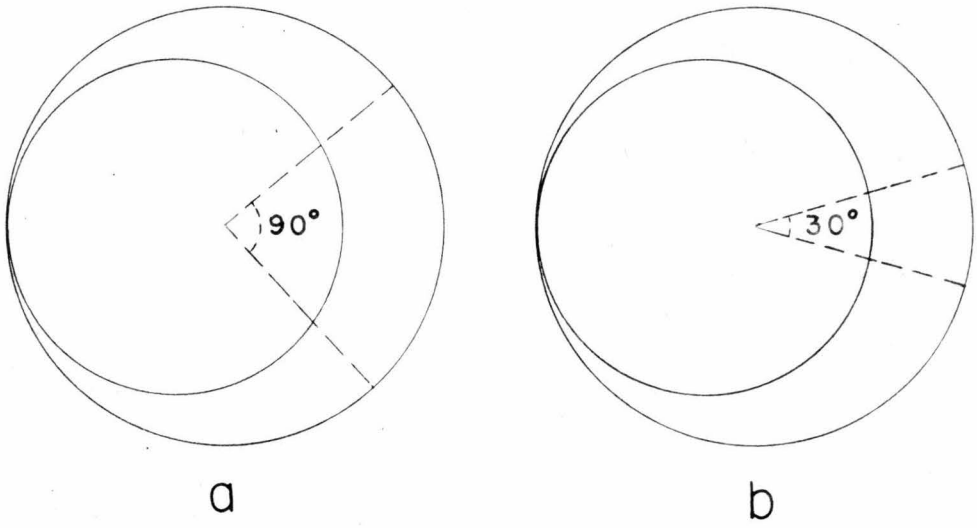


FIGURE 12. LIMITS OF INDENTATIONS SCORED AS "ONE".

minutes. In all the tests, the number of indentations appearing in the egg membrane in five minutes bears an inverse relation to the dilution of the extract. The results of these experiments are presented in Table XI. Each test was made using as diluent, sea water buffered at pH 8.1 with 0.02M barbiturate. The presence of barbiturate in this concentration appears to have no effect on lytic activity. All tests were performed at room temperature with 2-fold dilutions of the sperm extracts. Due to the difficulty of counting accurately the number of indentations where more than two or three appear in the membrane, only three dilutions of each extract were tested. A preliminary titration showed the approximate end-point. It should be observed here, that in most cases, it is easy to ascertain the presence or absence of a single indentation in the membrane. In a few instances where there ~~was~~ is doubt concerning the presence of an indentation, the practise has been to score these as zero. It is seen from the data presented in the table that in tests in which eggs are treated in several 2-fold dilutions of an extract there is a consistent decrease in the observed number of indentations per membrane with increasing dilution. There is, furthermore, a pronounced tendency toward greater consistency in the recorded number of indentations per membrane per dilution in dilutions in which the mean number of indentations per membrane is on the order of one. Where multiple indentations are produced the scoring method becomes increasingly less accurate.

Relation between dilution, dissolution time, membrane swelling and number of indentations. In another experiment, 2-fold dilutions of an active extract of frozen-thawed Megathura sperm were made up in sea water buffered at pH 8.1 with 0.02M glycylglycine. Five to seven eggs were tested with each dilution. The data from this experiment are recorded in Table XII. These show that with increasing dilution the mean time

TABLE XI

Number of eggs showing 0,1,2,3 and > 3 indentations in each of 3 2-fold dilutions in 5 minutes. Assays of 6 different extracts. Dilutions are numbered consecutively, no. 1 being the lowest and no. 3 the highest.

		Dilution														
		1					2					3				
	No. of indent.	0	1	2	3	>3	0	1	2	3	>3	0	1	2	3	>3
Assay	1	0	3	4	1	2	0	10	0	0	0	9	1	0	0	0
	2	0	3	5	2	0	1	8	1	0	0	7	1	0	0	0
	3	0	0	5	1	3	0	7	3	0	0	4	5	1	0	0
	4	0	4	1	4	0	0	10	0	0	0	9	1	0	0	0
	5	0	3	5	2	0	1	7	2	0	0	5	5	0	0	0
	6	1	5	5	2	2	2	12	1	0	0	12	3	0	0	0

TABLE XII

Dissolution time, membrane swelling and number of indentations in 2-fold dilutions of a sperm extract.

Dilution	Mean D.T. (min.)	Mean percent increase in diameter			Mean no. indentations in 5 min.
		½ hr.	1 hr.	3½ hrs.	
1	3				
2	8				
4	13				
8	28				
16	80				
32	145				
64	-			150	3½
128	-		35	100	2
256	-	15	23	35	1
Control (buffered sea water)	-	-	-	-	-

for complete dissolution increases correspondingly. At a 64-fold dilution, the membranes were not dissolved in 3½ hours, but they showed an average increase in diameter of 150 percent in this time. In this dilution, 3 to 4 indentations appeared in the membranes in five minutes. In the next higher dilution (128) the mean number of indentations appearing in the membranes at five minutes was 2. In 3½ hours, these eggs showed a mean increase in membrane diameter of 100 percent. In the highest dilution tested (256) the mean number of indentations per membrane at 5 minutes was one. The mean increase in membrane diameter in 3½ hours was 35 percent. The eggs in this dilution were observed at 20 hours. The membranes were still present, but now showed an average increase in diameter of 90 percent. These data are representative of numerous observations, all of which have shown that in time a significant increase in membrane diameter is produced by EML solutions whose activity is sufficient to cause but one indentation in five minutes. Membranes of eggs in buffered, or unbuffered sea water show neither indentations nor swelling in periods up to 24 hours.

It is apparent from a consideration of these data that activity equivalent to one lysin unit is not the least that can be determined. It is, however, an amount that can be conveniently determined in a time which makes practicable routine application of the indentation method.

Correlation between total nitrogen per lysin unit in different EML preparations. Evidence to be presented later indicates that EML is of protein nature or associated with protein. Lytic extracts prepared in the same way by essentially identical procedures might be expected to contain approximately equivalent amounts of total nitrogen per lysin unit. Several extracts were prepared by similar procedures, titrated by the standard indentation method, and total N determinations made by the

TABLE XIII

Total nitrogen per ml per lysin unit for each of 5 EML preparations after dialysis against sea water.

Extract	Non-dialyzable N mg/ml	Apparent titer	Total N/ml/lysin unit, γ/ml
1	0.016	24	0.7
2	0.029	32	0.9
3	0.038	100	0.4
4	0.095	256	0.4
5	0.050	64	0.8

micro-Kjeldahl procedure. Total nitrogen per lysin unit was obtained by dividing the total nitrogen per ml of the undiluted extract by the titer. The value so obtained is actually total nitrogen per ml per lysin unit, but for convenience is expressed simply as nitrogen per lysin unit. As shown in Table XIII the values for 5 different preparations range from 0.4 to 0.9 micrograms N per lysin unit. The greatest variation is thus slightly more than 2-fold. The results suggest, therefore, that within the limits of the titration method, approximately equivalent amounts of nitrogen per lysin unit are found in different extracts prepared under comparable conditions.

Reproducibility of assays by the indentation method. For the purpose of determining the reproducibility of assays made by the indentation method, a number of assays of each of several EML preparations were made using from 3 to 10 eggs for each. Titer was taken as that dilution in which 2/3 or more of the eggs tested showed one indentation in the membrane in 5 minutes. As shown in Table XIV, identical titers were obtained in all but 2 sets of assays.

Modification of the indentation assay method

In connection with some experiments to be described in a later section, it was desirable to attempt to differentiate between smaller than 2-fold differences in lytic activity. For this purpose, a preliminary titration was made by the standard procedure and the apparent titer determined. Three two-fold dilutions were then prepared, one corresponding to that giving the titer and the others the next higher and lower dilutions respectively. Each of these 3 dilutions was tested with 10 to 20 eggs and the number of indentations appearing in each egg in each dilution recorded. The mean number of indentations per egg per dilution was calculated and the values so obtained were plotted on graph paper with

TABLE XIV

Multiple assays of each of several different EML preparations. The numbers in parentheses indicate the number of assays giving the indicated titer.

Extract	No. of assays	Titer from assays
1	7	128 (5) 128 (2)
2	3	64
3	3	16
4	3	32
5	3	256
6	6	128 (5) 128 (1)

dilution as the abscissa and mean number of indentations the ordinate. The points were connected by straight lines. The titer was taken as the point of intersection of the line drawn through two of these points and a line drawn through the ordinate at one (1 indentation per egg). The point of intersection of these two lines is also referred to as the "one-intercept".

Where it was desired to compare the activity of different preparations by this method, the "one-intercept" values were plotted with appropriate coördinates and the points connected by straight lines to give a curve. Several examples of the application of this method will be presented in detail later.

Determination of antifertilizin in sperm extracts.

One of the problems in this investigation was to attempt to determine if the presence of antifertilizin is necessary for lytic activity of sperm extracts. In this connection, two methods for the estimation of antifertilizin were used.

Determination of antifertilizin by the inhibition method. The reaction of Megathura antifertilizin in solution with homologous fertilizin in the jelly hull of eggs does not result in agglutination of the eggs nor in formation of visible precipitation membranes. Antifertilizin activity of sperm extracts, therefore, cannot be determined directly by action on eggs, but it can be determined by means of an inhibition test.

For the test, a drop of a 1 percent suspension of sperm is added to 2 drops of sperm extract in a Syracuse dish. Two drops of homologous egg water (fertilizin solution) are then added. If antifertilizin is present in the sperm extract, it will neutralize some of the added fertilizin and, as a result, agglutination of the sperm will be delayed in comparison with agglutination time of an equal volume of sperm in sea

water. With sufficiently active antifertilizin preparations sperm agglutination may be completely inhibited in the undiluted extract. In any event, the titer of antifertilizin in the sperm extract is given by the lowest dilution in which agglutination time equals that of the control within limits set by the scoring system.

Tyler (1940) has described a scoring system for Megathura sperm agglutination by homologous egg water which depends upon the time for the appearance of macroscopic agglutinates. In the present study, the time for appearance of microscopic agglutinates has been taken as the basis of the scoring system. If agglutination is visible in 10 seconds or less, the reaction is scored 4-plus; in 10 to 30 seconds, 3-plus; in 30 to 60 seconds, 2-plus; in 60 to 120 seconds, 1-plus; over 120 seconds, plus-minus. In practise, titrations have not often been performed, since the significant point was the presence of absence of antifertilizin in the undiluted extract. In the data presented in connection with extraction procedures, the presence of antifertilizin in an extract is indicated by plus and its absence by minus.

Determination of antifertilizin by reaction with sea-urchin egg jelly. It was found in connection with this study that extracts of frozen-thawed Megathura sperm cross-react with sea-urchin fertilizin. Eggs are agglutinated and precipitation membranes are formed on the jelly. These may contract to the egg surface. Since certain non-specific substances, e.g., basic proteins, agglutinate sea-urchin eggs and produce precipitation membranes on the jelly (cf. pg. 60), it was important to obtain evidence that the agent in Megathura sperm extracts responsible for these effects is not such a non-specific agent. A criterion was available by means of which it could be established whether the Megathura sperm agent is antifertilizin or a non-specific agent. This is the fact that antifertilizin

does not agglutinate homologous sperm (Metz, 1949).

A frozen-thawed extract of Megathura sperm, which produced strong precipitation membranes on eggs of S. purpuratus and also agglutinated them, was tested with homologous sperm, and also with sperm of S. purpuratus. In neither case were the sperm agglutinated.

Testing sperm extracts, then, with sea-urchin eggs provided a simple and rapid method for detecting the presence of antifertilizin in Megathura sperm extracts. As in tests with homologous sperm extracts, some, or all eggs in a test might be stuck to the dish without precipitation membranes being visible. Antifertilizin was considered to be absent in an extract, therefore, in case precipitation membranes were not apparent, only if no treated eggs stuck to the dish.

2. Extraction of EML from sperm. Separation of lytic and antifertilizin activities.

Extraction of EML and antifertilizin by freezing and thawing sperm

Tyler (1939) showed that an extract obtained by freezing and thawing a sea water suspension of Megathura sperm exhibits both lytic and antifertilizin activities. It was found that if such an extract is heated for 2 minutes at 60° the lytic activity is destroyed, but the antifertilizin activity is unimpaired.

A preliminary attempt was made to determine whether lytic and antifertilizin activities could be separated from an extract of frozen-thawed sperm by isoelectric precipitation.

Method. A 10 percent suspension of washed sperm (cf. Basic Methods) in sea water was frozen in a mixture of dry ice and acetone, and then allowed to thaw at room temperature. As a result of this treatment, the sperm were coagulated into a compact jelly-like mass. The supernatant was recovered by centrifuging in a refrigerated centrifuge. Its pH was about 6.5; it was somewhat opalescent, green in color and foamed readily. In order to remove any remaining sperm fragments, the supernatant was filtered through a layer of washed asbestos in a sintered glass funnel of medium porosity.

A 50 cc portion of the filtrate was dialyzed at 1° against 5 changes of distilled water. Upon removal from the dialysis bath a copious white precipitate was observed to have appeared. This was removed by centrifugation (all centrifugations were performed in a refrigerated centrifuge) and suspended in 50 cc of sea water, in which it did not immediately dissolve. The suspension (Fraction I in Table XV) was allowed to stand overnight in the refrigerator. In the morning, it was found that the precipitated material had not dissolved appreciably. The pH of the suspension

was now 6.5. The pH of the supernatant (Fraction II in table XV), which had also been left in the refrigerator overnight, was 5.2.

Attempts to dissolve the precipitated material of fraction I by continuous stirring in the cold for 6 hours, keeping the pH between 8 and 9 were not obviously successful. Tests were then made to determine lytic and antifertilizin activities of fractions I and II. At this time the indentation assay method had not been developed and tests for EML were made by estimating membrane dissolution time with a number of eggs. Antifertilizin was determined by the inhibition test.

For the tests the undissolved material in fraction I was removed by centrifugation, and the clear supernatant adjusted to pH 8. A portion of fraction II was diluted with an equal volume of double sea water. This is designated fraction IIa in Table XV. Both II and IIa were adjusted to pH 8.

Results of the tests. The results of these tests are summarized in Table XV. From the table, it is seen that neither fraction II nor IIa showed any detectable lytic activity, whereas fraction I exhibited about 1/12 the activity of the original extract. Antifertilizin activity, however, was found in both fraction I and IIa (fraction II was not used for antifertilizin determinations). Comparison of antifertilizin activity of fractions I and IIa were made by diluting I with an equal volume of sea water. Although inhibition titers were not obtained, agglutination of sperm occurred more slowly in I than in II, indicating that more antifertilizin was present in the former fraction.

Extraction of EML and antifertilizin from living sperm

An attempt was now made to determine if EML could be recovered from living sperm. There was good reason for thinking that antifertilizin might be extracted by treatment of sperm in acid sea water, since this

TABLE XV

Lytic and antifertilizin activity of a frozen-thawed sperm extract and of fractions obtained by isoelectric precipitation.

Fraction of extract	Mean D.T.	Antifertilizin activity
Original extract (pH adj. to 8)	1 min.	+
Fraction I	12 min.	+
Fraction II	-	*
Fraction IIa	-	+

*Not tested

method is effective in removing antifertilizin from sea-urchin sperm (Tyler and O'Melveney, 1941). It was of interest, therefore, to see if EML as well as antifertilizin could be obtained in this way from Megathura sperm.

Methods. A suspension of approximately 5 percent dry, washed sperm in sea water was adjusted to pH 4.5 and allowed to remain at this pH at room temperature for 30 minutes. At the end of this time the majority of the sperm appeared still active. The sperm were removed by centrifugation. The supernatant (Fraction I) was adjusted to pH 8.

The sperm residue was resuspended in the original volume of sea water and the pH adjusted to 8.5. After one hour at room temperature the suspension was centrifuged. The supernatant (Fraction II) was now at pH 7.3. This was adjusted to 8. Most of the sperm appeared still to be alive.

Results. When tested, fraction I (acid extraction) exhibited antifertilizin activity and no EML activity, whereas fraction II (alkaline extraction) showed EML activity but no antifertilizin activity.

Differential recovery of EML and antifertilizin from living sperm at alkaline and acid pH

The results of the preceding experiment suggested that it might be possible to extract EML and antifertilizin separately from living sperm by treatment in alkaline and acid sea water respectively. Whether initial treatment in acid sea water is necessary for recovery of EML was investigated in the following experiment.

Method. A suspension of washed sperm (ca. 5 percent) in sea water was adjusted to pH 8.2 and left overnight in the refrigerator. In the morning, the sperm were still alive, as shown by their being activated by egg water. The suspension was centrifuged, and the supernatant filtered

through washed asbestos in a sintered glass funnel of medium porosity. No sperm or sperm fragments could be seen in the filtrate, which was clear, green-tinged and slightly foamy. The pH of the filtrate was 7.8. It was tested for EML and antifertilizin.

Results. Lytic activity was present in the filtrate, but no antifertilizin could be detected by the inhibition test.

The results of this experiment indicated that preliminary treatment of sperm at acid pH is not necessary for removal of EML in alkaline sea water.

Several experiments were now performed in which the effect of pH on the extraction of EML and antifertilizin from living sperm was sytematically investigated. The aim of these experiments was to obtain an EML - antifertilizin "spectrum" over a wide range of pH.

Methods. In these experiments, different lots of sperm were used at different times. A standard procedure was followed, however, so that the results from different experiments are comparable. The only exception to this was where it was desired to extract sperm at pH higher than about 9.5. In this case, it was necessary to suspend the sperm in 3.3 percent saline rather than in sea water, since certain of the sea water salts precipitate at about pH 10.3.

To determine the effect of pH on extraction of EML and antifertilizin over the range from 3 to 9.5, 4 ml aliquot portions of a 5 percent suspension of washed sperm in sea water were placed in each of a number of 10 cc beakers. The pH of each sample was adjusted with NaOH or HCl. Rapid and fairly precise adjustment could be achieved by using 0.5N and 0.1N reagents. The beakers were then placed in a 22° water bath. Every 15 to 30 minutes the pH of each sample was checked and readjusted if necessary. In doing this, the suspensions were thoroughly stirred. After 4 hours, each sample was poured into a separate graduated 15 ml

centrifuge tube and made to a volume of 5 ml with sea water which had been used to rinse the beaker. The suspensions were centrifuged in a refrigerated centrifuge for 15 minutes at about 3000 rpm. The supernatants were filtered through asbestos, equal portions of asbestos being used for each filtration. The filtrate were adjusted to pH 8. Since this usually required not more than 4 or 5 drops of reagent, no attempt was made, as a rule, to equalize the volumes. The filtrates were tested immediately for EML activity by the indentation assay method. Tests for antifertilizin were made either by the inhibition method or precipitation membrane test with sea-urchin eggs.

The same procedure was used for the determination of the effect of pH above 9.5 except that the sperm were first washed two or three times with 3.3 percent saline, and the 5 percent suspension then made up using saline.

Results of the experiments. The results of the several experiments are summarized in Table XVI. The EML titers shown in the table represent average values from the results of 2 to 4 tests, except above pH 9.5. Only one experiment was done at pH higher than 9.5. In the table, the presence or absence of antifertilizin is indicated by a plus or a minus sign.

The data show that up to about pH 6, only antifertilizin is recovered, while above this, increasing amounts of EML are obtained but no antifertilizin that can be detected by either of the methods used. Later, it was found that with more concentrated sperm suspensions, e.g., 25 percent, some antifertilizin may be obtained up to pH 6.5. Even with these concentrated suspension, however, no antifertilizin is detectable in extracts if the pH is maintained above 6.5.

It is of interest that at pH 9.5 to 10.5, there is a large increment

TABLE XVI

Effect of pH on release of EML and antifertilizin from living sperm.

pH	EML Apparent titer	Antifertilizin activity
2-3	0	-
3-4	0	+
4-5	0	+
5-6	0	+
6-6.5	1	tr
6.5-7.5	4	-
7.5-8.5	8-16	-
8.5-9.5	32	-
9.5-10.5	64	-*

*Extract in 3.3% saline dialyzed vs. sea water before testing for antifertilizin.

(4-fold or more) in the amount of EML recovered, compared with the amount recovered at about pH 8. Above pH 10.5, the sperm are disintegrated into a gel-like mass from which very little fluid can be recovered by moderate centrifuging.

Effect of temperature on release of EML

It was known that "dry" Megathura sperm remain viable for at least several days if kept at a low temperature (1°). The possibility was considered, therefore, that sperm in a sea water suspension might remain alive for a relatively extended period if kept at 1°. Since it was desired to extract living sperm as exhaustively as possible to recover maximal amounts of EML, an experiment was undertaken to determine the effect of temperature on the release of EML.

Method. A 5 percent suspension of washed sperm was prepared in sea water buffered at pH 8.2 with glycylglycine (0.02M). Several aliquots were removed and each of these allowed to stand for 1 hour in a water bath at a given temperature. The temperatures tested were 1°, 5°, 10°, 18°, 22°, 30°, 40°. At the end of 1 hour, each sample was chilled in ice water, centrifuged and the supernatants filtered. The pH of each filtrate was checked with the glass electrode, and in no case was the pH below 7.9 or above 8.2. EML assays were performed immediately. The sperm from each sample were tested with egg water.

Results of the experiment. As shown in Table XVII, identical titers were obtained with the samples extracted at 5° to 30°. A significantly lower titer was shown by the sample extracted at 40°, and a questionably lower titer by the aliquot extracted at 1°.

When the sperm from each aliquot were treated with egg water, no difference in activation and agglutinability of the sperm that had been exposed to temperatures from 1° to 22° was found, all being 100 percent activated and agglutinated. On the other hand, sperm that had been treated at 30°

TABLE XVII

Release of EML at different temperatures.

Aliquot	Temperature, °C	EML titer
1	1	8-16
2	5	16
3	10	16
4	17	16
5	22	16
6	30	16
7	40	4-8

and 40° showed 50 percent and 0 activation and agglutination respectively.

It is a matter of some interest that approximately identical titers were obtained with sperm suspension extracted at temperatures of from 1° to 30°. A more rapid falling off of the titer might be expected at the highest temperature (40°) in view of the heat lability of EML. Inactivation at this temperature, however, may be balanced by release of lysin. The fact that lysin release is seemingly independent of temperature from 1° to 30° suggests that EML is not actively secreted by sperm.

The extracts were not tested for antifertilizin, so it is not known whether this was released from sperm treated at the higher temperatures. The progressive impairment of the capacity of these sperm to be activated and agglutinated by egg water may mean that antifertilizin had been extracted from them, or it may reflect impairment of their viability.

Longevity of sperm at different temperatures.

Sperm have been kept at 1° in a viable condition, as shown by activation when treated with egg water, for as long as 5 days in 5 percent suspension and 12 days in 20 percent suspension. In both cases, the experiments were terminated as a result of considerations other than death of the majority of the sperm. The author does not doubt that the sperm would have remained alive longer. On the other hand, fresh sperm in a 5 percent suspension survived less than 24 hours at 18°.

3. Examination of sperm treated in alkaline and acid sea water through the light microscope and the electron microscope

It was of interest to determine whether any difference could be seen in the microscopic appearance of sperm treated in sea water at alkaline and acid pH. Samples of treated sperm, fixed in 10 percent formalin in sea water, were therefore, examined. Observations were made both with a light microscope and the electron microscope*. A number of photomicrographs and electron micrographs were made. Samples of sperm fixed immediately after removal from the animal were taken as normal specimens.

In order to determine if fixation in formalin might produce artifacts in the appearance of the sperm, living and fixed (normal) sperm were compared under the light microscope at high magnification (to 1800X). So far as could be ascertained, fresh, untreated sperm fixed in formalin appeared no different from comparable living sperm.

Appearance of normal sperm. The overall length of a spermatozoon, exclusive of the tail, is about 4 micra. Of this, the pointed acrosome accounts for nearly 1/4, the head and midpiece the remainder. The greatest width is about 1 micron. In figures 13, 14 and 15 are shown a photograph and electron micrographs of normal sperm. It is seen from the pictures that the outline resembles that of an artillery shell. In the photograph it is apparent that the acrosome and midpiece are distinguished from the rest of the head in being of optically different material, thus appearing darker in unstained preparations. The long tail emerges from the midpiece. In figure 15 the tail is seen apparently emerging from the tip of the acrosome. This may represent an agglutination phenomenon, in which the tail is stuck to the head in the manner shown in the picture.

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*Studies with the electron microscope were made possible by the cooperation of Mr. Burton L. Henke, who operated the instrument and made the electron micrographs.

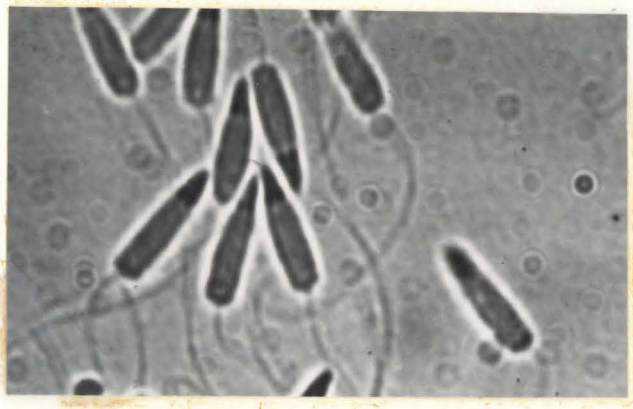

Figure 13. Photomicrograph of untreated sperm. X3800.

Figure 14. Electron micrograph of untreated sperm. X14000.

Figure 15. Electron micrograph of an untreated spermatozoon showing tail stuck to the head. X14000.

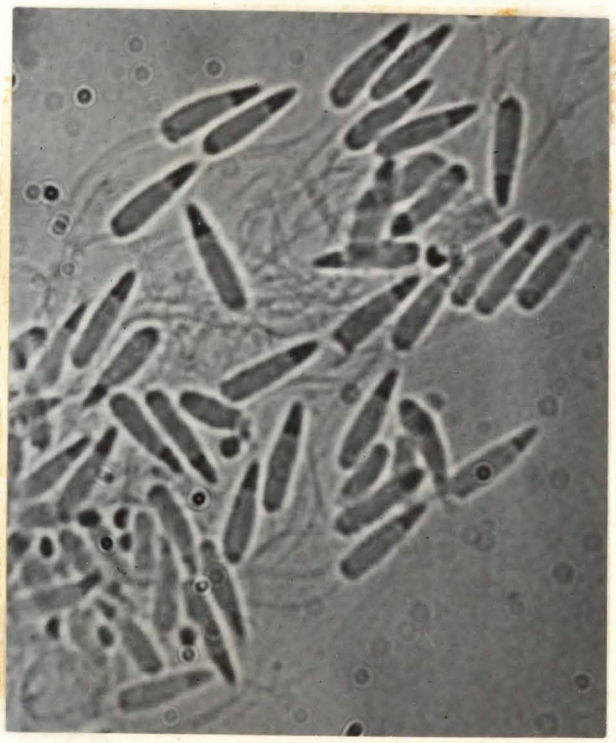

Figure 16. Photomicrograph of sperm treated at pH 7-8 for 4 days at 1°. X3800.

This is seen rather frequently.

Appearance of sperm after treatment in moderately alkaline sea water (pH 7-8). Figure 16 is a photograph of a portion of a sperm suspension that had been extracted in the pH range 7 to 8 with 5 changes of alkaline sea water at 1° over a period of 4 days. The sperm shown in the picture are representative of the majority of those of this and of comparable suspensions examined. It can be seen that most of the sperm appear no different from untreated sperm shown in figure 13. In two sperm shown in figure 16, however, the acrosome is clearly seen to be displaced. In some sperm, the midpiece as well as the acrosome may be broken off. This type of fragmentation is seen with higher frequency in suspensions treated at low and high pH than in the mildly alkaline range. Figures 17 and 18 are electron micrographs of sperm from and acid-treated suspension and show the acrosome and midpiece broken from the head. Fragmented sperm are occasionally seen in untreated samples. Less conspicuous aberrations that are found in all suspensions, but with less frequency in those exposed to slightly alkaline pH than in those treated at low and higher pH, are the appearance of light bands demarcating acrosome and midpiece from the head, and varying degrees of rounding of the acrosome (figures 19, 20 and 21).

In order to form an estimate of relative damage sustained by sperm treated at various pH's, counts were made of fragmented sperm (either acrosome or midpiece or both broken from head). No attempt was made to classify the different samples on the basis of smaller alterations in appearance. In untreated suspensions (normal sperm) not more than about 1 percent appeared fragmented in the samples examined. In suspensions treated at pH 7-8, 3 to 10 percent of the sperm were fragmented, depending, apparently upon the conditions of extraction. The higher percentages were

Figure 17. Electron micrograph of acid-treated, fragmented spermatozoon. X14000.

Figure 18. Electron micrograph of acid-treated fragmented spermatozoon. X14000.

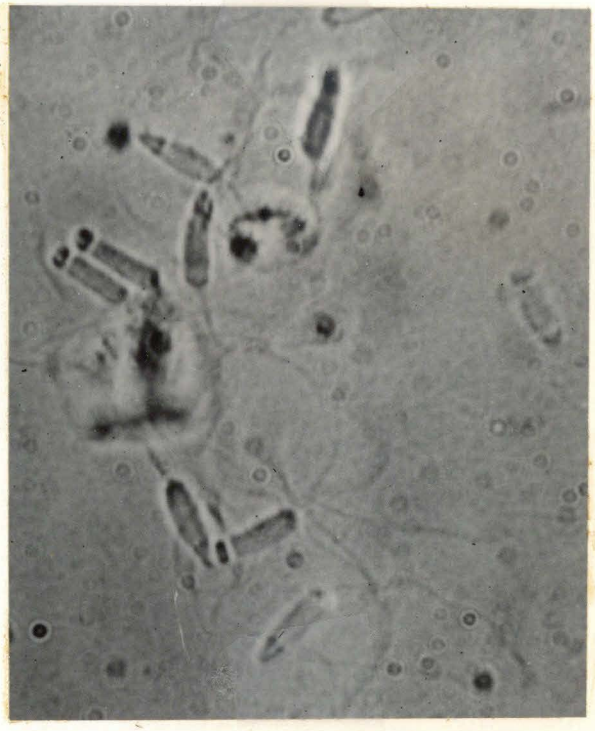

Figure 19. Photomicrograph of acid-treated sperm showing rounded acrosomes and light bands. X3800.

Figure 20. Electron micrograph of acid-treated sperm showing rounded acrosome. X14000.

Figure 21. Electron micrograph of acid-treated spermatozoon showing displaced, rounded acrosome. X14000.

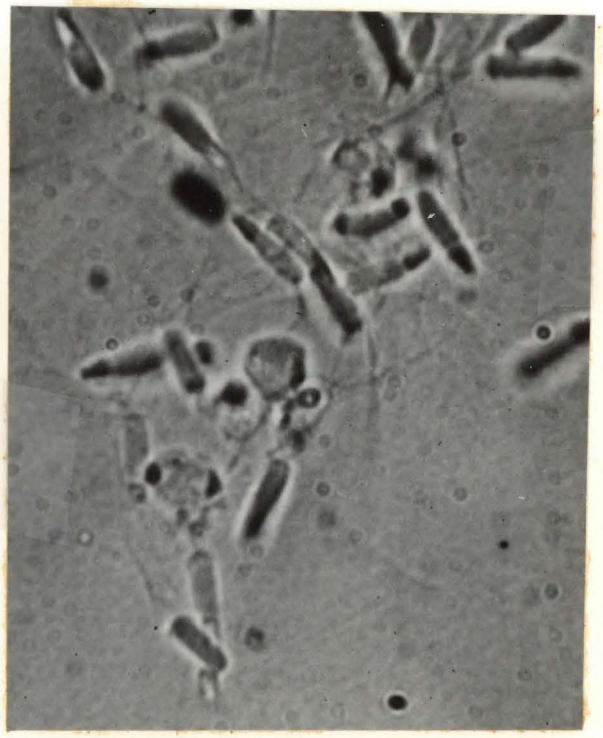

Figure 22. Photomicrograph of acid-treated sperm. Two sperm with disintegrated heads are shown. X3800.

observed in samples taken from suspensions that had been treated for longer periods. Other factors may influence the relative frequency of damaged and undamaged sperm in a suspension. Variations in pH, the concentration of the suspension and temperature, for example. No attempt was made to analyze the effect of these different factors.

Appearance of sperm after treatment at low and high pH. Samples of sperm were fixed after treatment for 1 hour at about pH 4 at 1°. The majority of these exhibited marked morphological changes as compared with sperm treated for even extended periods at pH 7 to 8. The percentage of fragmented sperm in acid-treated suspensions was on the order of 25 percent. Perhaps the most striking feature, however, was the tendency of acid-treated sperm to aggregate in large clumps in formalin-sea water. Sperm treated at pH 7-8 did not exhibit this tendency. Individual sperm were difficult to examine in these masses, but the appearance of those on the periphery and in thinner parts suggested that the majority were not obviously different from the relatively few scattered individuals found. Occasionally, sperm were seen in which the head had undergone disintegration, as shown in figure 22. These were comparatively rare. Many of the non-fragmented sperm showed light bands separating the head from the acrosome and midpiece (figure 20). Also common among the non-fragmented acid-treated sperm were individuals in which the acrosome was conspicuously rounded, as in figures 19, 20 and 21. These aberrant types were more frequent among acid-treated sperm than among those treated at pH 7 to 8.

Sperm fixed after 4 hours treatment at pH 9.5 to about 10.5 (in 3.3 percent NaCl solution) at 22° showed, on the whole, rather striking similarity to acid-treated sperm. The percentage of fragmented sperm in these suspensions ranged from about 30 percent at pH 9.5 to about 75 percent at the higher pH. Here again, the sperm were aggregated into large masses in

formalin sea water and many small aberrations were seen. Sperm treated at the higher pH appeared, in general, to be more severely damaged than those treated at pH 9.5. In the former case, the nuclear region of the head of the majority of the sperm seemed qualitatively different from sperm heads exposed to lower pH's. After treatment at the high pH, the head appeared less dense and of a more granular nature. No satisfactory photographs were obtained of these specimens. In suspensions exposed to pH 10.5, many disintegrated sperm heads were seen. At still higher pH (about 11), sperm were completely disintegrated into a jelly-like mass.

Discussion. The observations presented here show that extraction in sea water at low temperature, even for comparatively long periods of time, at mildly alkaline pH, does not result in extensive visible damage to the majority of Megathura sperm. Treatment in both acid sea water (pH 4) and more strongly alkaline solution (pH 9.5 to 10.5) results in visible damage to a much greater percentage of the sperm. The percentage estimates of damaged sperm were based on counts made of fragmented specimens in different samples. If smaller aberrations were taken into account, the percentage of damaged sperm in these supensions would be higher.

There appeared to be no significant difference in percent of fragmented sperm in suspension treated at pH 4 and in those treated at pH 9.5. These, however, were not entirely comparable, since treatment at acid pH was at 1° for 1 hour, while at the alkaline pH treatment was at 22° for 4 hours. It is not improbable that at higher temperature and with longer treatment, the proportion of sperm damaged (fragmented) at pH 4 would have approached more nearly that seen in the suspension treated at pH 10.5 (75 percent).

The relative frequency of smaller aberrations shown by treated sperm, e.g., rounded acrosomes, appearance of light bands in the regions where the head joinds the acrosome and midpiece, was not estimated.

Of interest is the fact that sperm treated at both low and high pH exhibited a tendency to aggregate in large masses, as though their surface had become sticky. Sperm from suspensions treated at neutral and mildly alkaline pH did not form such clumps. The masses showed no ordered arrangement - the sperm were seemingly disposed at random.

The qualitative difference observed in the head of sperm treated at pH 10.5 suggested that the sperm were near the threshold of disintegration. Treatment at $\frac{1}{2}$ pH unit higher, as a matter of fact, did result in disintegration.

With respect to release of EML, it can be said that at neutral and mildly alkaline pH - in a range in which EML is known to be released from sperm in sea water - the majority of individuals show no extensive visible damage. A small percentage, however, do exhibit some damage, and the frequency of damaged sperm increases with increasing pH in a manner which suggests a correlation with increased release of EML at high pH. At neutral and mildly alkaline pH, EML may be released mainly by the small fraction of visibly damaged and aberrant sperm. On the other hand, it might (also) be released from the surface of apparently undamaged sperm. Other evidence suggests that release of EML under mild pH conditions is not accompanied by death of many spermatozoa, since sperm have been extracted in sea water many days at low temperature, and the majority still respond to the stimulus of added egg water. It is likely that some EML is located at, or near the surface, and is released without visible change in the sperm, while more is situated beneath the surface, and release of this may entail more change in the sperm structure. This interpretation is supported by the fact that even after exhaustive extraction of sperm at low temperature in mildly alkaline sea water, significant EML activity is shown by extracts from the subsequently frozen and thawed sperm.

The increased frequency of damaged sperm observed at low pH suggests similar reasoning with respect to correlated EML release. Failure to detect EML here, may be due to irreversible inactivation of the lysin upon its release into the acid medium (section 6).

4. Release of EML and rate of oxygen uptake of sperm

A number of experiments was undertaken to determine if there is a correlation between release of EML and rate of oxygen consumption of the sperm. For this purpose, measurement of the rate of oxygen uptake was performed by use of the Warburg-Barcroft manometric method.

General methods

Preparation of sperm suspension. In order to be able to calculate rate of oxygen uptake per unit number of cells (ZO_2*), it was necessary to be able to estimate the number of sperm in a suspension. For this purpose, the method employed by Spikes (1948) in a study of the metabolism of sea-urchin sperm was utilized.

The density of sperm suspensions was determined with a Cenco photelometer. A calibration curve was prepared by measuring the density of various dilutions of a sperm suspension and plotting the values obtained (percent transmission) against the number of sperm per cm^3 determined by counting samples with a bright line hemocytometer. In order to utilize the middle range of the instrument, aliquots of the various suspension were appropriately diluted. A 20-fold dilution of a 5 percent suspension showed about 50 percent transmission using a distilled water blank. From the calibration curve which is shown in figure 23, it is possible to read directly number of sperm per cm^3, provided the percent transmission is known.

In order to obtain an estimate of the magnitude of error involved in making readings with the photelometer, several different samples of a suspension were taken, diluted in the same way, and the percent transmission of each read on the instrument. Each determination was made

*ZO_2 - ul gas exchange / 10^9 cells / hr. Conventional ZO_2 - rate / 10^8 cells. Due to the low respiratory rate of these sperm, 10^9 is a more convenient unit.

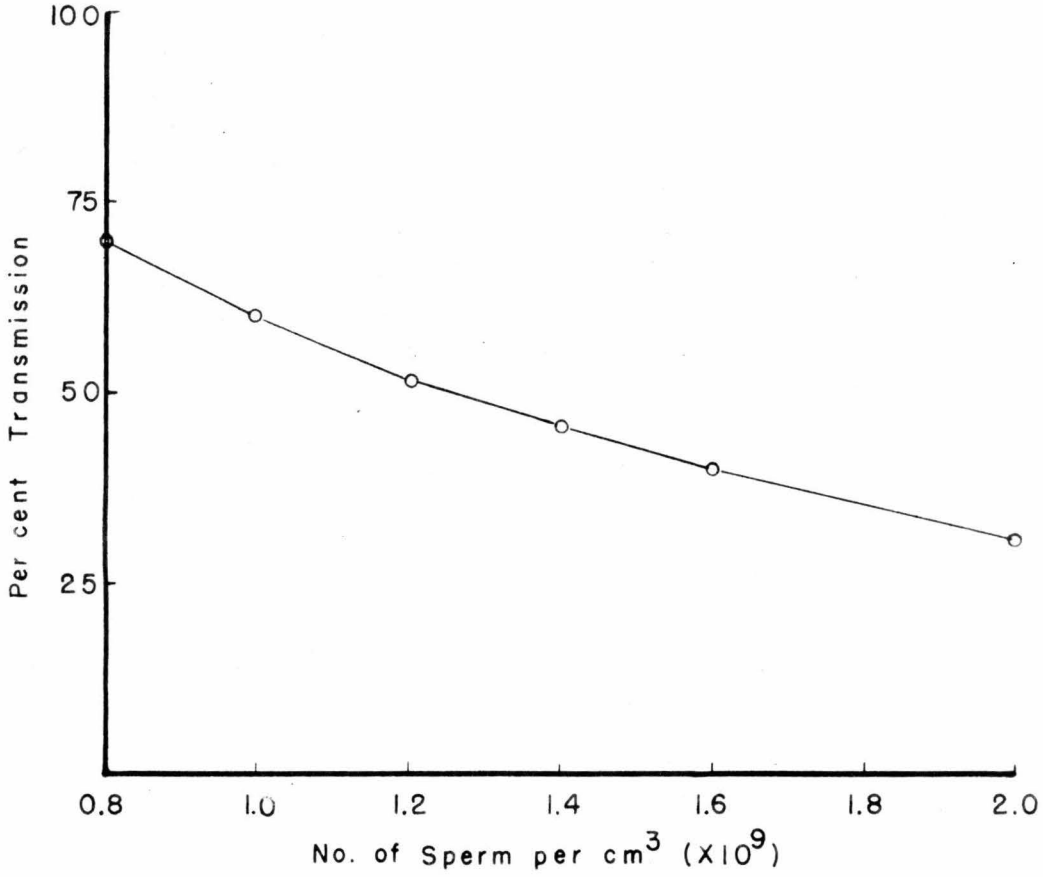

FIGURE 23. CALIBRATION CURVE. PER CENT TRANSMISSION VS. NO. SPERM PER CM^3.

rapidly, with no more care than would be used routinely. Table XVIII shows the results obtained. The average deviation from the mean is ± 1 percent.

For the runs, sperm suspensions were made up using artificial, carbonate-free sea water (formula developed by Tyler on basis of analysis by Lyman and Fleming, 1940), 0.025 molar in glyclglycine. This dipeptide is a suitable buffer to use with marine cells, since its pK' is 8.1 at 18.5° (Tyler and Horowitz, 1937). According to Tyler and Horowitz, in concentrations below 0.05M, glycylglycine has no particular effect on sea-urchin eggs. No data were available concerning its effect on Megathura sperm, but it was found in the present experiments, that it has no detectable effect in the concentration that was used. The most effective buffering range of glycylglycine is pH 7 to 9. Rather than attempt to use other buffers outside of this range, pH values lower than 7 and higher than 9 were attained by addition of sufficient HCl or NaOH to the suspensions containing the dipeptide. Variations in the pH of suspensions so treated were greater than those observed within the optimal buffering range, but, on the whole, were of small magnitude. This was especially true at acid pH.

Preparation of flask samples. To prepare the flask samples, 3 ml portions of a 5 percent sperm suspension were placed in beakers. The pH of each was adjusted to the desired value. The percent transmission of each sample was then determined with the photelometer. A 2 ml aliquot of each sample was placed in each manometer flask, except the thermobarometer, into which 2 ml of sea water were pipetted*. The same pipet was used for each transfer.

*A different procedure was followed in cyanide experiments (pg. 100).

TABLE XVIII

Determination of percent transmission of aliquot portions of an approximately 5 percent sperm suspension. Aliquots diluted 20-fold. Read on a Cenco photelometer with distilled water blank.

Sample	per cent transmission	Sample	per cent transmission
1	50	6	49
2	48	7	50
3	50	8	50
4	49	9	50
5	49	10	50

In the center well of each flask was placed a piece of fluted filter paper with 0.3 ml of 5 percent KOH. In experiments in which it was desired to add egg water, 1 ml was placed in the side arm. In controls, 1 ml of sea water was placed in the side arm.

All runs were made at 22° with constant rate and amplitude of shaking (86 cycles per min., 3.5 cm.). Readings were taken at 15 minute intervals. Most runs were of 4 hours duration.

At the conclusion of the run, the contents of each flask was pipetted into each of several beakers, and the pH determined with the glass electrode. For EML assays, the suspensions were then transferred to each of several 12 ml centrifuge tubes and centrifuged for 10 minutes at about 3000 rpm. The supernatant from each tube was recovered, and the pH adjusted to about 8. The volumes were equalized with that of the supernatant requiring the greatest amount of acid or base by addition of an appropriate number of drops of sea water to the other solutions. Any slight inequalities in volume were probably not significant in the EML assays, which were immediately performed.

Relation of respiratory rate and EML release to pH.

Results. Several runs were made using different lots of sperm at pH values ranging from about 4 to 9.5. The results with different lots of sperm were comparable, provided the sperm was used within 48 hours after removal from the animals and had been stored dry at 1° for this period. Representative data are presented in figure 24. It is seen that from about pH 6 to 7 there is a slow increase in ZO_2 with a steeper rise in the curve above pH 7 to a maximum between pH 8 and 9. Above pH 9 the curve drops sharply. Below pH 6 (to about 4.5) the respiratory rate is very low, being on the order of 1 to 2 ul of oxygen per 10^9 cells per hour.

EML assays of supernatants from the various suspensions used in these

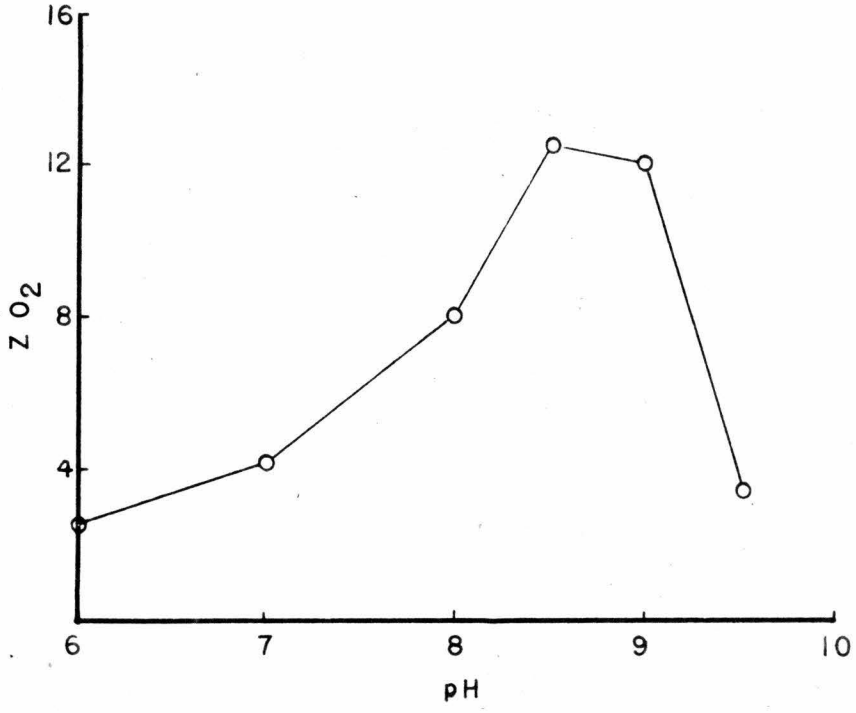

FIGURE 24. EFFECT OF pH ON RESPIRATORY RATE OF MEGATHURA SPERM. Z O_2 = μl PER HR. PER 10^9 SPERM.

experiments followed the same trend as in the pH experiments (section 2). Comparison of the data presented in this connection in section 2 with the curve shown in figure 24 indicates that EML release roughly parallels respiratory rate from pH 6 to about 9. Above pH 9, however, there is a marked difference. EML activity in extracts obtained above pH 9 shows a 4-fold or greater increase, whereas the respiratory rate drops sharply.

Discussion. The data from these experiments reveal a parallelism between respiratory rate and EML release from pH 6 to about 9, but above this there is a wide divergence. What this may mean with respect to interrelations between EML, other sperm constituents and metabolic pathways is a question, the answer to which is undoubtedly complex.

Nitrogenous materials other than EML are released from sperm under conditions favorable for EML release (alkaline sea water). The significance of these must be taken into consideration in any endeavor to evaluate the various factors involved in these relationships. The fact that a majority of sperm exposed to mildly alkaline sea water may show little, if any, visible structural modification does not mean that the loss of these substances may not be of significance in the economy of the sperm. One thinks immediately of the problem of sperm senescence. In connection with this, several experiments with aged sperm showed a significant diminution of the respiratory rate at physiological pH in comparison with fresh sperm. In other respects the data were incomplete and will not be presented here.

Among several possibilities that are suggested by the respiration-EML release experiments with respect to relation between the respiratory rate and release of EML, only one seemed easily susceptible to direct experimental attack. This was the possibility that release of EML is actually independent of the respiratory rate. Several experiments were performed

in order to obtain some evidence bearing on this. These are described in the following sections.

Two procedures were followed. In one, the respiratory rate of sperm cells was stimulated by addition of egg water, and EML release compared with that of non-stimulated sperm. In the other, respiration was inhibited by cyanide and comparison of EML release by treated sperm made with that by untreated sperm.

In preliminary experiments, it was found that EML activity is apparently not influenced either by egg water or by cyanide in the concentration in which this was used (10^{-4}M). It was assumed, therefore, that any significant difference in EML titer of supernatants from egg water or cyanide-treated suspensions, as compared with non-treated sperm, might be attributed to increased or decreased production related to metabolic activity of the cells.

Respiratory rate and EML release in the presence of egg water.

In two experiments, egg water was added to sperm suspensions in the manometer flasks. In one of these, the effect of egg water in the pH range 6 to 9.3 was investigated. In the second experiment samples were run only at pH 8. In each experiment, 1 ml of egg water at appropriate pH was tipped into the sperm suspension from the side arm of the flask one hour after the run was started. In controls, 1 ml of sea water was tipped into the sperm suspension.

The curves shown in figure 25 present the data concerning respiratory rate from the first experiment. It is seen that the rate was greatly increased over nearly the entire pH range in the presence of egg water. From pH 7 to 8 the increase was approximately 4-fold. Fresh sperm were used for these experiments.

In Table XIX are presented the results of the EML assays. Within the

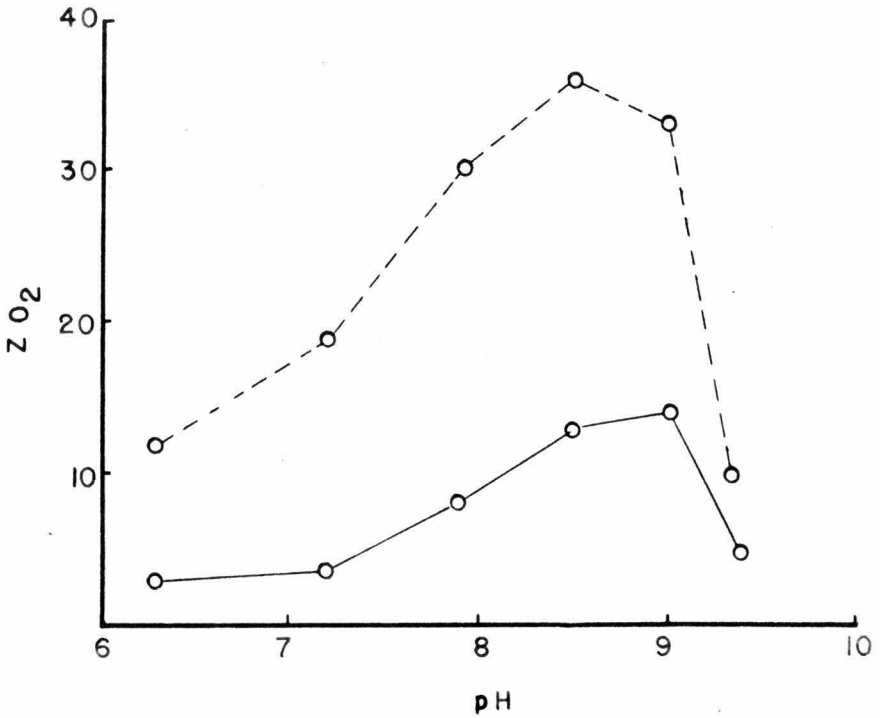

FIGURE 25. EFFECT OF ADDED EGG WATER ON RESPIRATORY RATE OF MEGATHURA SPERM. SOLID LINE = WITHOUT EGG WATER, BROKEN LINE = WITH EGG WATER. Z O_2 = μl O_2 PER HR. PER 10^9 SPERM.

TABLE XIX

EML titers of supernatant fluid from egg water-treated and non-egg water-treated sperm suspension.

	EML titers	
pH	without egg water	with egg water
6.3	1	1
7.2	4	2
7.9	4	4
8.5	8-16	8-16
9.0	8-16	16
9.3	not tested	not tested

TABLE XX

Respiratory rate (ZO_2) and EML titer of sperm suspensions treated with egg water and with cyanide (10^{-4}M).

With egg water			With cyanide		
Sample	ZO_2	EML titer	Sample	ZO_2	EML titer
1	13.4	16	1	1.8	16
2	12.9	16	2	1.2	16
control #1	6.2	16	3	1.5	16
control #2	6.8	16	control #1	6.5	16
			control #2	5.3	16

limitations of the assay method, it is seen that essentially similar EML activity was found in supernatants from egg water-treated and from control sperm.

Sperm used for the second experiment had been stored dry at 1° for about 60 hours. One run was made in which two flasks were run at pH 8 with added egg water and two with added sea water. At the conclusion of the run EML assays were made. The data obtained from this experiment are presented in Table XX. It is seen from the table that the addition of egg water resulted in but a two-fold increase in respiratory rate as compared with the controls. The EML activity of the supernatants, however, was again essentially the same, as indicated by the titers. These results are of especial interest since it was with sperm of this same lot that the cyanide experiment was performed on the same day under comparable conditions.

Respiratory rate and EML release in the presence of cyanide

The method proposed by Robbie (1946) was followed in preparing the sperm suspensions and flasks. No data were available concerning the effect of cyanide on Megathura sperm. Spikes (1948) reported that maximum inhibition of sea-urchin sperm respiration is produced by 10^{-4}M CN. It was decided to use this concentration in the present experiment.

For the experiment, 2 ml of sperm suspension were placed in each flask (except the thermobarometer). As in other experiments, the suspensions were prepared in artificial carbonate-free sea water, 0.025M in glycylglycine. In the present case, all flasks were run at pH 8. Into each of four of the flasks was pipetted 0.3 ml 10^{-3}M HCN. The volume of each of these suspensions was made to 3 ml by adding 0.7 ml of artificial sea water. To each of two control suspensions, 1 ml of sea water was added.

In order to achieve proper HCN equilibrium between the experimental

fluid within the flask and the center well liquid, Robbie found that at 22°, and with a concentration of 10^{-4}M HCN in the flask fluid, the center well fluid should be a mixture of 1.03M KCN, 0.5M in KOH. With 3 ml of experimental fluid, Robbie suggests that 0.6 ml of the KCN-KOH mixture be added to the center well containing two fluted filter paper strips. This procedure was followed. In the control flasks, the center well contained 0.6 ml of 0.5M KOH with double filter paper strips.

At the conclusion of the run, each supernatant was assayed for EML activity.

The results of this experiment are recorded in Table XX. It is seen that in the different CN suspensions, the rate of oxygen uptake ranged from about 15 to 35 percent of the control values. Taking the mean value as about 25 percent and comparing this with the approximately 100 percent increase in respiratory rate shown by the egg water-treated sperm in the preceding experiment, there is seen to be an 8-fold difference in rate of oxygen consumption. Comparison of EML activity of the various extracts, however, shows that this was of the same order in each case, since identical titers were obtained.

Discussion. The results of experiments in which the respiration of sperm was stimulated with egg water and inhibited by cyanide suggest that EML release is independent of the respiratory rate under the conditions of the experiments. This is in harmony with the data obtained from an earlier experiment in which the effect of temperature on EML release was investigated. It will be recalled that this experiment showed that from 1° to 30° the release of EML appeared to be essentially independent of the temperature. It was concluded from these results that EML is not released by an active secretory process which, presumably, would be sensitive to temperature differences.

It may be justifiable to assume as a working hypothesis, that in sperm suspensions of the concentration used in the experiments (5 percent), release of EML in sea water at physiological temperatures is mediated primarily by the pH of the medium.

5. Effect of pH, temperature and salt concentration on action of EML. Nature of the lytic reaction

The possibility that EML may act catalytically in lysis of the egg membrane was considered. In this connection, it was of interest to determine whether the action of EML is sensitive to environmental pH, temperature and salt concentration. It was also important to attempt to determine whether EML is used up in the lytic reaction. Experiments undertaken in order to try to elucidate these points are described in this ~~chapter~~ section.

Effect of pH, temperature and salt concentration on the action of EML

Procedures. In these experiments the action of EML was determined by means of the modified indentation assay method described in section 1. This method was designed to give an estimate of smaller than 2-fold differences in titer. A preliminary test was made of the reproducibility of results obtained. For this purpose ten separate assays were made of a preparation buffered at pH 8 with Michaelis' universal buffer (see below). Titers ("one-intercept" values) ranging from 75 to 100 were obtained. The mean of the ten titers was 88 and the mean deviation from this was on the order of ± 10 percent.

For the pH experiments a modification of Michaelis' universal buffer (Michaelis, 1931) was used. The modification consisted in making the buffer with sea water. For the temperature experiment veronal-buffered sea water was used, and for the salt concentration experiment veronal buffer in distilled water was made to the desired salt concentrations by addition appropriate amounts of NaCl. In each experiment the test eggs were equilibrated in the buffer soltuion used. Eggs in buffer served as controls. Except in the temperature experiment, all tests were carried out at room temperature (21-23°).

For the pH and temperature experiments the stock EML solution was

diluted 10-fold with buffer. For the temperature experiment, the EML solution was diluted 100-fold.

Technique of the tests. Washed eggs taken from the stock of formalin-preserved eggs were suspended in the appropriate buffer. For each test, 0.5 ml aliquots of the EML preparation were pipetted into each of 10 separate salt cellars. These were lined up in a convenient way and at 30 second intervals an egg was added to each dish. In this way, the first egg was ready for examination 30 seconds after the last was placed in the solution. All tests were made in duplicate, so that a total of 20 eggs was used in each.

A preliminary titration of the EML solution at each pH, temperature and salt concentration was made. Three 2-fold dilutions, one the same as that giving the titer in the preliminary test, the other two the next higher and lower dilutions respectively were then prepared and tested as outlined above. The number of indentations in the membrane of each egg after five minutes treatment was recorded. The mean number of indentations per egg per dilution was plotted as described in section 1.

Effect of pH. For these experiments, buffer was prepared by dissolving 9.7 gm sodium acetate $3 \cdot H_2O$ and 14.7 gm veronal in sea water brought to pH 9 with 10N NaOH and the volume made to 500 cc. To 5 cc of this solutions, "a" cc of either 0.1N HCl or 0.1N NaOH were added plus (20 - a) cc sea water. For each desired pH, "a" was determined with the glass electrode. The pH of the final solutions was checked with the pH meter.

Assays were made at each of the following pH values: 5.1, 5.6, 6.2, 6.9, 7.9, 8.1, 9.5. Figure 26 shows a plot of titers ("one-intercept" values) at each pH. In the figure, the ordinate is dilution of the 10-fold dilution of the stock extract. The points in the figure have been connected to give a curve. It is seen that this rises from a low value

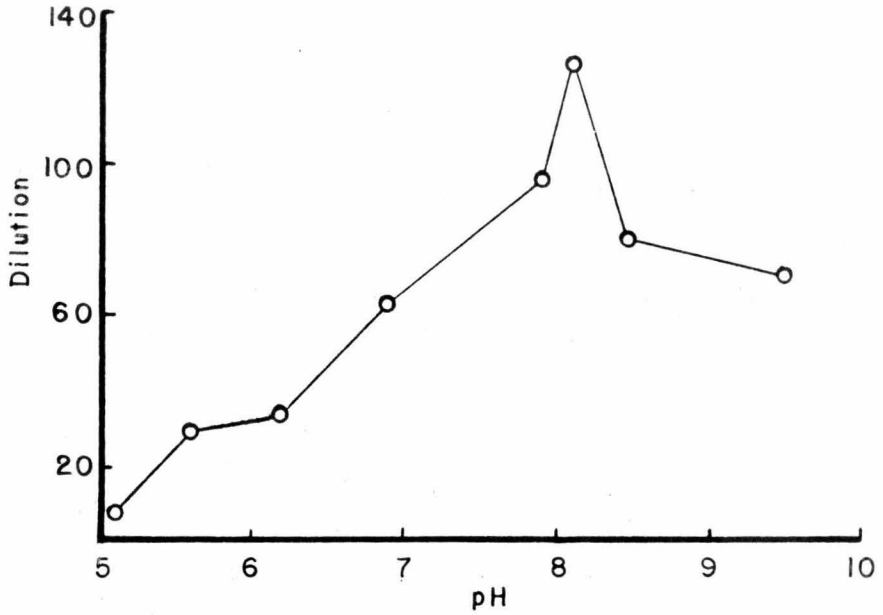

FIGURE 26. EFFECT OF pH ON TITER OF EML.

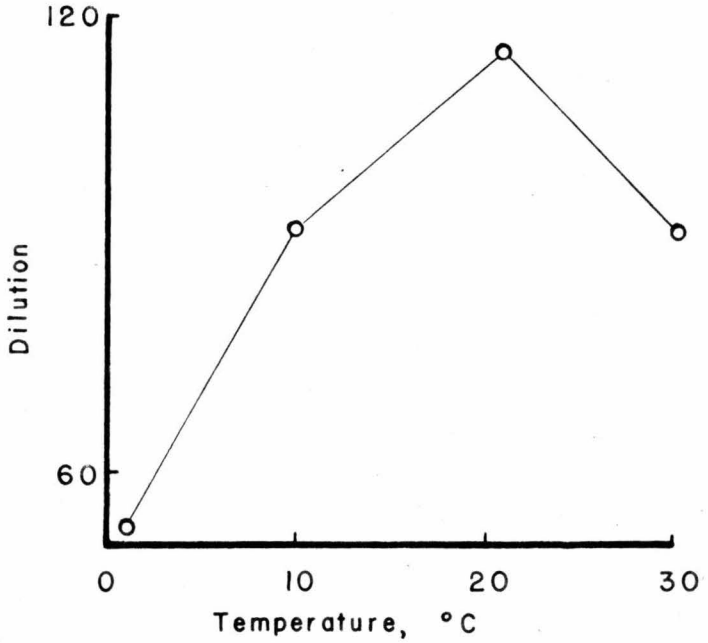

FIGURE 27. EFFECT OF TEMPERATURE ON TITER OF EML.

at pH 5.1 to a maximum at 8.1, involving a 16-fold difference in titer. Above pH 8.1 the curve falls.

Effect of temperature. For this experiment buffer was prepared by combining 1.82 gm veronal and 5.5 ml N NaOH and making to a volume of 250 cc with sea water. The EML extract was diluted 10-fold with buffer and the pH of the final solution adjusted to 8.1.

Assays were performed at 1°, 10°, 21° and 30°. The tests at the two lower temperatures were done in cold rooms maintained at 1° and 10° respectively. The temperature of the laboratory was 21° and a room warmed to 30° was available for the test at this temperature. The egg suspension, EML solution and all glassware were equilibrated at each temperature before the tests were made. For the assay at 30°, EML dilutions were prepared at normal room temperature, brought to 30° in a water bath immediately before the tests were made and aliquot portions transferred to salt cellars equilibrated to 30°.

The results of the tests are presented in figure 27. Dilution is plotted against temperature. The tiers show a low value at 1°, intermediate values at 10° and 30° and a maximum at 21°.

Effect of salt concentration. The buffer used in this experiment was prepared by combining 1.82 gm. veronal and 5.5 ml 1N NaOH and making to 250 cc with distilled water. The ionic strength of this solution was approximately 0.02. The EML preparation was diluted 100-fold with the buffer. This resulted in dilution of the sea water salts to a value approximately equivalent to 0.03 percent NaCl.

Buffered EML solutions were made to the desired salt content by addition of appropriate amounts of NaCl. The final pH of the solutions was checked with the pH meter and in each case was about 8. The total salt content of the different solutions, expressed as ionic strength, was, res-

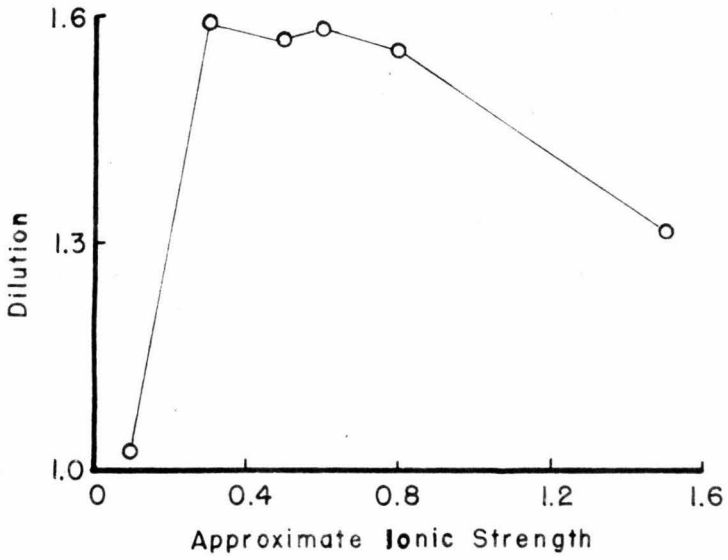

FIGURE 28. EFFECT OF SALT CONCENTRATION ON TITER OF EML.

pectively, about 0.1, 0.3, 0.5, 0.6, 0.8, and 1.5.

The results of this experiment are presented in figure 28. Dilution is plotted against salt content (expressed as ionic strangth). In the solution of lowest salt content the titer was about 1. Maximal, and approximately equal titers were observed where the salt content was equivalent to ionic strength of 0.3 to 0.8. The drop in titer shown by the solution of highest salt content is of unassessed significance. It is not unlikely that high salt concentration may influence EML activity unfavorably, though this was not tested further.

Nature of the lytic reaction

It was important to try to determine whether EML acts catalytically. Sensitivity of the lytic reaction to pH, temperature and salt concentration suggests that this might be the case. This possibility was tested as described below.

Experiments. In the first experiment, a small quantity of active EML preparation was allowed to react with a large number of eggs (membranes). Supernatant fluid was withdrawn at intervals and assayed for EML activity by the standard indentation method. Titers were compared with that of a control sample which contained no eggs.

Procedure. A quantity of formalin-preserved, stock eggs was washed with several changes of fresh sea water. Washing was accomplished by mixing the eggs with about 10 times their volume of sea water, centrifuging the suspension lightly and pouring off the supernatant fluid.

After the final washing, equivalent amounts of the egg suspension were added to each of 3 7X75 mm test tubes. These were centrifuged and most of the remaining sea water removed. Examination of eggs after this preliminary treatment showed that the membrane was intact on practically all.

An EML solution, obtained by extraction of living sperm with alkaline sea water and dialyzed against sea water, was used for the tests. The pH was adjusted to 8.1. Assay showed that it had a titer of 32.

To each of the three tubes, containing approximately ½ cc of packed eggs, 1 cc of the EML solution was added. To a 4th tube without eggs, an equal quantity of EML was added. Each tube was capped with a piece of parafilm and at frequent intervals was gently inverted in order to mix the contents. The experiment was performed at room temperature (ca. 23°).

At intervals after the experiment was started, successive tubes were centrifuged sufficiently to pack the eggs and the supernatant solution poured off. Each supernatant was immediately assayed for EML activity. The control solution was assayed at the conclusion of the experiment, so that any inactivation resulting from standing at room temperature would be detected.

Results. The results of this experiment are presented in Table XXI. The data show that when the test solution was recovered from the egg within 10 minutes, there was no significant difference in titer as compared with that of the original preparation. Examination of the treated eggs showed that the membrane was present in essentially 100 percent and but slightly thinned and distorted. The solution assayed after being in contact with eggs for 35 minutes showed only about ¼ the original activity, and that assayed after 85 minutes showed 1/8 to ¼ the original activity. Examination of eggs treated in these solutions revealed no marked difference in the condition of the membranes (present on practically 100 percent of the eggs in each case), but compared with those from the first tube, those in each of the last tubes were markedly thinner, more swollen and distorted.

Assay of the control after 100 minutes showed that its titer was the same as at the start of the experiment.

TABLE XXI

Assay of EML solutions after different periods of treatment with eggs.

Sample	Period of treatment with eggs	Titer
Control	-	32*
"	-	32*
1	8 min.	32
2	35 min.	8
3	85 min.	4-8

*Titer at start and after 100 min.

Failure of the great majority of egg membranes to be dissolved in 85 minutes of treatment suggested the desirability of determining the dissolution time of single egg membranes by treating individual eggs in 0.5 cc portions of the EML solution. The membrane of each of three eggs so treated was dissolved in less than 30 minutes.

The second experiment was designed to test the possibility that material released from the eggs might be responsible for inactivation of the lysin.

Procedure. A quantity of washed eggs from the stock suspension was treated in an excess of approximately 1N thioglycolate at pH 9 (cf. section 8). This treatment was repeated with a number of changes of fresh thioglycolate, centrifuging to remove the supernatant, until the membranes were much weakened and softened. With repeated violent shaking it was possible to free many eggs from their membranes. The eggs were then washed with 8 or 10 changes of fresh sea water, the eggs being well shaken each time. Finally, examination showed that at least 50 percent of the eggs were denuded of their membrane, and the remaining membranes were largely disintegrated.

After a final washing, the packed eggs, amounting to about 0.5 cc, were suspended in 2 cc of sea water and homogenized. This was followed by rapid freezing and thawing. Upon centrifugation of the thawed suspension, a clear solution was obtained. The pH of this was adjusted to 8.1.

For the tests, a portion of the same EML solution used in the 1st experiment was diluted with an equal volume of the egg extract. For a control, another portion of the EML preparation was equally diluted with sea water. Assay of the two solutions gave identical titers.

A third experiment was performed which was essentially a repetition of the first. A slightly different procedure was followed. This is des-

cribed below.

Procedure. A quantity of eggs from the stock suspension was washed as before. After the final washing, the packed eggs were removed from the tube with a minimal amount of water and placed in a shallow covered dish. Into each of 5 salt cellars, 0.5 ml of the same EML solution used before was pipetted. With a fine dropping pipet successive drops of eggs were added at intervals of 5 to 10 minutes to each of 3 of the EML solutions. To the other 2, the same number of drops of sea water, delivered with the same pipet (after rinsing it) were added. After the last drop was added, the suspensions were transferred to separate 10X75 mm test tubes, and centrifuged in a microcentrifuge to pack the eggs. From each of the supernatants, 5 drops of solution free of eggs were recovered. These were placed in different salt cellars and 3 to 5 freshly washed eggs from the stock suspension were added in a single drop to each dish. A similar number of eggs was added to 5 drops of each of the two control solutions. Membrane dissolution time was then determined as closely as possible for the different eggs in each dish.

Results. To each of the 3 experimental dishes, a total of 8 drops of packed eggs was added. The same number of drops of sea water was added to each of the two control dishes. The last drop was added approximately 1 hour after the first.

The mean time for complete dissolution of the membrane of eggs added to each of the control dishes at the conclusion of the experiment was, in each dish, 20 to 30 minutes, as shown in Table XXII. In contrast, as the data show, none of the egg membranes in the 3 experimental dishes were dissolved in a period of 2 hours that observations were continued. All of these membranes were clearly visible for this time, although thinned and distorted to varying degrees.

TABLE XXII

Estimated mean membrane dissolution time with EML solutions after treatment with eggs.

Sample	Estimated mean D.T. after treatment with eggs
Control 1 & 2	20 min.
1	>2 hrs.
2	"
3	"

Discussion. The results of experiments presented in this chapter show that the action of EML is influenced by pH, temperature and salt concentration. The optima for these factors are, respectively: pH 8.1; 10° to 20°; salt content equivalent to 0.5 to 0.8M NaCl. It is of interest that these optima duplicate very closely the conditions prevailing in the natural habitat of Megathura.

The evidence from experiments in which EML solutions were allowed to react with relatively large quantities of eggs and then tested for lytic activity by either the standard indentation method or by estimation of membrane dissolution time, indicates that activity is lost in the reaction. No evidence was obtained that lysin-inactivating material is released from eggs. The results suggest that EML does not act catalytically.

The sensitivity of the lytic reaction to environmental factors must be taken into account in considering the nature of the reaction. It appears, however, that these act in some other way than by influencing the rats of a catalytic process.

Further data concerning the nature of the lytic reaction will be presented in later sections.

6. Inactivation of EML by various treatments

Tyler (1939) found that EML activity is destroyed in frozen-thawed extracts of Megathura sperm as a result of brief heating at 60°. This suggested that the agent may be of protein nature. Further evidence in favor of this supposition was adduced on the basis of inactivation of lysin by crystalline trypsin and chymotrypsin.

In this chapter experiments will be described in which inactivation of EML was achieved by a variety of treatments.

Inactivation of EML at different temperatures.

In confirmation of the results reported by Tyler (1939), it was found that lytic activity of an extract of frozen-thawed sperm was reduced as a result of heating at 60° for 2 minutes. Inactivation was not complete, however, since it was observed that the membrane of eggs left in the extract for about an hour showed some thinning and swelling. In the untreated extract, egg membranes were completely dissolved in 3 to 5 minutes. An experiment was then undertaken to determine the degree of inactivation caused by exposure of EML solutions to different temperatures.

Procedure. For the experiment, an extract obtained from living sperm was used. Antifertilizin could not be detected in this extract. In order to control the pH, the preparation was dialyzed at 1° for 18 hours against a large volume of sea water, 0.01M in veronal at pH 8.2. After dialysis, the pH of the sample was checked with the pH meter and found to be 8.2.

From the EML sample, 8 aliquot portions were removed and exposed for varying times to each of 8 different temperatures. On sample served as a control and was kept at 1° during the experiment. Of the other portions, 1 was kept at room temperature (21.5-22.5°) for the duration of the experiment (about 2 hours); 1 was heated at 30° for 1 hour; 1 heated at 40°

for 30 minutes; 1 heated at 50° for 15 minutes; 1 heated at 60° for 5 minutes; 1 heated at 80° for 1 minute; 1 brought rapidly to boiling and boiled 10 seconds. At the end of the heating period, each sample was chilled in ice water and kept at 1° until assayed. EML activity was determined by the standard indentation assay method.

Results. The results of the experiment are presented in Table XXIII. From the table it is seen that there was no detectable inactivation at room temperature. At 30° the sample had lost 25 percent of its original activity after 1 hour. At higher temperatures inactivation occurred more rapidly. At 60°, approximately 97 percent inactivation occurred after 5 minutes at this temperature, and a comparable degree of inactivation after 1 minute at 80°. It is of interest that in the boiled sample, more than 5 percent of the original activity was present. The samples treated at 40° and above showed definite turbidity. The other aliquots remained clear.

Inactivation of EML at acid pH

It has been shown that EML is not as a rule detected in extracts obtained from sperm at acid pH. It has been suggested that EML may actually be released from sperm at acid pH, but is irreversibly inactivated under these conditions. Two experiments were accordingly, performed to obtain evidence bearing on this possibility.

Procedure. An extract obtained from living sperm was used in the first experiment. From the stock solution in sea water, 4 ml aliquots were placed in each of several beakers and the pH adjusted to the desired value. Each sample was then transferred to a 5 ml volumetric flask and made to volume with sea water of the appropriate pH. After mixing, the pH of the solution was checked with the pH meter. Samples at each of the following pH's were prepared: 8.1, 6.0, 5.7, 4.8, 3.4. The sample at

TABLE XXIII

Effect on EML activity of heating at different temperatures for various times

Sample	Temp. (°C)	Time of treatment in min.	Titer after treatment	percent activity lost
1	30	60	24	25
2	40	30	16	50
3	50	15	8	75
4	60	5	1	97
5	80	1	1	97
6	100	1/6	2	94
7	21.5-23	180	32	0
Control	1	180	32	0

pH 8.1 served as a control.

After standing 1 hour at room temperature the various samples were assayed without adjusting the pH. When checked with the pH meter it was found that the pH of each sample was the same as at the beginning of the experiment. After this assay, 4 ml aliquots of each of the samples were transferred to each of several beakers and the pH of each adjusted to 8. The control sample at pH 8 was treated similarly except that the 4 ml aliquot removed from it did not require adjustment in pH. EML assays were made as soon as possible.

Results. As shown in Table XXIV, EML activity showed a progressive decrease with lowered pH. No activity was detectable in the samples treated at pH 3.4 and 4.8 when these were assayed at the acid pH. These two portions each appeared definitely turbid in comparison with the control. The sample at pH 5.7 showed some opalescence. The control, as well as the sample at pH 6, was clear. Assays made with the samples after readjusting the pH of the acid-treated ones to 8 showed that those which had been treated at pH lower than 5 were still almost completely inactive. Activity of the sample treated at pH 6 was now essentially equal to that of the control, while that of the sample treated at pH 5.7 was about one-half that of the control.

The second experiment was done in the same way, using an extract of frozen-thawed sperm (containing antifertilizin). At pH 8 the EML activity of this preparation was sufficient to cause membrane dissolution in 2 to 3 minutes. Data from this experiment are recorded in Table XXV. It is seen that in this case also, EML activity was not detectable at low pH. After adjustment of the pH of the acid-treated samples to 8, however, definite activity was recorded for the sample that had been treated at pH 3.5, but this was much less than that of the control and less than that

TABLE XXIV

EML titers of acidified preparations. In the first assay, pH of solutions not adjusted; in the second, pH adjusted to 8.

Sample	Initial pH	pH of 1st assay	Titer from 1st assay	% of control activity	Titer when pH adjusted to 8	% of control activity
Control	8.1	8.1	64	100	64	100
1	6.0	6.1	8	12	64	100
2	5.7	5.7	4	6	32	50
3	4.8	4.8	0	0	4	6
4	3.4	3.4	0	0	1	1.5

TABLE XXV

Mean dissolution time of acidified preparations. For 1st assay, pH of solutions not adjusted; in 2nd, pH adjusted to 8.

Sample	Initial pH	pH of 1st assay	Mean DT of first assay	% of control activity	Mean Dt when pH adjusted to 8	% of control activity
Control	8.0	8.0	3 min.	100	3 min.	100
1	6.0	6.2	9 min.	33	3 min.	100
2	5.7	5.8	35 min.	8	6 min	50
3	3.5	3.5	----	0	52 min.	6

of the sample treated at pH 5.7, which, in its turn, appeared to be less than that of the control.

Discussion. The results of these two experiments show that at room temperature, EML is inactivated at a pH below 6, and a percentage of the original activity is not recovered upon subsequent raising of the pH. At about pH 3.5, less than 10 percent of the initial activity may be recovered. Samples treated at pH lower than about 6 exhibit opalescence or turbidity. The effect of different duration of exposure to acid pH was not tested, but it seems likely that this would be a factor in the inactivation of the lysin.

It can be concluded that EML may, in part, be irreversibly inactivated at acid pH, at room temperature, to the extent that initial activity is not recovered within a comparatively brief period after the pH of an acidified solution is raised to about 8. The appearance of opalescence, or turbidity in acidified EML solutions suggests that inactivation may be related to change in solubility of some constituent of the solutions. Failure to detect EML activity in extracts obtained from sperm at acid pH may in part be accounted for by irreversible inactivation of EML that may be released under these conditions. The present evidence, of course, does not show that EML actually is released from sperm at acid pH.

Inactivation of EML by proteolytic enzymes

EML solutions (pH 8) were mixed with equal parts of (a) a saturated solution of trypsin in sea water at pH 8, or (b) a 0.5 percent solution of chymotrypsin in sea water at pH 8. The enzymes used were in crystalline form. Controls consisted of EML solutions mixed with equal parts sea water at pH 8, and undiluted enzyme solutions.

Eggs were treated in samples of the different solutions at intervals for EML assay. The experiments were carried out at room temperature.

The results of the experiments are presented in Table XXVI. It is seen from the table that the activity of EML treated with either trypsin or chymotrypsin was rapidly reduced in comparison with the control samples of EML solution without enzyme. When eggs were left in the different samples beyond the time required for assay by the indentation method, it is seen that in EML solutions diluted with sea water dissolution of membranes occurred in 20 to 30 minutes, whereas egg membranes in the EML - enzyme mixtures exhibited no detectable change in 1 hour. In the same period, membranes of eggs treated in undiluted enzyme solutions underwent no apparent change.

These data may be taken as confirming the results reported by Tyler (1939), who found that EML activity is lost in the presence of trypsin and chymotrypsin.

Inactivation of EML by other treatments and agents

The preceding experiments have shown that EML activity is related to some constituent(s) of sperm extracts which is sensitive to agents whose biological effects are commonly attributed to action on protein. Thus, for example, heat and acid are known to induce changes in protein molecules ("denaturation"), rendering them in some way different from the native condition. The enzymes trypsin and chymotrypsin act specifically on some proteins by attacking the peptide bond. It has already been implied that EML is associated with a non-dialyzable constituent of sperm extracts, which is thus of large molecular size. More definitive evidence on this point will be presented later. It seems safe to assume that the lytic agent is a protein, or that lytic activity of sperm extracts is a property of protein constituent(s).

It was of interest to test the effect on EML, of other treatments and agents which are known to cause changes in proteins. Because of con-

TABLE XXVI

Effect of trypsin and chymotrypsin on action of EML. All solutions at pH 8 in sea water.

Sample	Titer		Mean DT of membranes left in dishes after assay at 30 minutes.
	At 10 min.	At 30 min.	
EML + trypsin	4	0	no change in 1 hr.
EML + chymo trypsin	4–8	0	no change in 1 hr.
EML + sea water	32	32	20–30 min.
Trypsin (sat'd sol'n)	0	0	---
Chymotrypsin 0.5% sol'n	0	0	---

siderations of space and time, experimental procedures will not be presented in detail. In the different experiments, the activity of EML solutions subjected to the various treatments was compared with that of comparable untreated samples. The data (Table XXVII) in each case are expressed as percent activity of treated EML compared with that of the appropriate control as 100.

The treatments and agents whose effects on EML were investigated were: (1) urea, (2) detergent (Duponol C), (3) heavy metals (Fe, Cu, Hg), (4) mild shaking, (5) irradiation with ultra-violet light.

The data from these experiments are summarized in Table XXVII. As shown in the table, EML activity was adversely affected by each of the treatments.

EML activity is apparently not affected by saturation of the solution with toluene. The possibility of storing samples at 1° under toluene was investigated, but under these conditions bacterial growth was not prevented.

In lieu of more definitive criteria to establish the protein nature of EML, the evidence presented in this chapter can be taken as support for the supposition that EML activity is associated with protein in sperm extracts. It should be noted that tests of active EML solutions for carbohydrate (Molisch test) have yielded only negative results. Evidence for the presence in lytic solutions of some lipid material will be presented in the next section.

The immediate problem at this point appeared to be to obtain concentrated solutions of EML for further characterization of the active agent. In this connection, it was important to make dry weight determinations of extracts, and desirable to obtain some preliminary chemical characterization of material with which EML is associated. Experiments on these lines are described in the next section.

TABLE XXVII

Effect of various treatment on activity of EML

Treatment	% activity after treatment
S_{at}'d with urea at room temperature	0
1% in Duponol	0
0.01M in $FeCl_3$	35
0.01M in $HgCl_2$	0
0.01M in $CuSO_4$	20
(0.01M in Na_2SO_4	100)
Shaking at room temperature 5 min.	35
Irradiation with ultra-violet light (20 min. at 5 cm.)	6

7. Fractionation of sperm extracts. Chemical analyses

Preliminary experiments

Information at hand suggested several procedures that might be utilized in attempts to fractionate sperm extracts. First, the possibility of obtaining extracts containing EML activity but without detectable antifertilizin activity by treatment of living sperm in alkaline sea water, indicated that initial extracts might best be prepared in this manner. Secondly, the available evidence that EML activity is associated with non-dialyzable material suggested that dialyzable components of sperm extracts could be eliminated without affecting lytic activity. The apparent large molecular size of active material further suggested the possibility of employing differential centrifugation to concentrate the activity. For this purpose, it was also thought that lyophilizing might prove of value. Fractionation by precipitation procedures (isoelectric precipitation, salting-out with ammonium sulfate, alcohol precipitation) had been attempted in early experiments but with discouraging results; these methods remove EML from extracts, but at the same time inactivate it to a large degree. Reprecipitation by the same procedures results in further inactivation so that in no case was it possible to concentrate activity. In general, the lability of EML activity imposes the necessity of utilizing only mild procedures.

Direct evidence that EML is associated with nondialyzable material in sperm extracts was obtained by dialyzing samples against relatively large volumes of sea water at 1°. In order to eliminate the possibility that alterations in pH during dialysis might effect some inactivation, samples were also dialyzed against sea water buffered at pH 8.1 with 0.01M veronal. Use of veronal as a buffer had the further advantage of appearing to inhibit bacterial growth. Samples dialyzed against sea water showed

no loss of EML activity even where dialysis was continued for several days in veronal-buffered solution. These experiments showed not only that EML is non-dialyzable but also that removal of dialyzable constituents does not effect loss of lytic activity under the conditions of the experiments. The presence of dialyzable components of the extracts was suggested by the fact that the initially greenish solutions were colorless after dialysis.

Some further experiments of a preliminary nature were undertaken to investigate the effect of high-speed centrifugation and lyophilizing on preparations that had been dialyzed against sea water.

For the centrifuging experiments a sample was first dialyzed against veronal-buffered sea water at pH 8.1. This was centrifuged in a multi-speed head, using a refrigerated centrifuge, at 18000 rpm (25,000 X G.) for one hour. A slight precipitate was thrown down (the sample was initially slightly opalescent) but the clear supernatant was no less active than a control which was not centrifuged.

To determine the effect of lyophilizing, a dialyzed sample (non-buffered) showing fair activity (titer 128) was lyophilized to dryness. The dry material was taken up in distilled water to $\frac{1}{4}$ the original volume. After dialysis against sea water the preparation was inactive. The experiment was repeated twice with similar results. In each test the sample was frozen rapidly in acetone-dry ice mixture and was not permitted to thaw during the drying process.

In view of the results of the preliminary experiments, efforts were now made to obtain extracts with high EML activity from living sperm. In the following sections the results of later experiments are presented.

General procedures. In order to obtain concentrated EML extracts, 20 percent suspensions of washed sperm in sea water were prepared. The

pH of the suspensions was maintained in the range 7 to 8.5 by occasional additions of 0.5N NaOH. At 1° the sperm remained alive for as long as 12 days, as shown by reaction to egg water. Removal of supernatant fluid was accomplished by centrifugation in the cold at about 3500 rpm for 30 minutes. The supernatant was then filtered with suction through washed asbestos in a sintered glass funnel of medium porosity. The filtrate so obtained was green-colored, clear and free of sperm fragments. The usual practise was to resuspend sperm in sea water to the original volume after recovery of one supernatant, continue extraction and recover more active supernatant. This was repeated, with different lots of sperm, 3 to 6 times.

Filtered extracts were tested for EML and antifertilizin. When extraction was carried out in the manner described, the filtrates showed no antifertilizin activity. EML activity in different filtrates from various lots of sperm ranged from titers of 32 to 512. It is of interest to note that using sperm suspensions of 20 percent, the EML titer of successive extracts exhibited a tendency to increase in later ones. Representative data are presented in Table XXVIII which show that, where equal volumes of sea water were used for the extractions, the EML titer of the first filtrate was 32, while that of the last one recovered, was 256-512. The extraction periods were approximately of the same duration, and the pH maintained in the same range.

Following assay of a filtrate, a sample was removed for total nitrogen determinations (microKjeldahl) and for examination with the spectrophotometer. The bulk of the solution was then dialyzed against several changes of sea water at 1°. After dialysis, samples were removed for assay, total nitrogen, spectrophotometric determinations, and, in two instances, dry weight determinations and elementary analyses.

TABLE XXIX

Total nitrogen per ml of dialyzed and non-dialyzed extracts obtained from living sperm in alkaline sea water.

Extract	Total N/ml (mg/ml) Before dialysis	After dialysis	Percent of total represented by non-dial. N
1	.160	.037	23
2	.191	.059	31
3	.181	.061	33
4	.190	.080	42
5	.160	.050	31
6	.780	.120	15

TABLE XXVIII

Titers of successive supernatants removed from approximately 40 cc of packed sperm in a period of 12 days. Extraction at 1°, pH 7-8.

Supernatant	Volume of suspension	Titer of filtered supn't
1	200 cc	32
2	200 cc	64
3	200 cc	256
4	200 cc	256-512

Determinations of dry weight of material precipitable by heat (10 minutes at 60°) or removal or electrolytes (dialysis) were made with two extracts. In each case, precipitated material was removed by centrifugation, washed with distilled water and alcohol, then dried at 80° to constant weight. The supernatant, made salt-free by dialysis against distilled water, was evaporated to small volume on a steam bath, transferred to a weighed evaporating dish, evaported to dryness on the steam bath and dried to constant weight at 80°.

Elementary analyses of the dried precipitated material were obtained.* The analyses included determinations of C, H, N, and S. One of the dried samples was subjected to acid hydrolysis and the hydrolysate analyzed for cystine and cysteine.

Total nitrogen of non-dialyzed and dialyzed extracts. It is of interest to compare total nitrogen, determined by the micro-Kjeldahl method, of non-dialyzed and dialyzed extracts. Data from a number of such determinations are shown in Table XXIX. It is seen from the table that from 42 to as little as 15 percent of the total nitrogen of an extract obtained from living sperm in alkaline sea water may be associated with non-dialyzable constituents. The relative amount in an extract of dialyzable and non-dialyzable nitrogen probably depends upon a number of factors. These may include conditions of extraction, such as the concentration of the sperm suspension, variations in pH, number of extractions and length of extraction periods.

Absorption in the ultra-violet by non-dialyzed and dialyzed extracts. Samples of non-dialyzed and dialyzed extracts were examined spectrophotometrically with the Beckman spectrophotometer. The results of two tests are shown in figure 29. The samples examined were nos. 1 and 2 in Table

*Elementary analyses performed in micro-analytical laboratory.

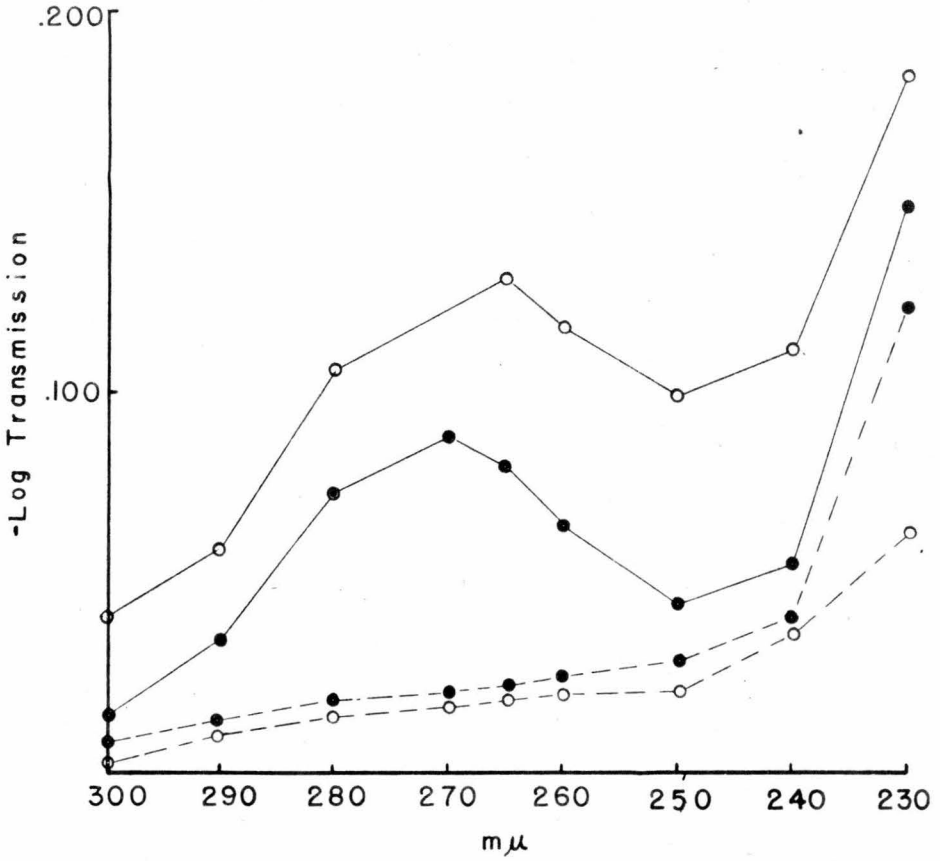

FIGURE 29. ULTRA-VIOLET ABSORBTION OF NON-DIALYZED AND DIALYZED EXTRACTS OF LIVING SPERM. CONCENTRATIONS ARE COMPARABLE. SOLID LINES = NON-DIALYZED, BROKEN LINES = DIALYZED. OPEN CIRCLES = EXTRACT NO. 1 IN TABLE XXIX, CLOSED CIRCLES = EXTRACT NO. 2 IN TABLE XXIX.

XXIX. It is seen in the figure that both non-dialyzed preparations exhibited a well-defined absorption maximum at 265 to 270 mu. After dialysis, in contrast, neither sample showed a peak in this region. A control sample of nucleic acid dissolved in sea water at the same pH (about 8) showed a sharp maximum at 260 mu (not shown in the figure).

The data presented in this and the preceding section show that low molecular weight nitrogenous material with a pronounced absorption maximum in the ultra-violet at 265-270 mu is released by sperm in alkaline sea water.

Absence of an absorption maximum at 260 mu in the dialyzed preparations indicates that nucleic acid is not associated with EML activity of sperm extracts.

Dry weight determinations. Results of dry weight determinations are presented in Table XXX. It is seen from the table that in two extracts, both dialyzed against sea water and comparable with respect to the concentration of the sperm suspension from which they were obtained and to other conditions of extraction, the total dry weight per ml was 0.45 and 0.54 mg respectively. Of the total dry weight of Extract I, in the table, about 90 percent, or 124 mg, was recovered upon precipitation by dialysis against distilled water. Extract II in the table, was heated for 10 minutes at 60°. Of the total dry weight of this extract, 81 percent (233 mg) was accounted for by the precipitate.

The two extracts were originally similar enough with respect to EML activity to give the same titer when assayed by the standard method. In each case this was 256. Dividing the dry weight per ml by the titer gives the dry weight per ml per lysin unit, or 1.8 and 2.1 micrograms respectively. In neither case, may the entire amount represent EML. In lieu of more definitive information, it may be assumed that these values are maximal.

TABLE XXXI

Constituents of dried material precipitated from dialyzed alkaline sea water extracts of living sperm.

	Percent of dry weight	
Substance	Extract I	Extract II
Carbon	51.8	53.9
Hydrogen	7.2	7.7
Nitrogen	10.9	9.3
Sulfur	1.5	1.4
Cystine	1.5	
Cysteine	0.15	
Cystine-cysteine sulfur	0.45	
Lipid	4	

TABLE XXX

Dry weight determinations of extracts of 2 lots of sperm. Each extract from 20 percent suspension extracted 4 days at 1°, pH 7-8. Supernatant filtered and dialyzed against sea water.

Extract	Original titer	Vol.	Total dry wt.	Dry wt. of mat. ppt'd by dialysis	Dry wt. of mat. ppt'd by heating	Dry wt. of mat. remaining in supn't.	% of total dry wt. represented by precipitated material
I	256	316cc	140mg (.45mg per ml)	124 mg	—	16 mg	90
II	256	530cc	288mg (.54 mg per ml)	—	233 mg	55 mg	81

These determinations are of interest primarily in establishing an order of magnitude for the weight of dry material which may be associated with one lysin unit.

Elementary and other analyses of dried precipitated material. Elementary analyses of dried precipitated material recovered from extracts I and II above, are presented in Table XXXI. Of the total S, approximately 30 percent is accounted for in extract I by combined cystine and cysteine. These amino acids were determined in an acid hydrolysate of this material by the method of Vassel (1941). As shown in Table XXXI, cystine accounted for about 1.5 percent of the dry weight and cysteine for about 0.15 percent.

A portion of dried precipitated material of extract I was extracted with boiling ethanol-ether mixture. The material (lipid) extractable in this way was calculated to amount to about 4 percent of the total dry weight. It is of interest in this connection to note that gentle mixing of an EML preparation with an equal volume of ether for 5 minutes at room temperature did not reduce the titer of the sample as compared with a control mixed similarly but without ether.

Discussion. Features of interest in the analyses presented above are: (1) nitrogenous constituents of low molecular weight are present in alkaline sea water extracts of living sperm, and these may account for from about 58 to 85 percent of the total nitrogen; (2) dialyzed extracts do not show an absorption maximum in the ultra-violet (290 to 240 mu) and, hence, it appears that EML activity is not associated with nucleic acid; (3) dry weight determinations reveal an amount of dry material on the order of about 2 micrograms per ml per lysin unit; (4) sulfur is present in small amount in dried precipitated material, and about 30 percent of this is accounted for (in one extract) by combined cystine and cysteine; (5) alcohol-ether soluble material accounts for about 4 percent of the dry weight

(extract I).

Further attempts to characterize the lysin were based upon the assumption that extracts more concentrated than are seemingly obtainable from living sperm at mildly alkaline pH would have to be used. Two procedures appeared to be of promise in this respect. These were (1) extraction of sperm at high pH; (2) extraction by freezing and thawing. In the time available it was possible to investigate only the latter.

In view of earlier experiences with attempted fractionation of frozen-thawed sperm extracts (cf. section 2) it was realized that two major problems would have to be overcome. These are (1) separation from antifertilizin; (2) loss of EML activity. The method that was tried is described in the following section.

Fractionation of frozen-thawed extracts

Procedure. A 10 percent suspension of sperm in sea water was frozen in dry ice-acetone mixture and allowed to thaw to room temperature. The supernatant was clarified by centrifuging (cold) and filtering. The filtrate was assayed for EML and antifertilizin. The pH was brought to about 10.4 with 2.5N NaOH, which resulted in precipitation of sea water salts. The suspension was allowed to stand at 1° for 1 hour, when the precipitate was removed by centrifuging. The supernatant (#1 in table XXXII) showed but slight EML activity and strong antifertilizin activity. It was discarded. The precipitate (#1) was washed with 3.3 percent NaCl solution at pH 10.4 and the washings discarded, as they showed no EML activity. The precipitate was suspended in 3.3 percent saline equal to about 1/10 the original volume and the pH adjusted to 8. The precipitate did not dissolve completely and after 1 hour the sediment was removed by centrifuging. The EML activity of the supernatant was less than that of the original extract (see Table XXXII) and it showed only slight antifertilizin activity.

Reprecipitation at pH 10.4 was then performed. The supernatant (#2) showed antifertilizin activity and no EML activity and was discarded. The precipitate (#2) was taken up in the same volume of 3.3 percent NaCl solution and the pH adjusted to 8. Some of the precipitate failed to dissolve and the sediment was removed after 1 hour. The supernatant showed no antifertilizin activity and slight EML activity.

The same procedure as above was repeated with another portion of the same extract. This time, however, all operations were carried out in a cold room at 1°. The results of the second experiment were essentially the same as the first.

Results. The results of the first experiment are summarized in Table XXXII. It should be pointed out that the supernatants from pH 10.4 precipitation were always clear. Antifertilizin activity was removed in these. It appears from these results that a method for the separation of EML and antifertilizin activities may have been found, since EML appears to have been precipitated or adsorbed on the precipitated sea water salts, while the bulk of the antifertilizin remained in solution. Again, however, this method is of little value with respect to concentration of EML activity since this is largely destroyed in the process.

Discussion. The ease with which EML is inactivated by treatments which change its equilibrium state in sea water renders difficult attempts to obtain active solutions of sufficient concentration to permit its characterization. Due to the pressure of time and the necessity of investigating other phases of the general problem, procedures other than those described were not investigated.

In the next section experiments are described in which were tested the effects on the egg membrane of agents other than EML.

TABLE XXXII

Fractionation of frozen-thawed sperm extract by precipitation at high pH. Antifertilizin activity determined by testing with sea-urchin eggs.

Fraction	pH for assay	EML activity	Antifertilizin activity
Initial extract	8	DT = 2 min.	++++
Supn't 1	8	titer = 1	++++
Supn't from resuspended ppt 1	8	DT = 12-15 min.	++
Supn't 2	8	---	++
Supn't from resuspended ppt 2	8	titer = 8	0

8. Action of various agents on the egg membrane

Action of proteolytic enzymes

As a starting point for these experiments, eggs were treated with solutions of trypsin, chymotrypsin and pepsin. The first two enzymes were in crystalline form. Preserved eggs were exposed to a saturated solution of trypsin and 0.5 percent solution of chymotrypsin and pepsin in sea water. For the first two, the pH was adjusted to 8; for pepsin, the pH was adjusted to 2. After 24 hour exposure to these enzymes at room temperature, egg membranes were apparently unaffected by any of them.

Somewhat later the experiment was repeated using fresh eggs and they were incubated with the enzymes at 37° for a period of 60 hours. The solutions were saturated with toluene. The results of the second experiment are presented in Table XXXIII. Compared with eggs left in sea water saturated with toluene under the same conditions, it is seen from the table that egg membranes in trypsin showed significant increase in diameter (the error involved in measuring membrane diameters had earlier been established as about ± 5 percent); chymotrypsin had somewhat less effect and pepsin had none. It was concluded from this experiment that the egg membrane may be slightly susceptible to attack by trypsin and to a less extent by chymotrypsin, but that the action of neither of these enzymes is comparable to that of EML.

Action of hyaluronidase

A 1 percent solution of purified hyaluronidase (Schering) in sea water at pH 6 had no observable effect on membranes when eggs were treated at room temperature for 24 hours.

Action of thioglycolate

In connection with the original experiments with proteolytic enzymes some eggs were treated first with 0.01M thioglycolic acid in sea water at

TABLE XXXIII

Effect of proteolytic enzymes on egg membranes. Incubated 60 hrs. at 37°.

Mixture	pH	Mean % increase in membrane diameter*
Eggs in sat'd sol'n of trypsin, with toluene	8	25
Eggs in 0.5% sol'n of chymotrypsin with toluene	8	17
Eggs in 0.5% solution of pepsin with toluene	2	0
Eggs in sea water sat'd with toluene	8	0

*Variation of less than ± 5 percent considered as no effect.

pH 6. Monroy and Runnström (1948) reported that the fertilization membrane of sea-urchin eggs is "softened" by thioglycolic acid in this concentration and pH then attacked by trypsin and chymotrypsin. The results with Megathura egg membranes were pronounced. Membranes were rapidly attacked by the acid and in a period of 10 to 20 minutes were noticeably thinned and swollen. Subsequent treatment with proteolytic enzymes had no further effect, however (at room temperature, using formalin-fixed eggs).

Further experiments with thioglycolic acid showed that pH is a critical factor in the action of this reagent on the egg membrane. The results of tests at different pH's are summarized in Table XXXIV. From these data it is seen that the action is accelerated at alkaline pH. Experiments with fresh (living) eggs gave results similar to those with fixed eggs.

Action of other SH compounds

Data from tests with other SH compounds are summarized in Table XXXV. It was found that at alkaline pH, cysteine, glutathione and thiosalycylate were each effective in causing dissolution or significant thinning and swelling of the membrane.

Action of KCN

In relatively low concentration at about pH 9 and above, KCN was found to attack the egg membrane (see Table XXXV). Compared with SH compounds (thioglycollate, cysteine and glutathione particularly) somewhat higher concentrations of KCN appeared to be required to effect complete membrane dissolution.

Action of NaOH

Some early experiments in which formalin-fixed eggs were treated for a period of 30 minutes at 22° in different concentrations of NaOH (0.5N

TABLE XXXIV

Effect of pH on action of thioglycolic acid on egg membrane. Egg membranes measured after 1 hour treatment at room temperature.

	pH	Percent increase in membrane diameter
Thioglycolic acid, 0.01M	4.0	0
	5.1	0
	5.9	5-10
	7.1	20-30
	8.0	50-100
	9.1	100-150
Sea water	4.1	0
	9.0	0

TABLE XXXV

Effect of SH compounds and KCN on the egg membrane at room temperature.

Substance	Concentration	pH	Effect on membrane: Percent increase in membrane diamter at 1 hr.	Effect on membrane: Estimated DT (min.)
Thioglycolic acid	0.1M	8	...	15-45
	0.1M	8	50-100	...
Thiosalycylic acid	0.1M	7.5	50-75	...
Cysteine	0.1M	8	...	20-40
	0.01M	8	50-100	...
Glutathione	0.1M	8	...	20-50
	0.01M	8	50	...
KCN	0.1M	9.5	....	60-120
	1.0M	9.5	...	10-15

to 2.5N) showed that some degree of thinning and swelling of the membrane occurred, being most pronounced in the highest concentration of the reagent. These experiments were repeated with fresh eggs. Data from these tests are presented in Table XXXVI. The data show that the action is more pronounced in 2.5N solution than in either 0.5N or 8N. In 2.5N NaOH the membrane was almost completely disintegrated in 10 minutes and entirely dissolved in 1 hour at room temperature. It should be noted that for these tests eggs were transferred from sea water to 3.3 percent NaCl solution prior to testing with NaOH. Control tests with solutions of similar concentrations of NaCl showed that hypertonic neutral salt solutions have no effect on the membrane.

Action of acids

Membranes of formalin-fixed eggs treated in HCl in concentrations up to 12 normal for periods up to 24 hours were not visibly affected. Identical results were observed when fresh eggs were treated in 1N and 8N HCl for 6 hours at room temperature. The results of these tests are summarized in Table XXXVI. Also shown in the table are data obtained from tests with dilute and concentrated H_2SO_4, concentrated HNO_3 and glacial acetic using formalin-fixed eggs. Of these acids, only concentrated HNO_3 was observed to effect definite thinning and swelling of the membrane. Action of the acid on the eggs made observation difficult, but the membranes may have been nearly dissolved in 1 hour. Glacial acetic and concnetrated H_2SO_4 also attacked the egg, but as well as could be determined neither of these effected noticeable disintegration of the membrane in 24 hours. Dilute H_2SO_4 (3N) was without effect in the same period.

TABLE XXXVI

Effect of alkali and various acids on the egg membrane. Treatment at room temperature. Data for NaOH and HCl obtained using fresh eggs, others using fixed eggs.

Reagent	Conc.	Effect on egg membrane
N_aOH	0.5N	Largely disintegrated in $3\frac{1}{2}$ hrs. but not dissolved
	2.5N	dissolved in about 1 hour
	8N	Disintegrated but not completely dissolved in $3\frac{1}{2}$ hours
HCl	8N	None (6-24 hrs.)
	3N	None (6-24 hrs.)
H_2SO_4	36N	Probably none in 24 hrs.
	12N	None in 24 hrs.
HNO_3	conc.	Thinned and swollen in 1 hr.
HAc	glacial	Probably none in 24 hrs.

Action of organic solvents

Alcohol (95 percent), toluene, ether, petroleum ether, chloroform and benzene did not appear to dissolve or otherwise attack the egg membrane. Observation in these solvents was difficult due to their action on the eggs, but in spite of this it was possible to see that the membranes apparently remained intact without thinning or swelling or other indication of disintegration.

Minimal effective concentrations of SH compounds and KCN.

It was of interest to determine the minimal concentration of SH compounds that would be effective in producing an observable effect on the egg membrane. For comparison, the minimal effective concentration of KCN was also determined.

At this time, it must be pointed out that the action of these compounds on the egg membrane duplicates that of EML not only in causing dissolution (in adequate concentration and at suitable pH) but also in producing indentations in the membrane in low concentration. Depending upon the concentration and the pH, appearance of indentations may be followed or accompanied by observable thinning, swelling and eventual dissolution. The importance of the pH is shown by the data in Table XXXIV from which it is seen that at pH 4, 0.01M thioglycolic acid produced no observable effect (indentation). At pH 6 the same concentration produced 1 or 2 indentations per egg (not indicated in the table) followed by swelling, and at pH 7 the membrane was initially collapsed and later became thin and swollen. The same phenomenon was observed with cysteine. Thus, at pH 4.3, 0.01M cysteine in sea water produced no observable effect on the egg membrane. At pH 5.7 the same concentration of cysteine produced 1 or 2 indentations per membrane and some thinning and swelling. At higher pH (6.3 to 8.5) the membranes rapidly thinned and became swollen and distorted

in outline. Adequate controls showed that the pH alone was not responsible for these effects.

It seemed probable that indentation of the membrane is an osmotic effect (cf. discussion following). When eggs are transferred from sea water to distilled water, for instance, the membranes are temporarily indented or collapsed. As osmotic equilibrium is established, they round out and in 10 to 20 minutes have regained their spherical shape. When transferred from sea water to concentrated NaCl solution, however, no indentations appear. This indicates that the membrane permits rapid diffusion of ions. In connection with the present experiments it was pertinent to observe the effect of sucrose (mol. weight 342) on the membrane. It was found that in concentrations of 25,000 mg per liter (73 mM) and higher, in sea water at pH 8, sucrose causes indenting or collapse of the membrane (see Table XXXVIII). In lower concentration it has no observable effect.

In the experiments with SH compounds and KCN the minimal effective concentration was taken as that which causes the appearance of a single indentation in the egg membrane in a period of 5 minutes. By adopting this criterion, comparison with EML, sucrose and other substances could then be made on a quantitative basis.

In the experiments the SH compounds were tested at pH 8, KCN at pH 9.5. The results are summarized in Table XXXVII. It is seen from the table that SH compounds and KCN are many times more effective than sucrose in their action on the membrane, whether compared on a basis of weight per volume or molarity. On the other hand, on a weight per volume basis, these compounds are many times less effective than EML, one lysin unit of which corresponds to about 2 mg per liter dry weight (cf. section 7).

In column 6 of Table XXXVII are shown the minimum concentrations of

TABLE XXXVII

Minimal effective concentration of KCN and SH compounds at room temperature

Compound	Mol. Wt.	pH	Minimal effective concentration mg/liter	Minimal effective concentration millimoles	Minimal conc. required to cause 10% increase in membrane diam. in 1 hr. (millimoles)
KCN	65.11	9.5	1,302	20	---*
Thioglycolic acid	92.09	8	460	5	---*
Cysteine	121.12	8	60	$5X10^{-1}$	5
Glutathione	290.18	8	870	3	10

*Not tested.

cysteine and glutathione in which egg membranes showed at least 10 percent increase in diameter in 1 hour at room temperature. These values are somewhat higher than those for the minimal effective concentration producing indentation. In this respect these substances differ from EML, then, since 1 lysin unit of the latter is effective in producing significant enlargement of the membrane in this time.

Effect of high molecular weight substances on the membrane

The effect on egg membranes of a number of proteins was tested by the same criteria employed with EML, SH compounds, etc. It was found, as shown in column 3 of Table XXXVIII that, for production of indentations, the minimal effective concentration of bovine serum albumin, bovine serum globulin, egg albumin, Baker's gelatin and pepsin, in sea water at pH 8, was in each case of the same order when calculated on a weight per volume basis. On a molar basis, the proteins are relatively more effective than SH compounds. This probably is a consequence of the molecular weight of the proteins (molecular weights from Cohn and Edsall, 1943). None of the proteins, however, showed a lytic effect on the membrane, i.e., thinning, swelling or dissolution, in concentrations as high as 1 percent.

The only non-protein colloid that was tested for minimal effective concentration was the dye congo red. It was necessary to test this in distilled water, since in the presence of salts it precipitates. Eggs were, accordingly, washed in distilled water and allowed to equilibrate in this medium before being transferred to solutions of the dye. The minimal effective concentration was found to be 20 mg per liter (see Table XXXVIII). The molecular weight of congo red is 696 (Taylor, 1923) but it behaves in many respects as a high molecular weight colloid (Zsigmundy and Spear, 1917). Calculation shows that 20 mg per liter is equivalent to about $3X10^{-2}$ mM. Congo red, therefore, is seen to approach

TABLE XXXVIII

Minimal effective concentration of sucrose, congo red, proteins and EML. Room temperature.

Substance	Mol. Wt.*	mg/liter	Minimal effective concentration millimoles
sucrose (in sea water)	342	25,000	73
congo red (in dist. water)	696	20	$3X10^{-2}$
bovine serum albumin	70,000	500	$7X10^{-3}$
bovine serum globulin	174,000	500	$3X10^{-3}$
egg albumin	43,000	500	$12X10^{-3}$
Baker's gelatin		500	
pepsin	36,000	500	$14X10^{-3}$
EML	---	2**	---

*Molecular weight of proteins from Cohn and Edsall (1943).
**This value from dry weight determination, section 7.

the proteins with respect to the order of magnitude of the minimal effective concentration in millimoles. To make the comparison more valid, serum albumin was tested in distilled water. Essentially identical results were obtained as when the protein was dissolved in sea water. As with the proteins, congo red did not effect swelling, thinning or dissolution of the membrane.

Effect of combined cysteine and serum albumin on the membrane

It was of interest to determine whether a mixture of cysteine and serum albumin would be more effective, in terms of minimal concentration of each component, than either of these substances separately. A solution was made up containing 500 mg serum albumin and 60 mg cysteine respectively, in sea water at pH 8. These amounts, it will be recalled, are the minimal effective concentrations for each of the components separately. Other solutions were prepared containing, respectively, 250 mg albumin per liter and 30 mg cysteine per liter, and 150 mg albumin per liter and 18 mg cysteine per liter.

The results of the tests are shown in Table XXXIX. It is seen that the minimal effective dosage of the mixture of albumin and cysteine was that containing 250 mg per liter albumin and 30 mg per liter cysteine, or each component was represented by an amount equal to one-half the minimal effective concentration separately.

This experiment shows that a mixture of albumin and cysteine does not approach EML in effectiveness.

Effect of pH and heat on action of proteins and cysteine

Tests of the effect of pH and heat on the action of proteins and cysteine were performed with amounts of these substances larger, in each case, than the minimal effective concentration. The criteria of action used here were: (1) indenting or collapse of the membrane in 5 minutes

TABLE XXXIX

Minimum effective concentration of combined bovine serum albumin and cysteine in sea water at pH 8. Room temperature.

Concentration in mixture				Indenting in 5 min.	% increase in membrane diameter in 5 hrs.
Albumin		Cysteine			
mg/liter	nM	mg/liter	nM		
500	$7X10^{-3}$	60	$5X10^{-1}$	1	10-20
250	$3.5X10^{-3}$	30	$2.5X10^{-1}$	1	0
150	$1.5X10^{-3}$	18	$1.5X10^{-1}$	0	0

(initial action); (2) with cysteine, thinning, swelling or dissolution within 1 to 24 hours. As a matter of course, all the test dishes were examined at intervals up to 1 hour and after 24 hours. In the case of the proteins, negative results actually mean that no effect (indenting) was observed up to at least 1 hour after eggs were placed in the solutions. For comparison, data from Table XXIII (section 6) relating to the effect of heat on EML are included with the data obtained from the present experiment.

Proteins used in this experiment were: serum albumin, 0.1 and 0.5 percent; serum globulin, 0.1 percent; egg albumin, 0.1 percent; pepsin, 0.1 percent. Cysteine was used in a concentration of 0.01M. All the solutions were prepared in sea water.

Results of the tests are presented in Table XL. The data show that the proteins in each case were "inactivated" only be treatment which also resulted in the appearance of a precipitate. Thus, for example, serum albumin was effective in causing membrane indentation at room temperature at pH 3.5 to 8; solutions at pH 5 to 8 were likewise effective after being heated at 60° for 5 minutes, but when a solution at pH 3.5 to 4 was heated 5 minutes at 60° it was then not effective. All solutions except the latter remained clear; this one became turbid upon being heated. Similar results were obtained with each of the other proteins except serum globulin, which, however, was tested only at pH 6 and 8.

In contrast to the results with proteins, cysteine was not "inactivated" at pH 8 even by boiling for 5 minutes. There was no point in testing the effect of heat on cysteine at low pH because it is effective only at neutral or alkaline pH.

TABLE XL

Effect of heat at different pH's on action of proteins on egg membrane. Effect of heating cysteine at pH 8. Data for EML from Table XXIII.

Substance	% Conc.	pH	Temp. (°C).	Time (min.)	Visible change	Action on membrane initial	Action on membrane 1-24 hrs.
Serum albumin	0.5	6-8	21-23	...	none	collapse	rounded out
	0.5	3.5-4	21-23	...	none	collapse	collapsed
	0.1	3.5-4	21-23	...	none	collapse	collapsed
	0.1	5-8	60	5	none	indented	rounded out
	0.1	3.5-4	60	5	ppt.	none	...
Serum globulin	0.1	6-8	21-23	...	none	indented	indented
	0.1	8	60	5	none	indented	indented
Egg albumin	0.1	8	21-23	...	none	indented	rounded out
	0.1	8	60	5	none	indented	rounded out
	0.1	3.5-4	21-23	...	none	indented	not obs.
	0.1	3.5-4	60	5	ppt.	none	...
Pepsin	0.1	8	21-23	...	none	indented	rounded out
	0.1	8	60	5	ppt.	none	...
Cysteine	0.1	8	21-23	...	none	collapse	dissolved
	0.1	8	60	2	none	collapse	dissolved
	0.1	8	100	5	none	collapse	dissolved
EML	...	8	60	5	ppt	ca. 97%	inactivated
	...	8	100	1/6	ppt	ca. 94%	inactivated

Discussion. Some conclusion of a general nature can be drawn from the results of the experiments presented in this chapter. The first of these concerns the nature of the egg membrane.

Of primary significance, perhaps, is the fact that the egg membrane is dissolved by SH compounds, KCN and dilute NaOH at room temperature. Comparison of relative concentration of these substances required to effect dissolution of the membrane shows that SH compounds and KCN are much more effective than alkali. The difference appears to be of the order of magnitude of 50 to 250 fold. It should also be pointed out that there seems to be a qualitative difference in the action of SH compounds, KCN and alkali. The former act in a manner similar to EML. Depending upon the concentration and the pH these may cause indentation or collapse, and thinning, swelling and dissolution of the membrane. In relatively high concentration (0.1M to 0.5M) at pH 8-9.5, the membrane may thin and dissolve rapidly in a manner analogous to that observed with active EML preparations. Alkali, on the other hand, in low concentration (0.5N) seems to effect only some degree of disintegration without dissolution. In higher concentration (2.5N) the membrane swells without indenting and more or less rapidly is disintegrated to an amorphous, tenuous mass which may eventually dissolve.

It seems possible to infer that the effect of SH compounds and KCN may be attributable to reduction of -S-S-bonds in the egg membrane, associated, perhaps, with cystine. Alkali, on the other hand, may act on other bonds. It is suggestive, that Goddard and Michaelis (1934, 1935) have shown that wool keratin is dissolved by thioglycolate at alkaline pH. These authors attribute this effect to reduction of -S-S- bonds of cystine. According to Goddard and Michaelis, the products of the reaction of keratin and thioglycolic acid are true proteins and are precipitated

by ordinary protein precipitants. These products are given the name "kerateine". Re-oxidation of kerateine does not restore the pattern of the original keratin, however. Re-oxidized kerateine is called "meta-keratin" and it is soluble in weak alkali and digestible by proteolytic enzymes. It is also reduced by thioglycolate at neutral and alkaline pH and is slightly soluble in dilute HCl.

Keratins, it should be pointed out, are not all the same with respect to their resistance to proteolytic enzymes. Block and Bolling (1939) classify as "eukeratins" insoluble proteins resistant to enzymatic digestion, and yielding histidine, lysine and arginine in molecular ratios of approximately 1:4:12; as "pseudokeratins" proteins insoluble in ordinary protein solvents, but dissolved to the extent of from 25 to 60 percent by trypsin and pepsin, and yielding histidine, lysine and arginine in some other ratio than 1:4:12.

The Megathura egg membrane may be termed "keratin-like" with respect to a number of its properties.

A report by Monroy and Runnström (1948) has been cited (pg. 126) in which it is stated that the fertilization membrane of sea-urchin eggs is attacked by thioglycolic acid. These authors suggest that this membrane is of a keratin-like nature. Runnström, Monne and Broman (1943) showed that the sea-urchin fertilization membrane is also attacked by cysteine. Monroy (1948) has presented evidence of an indirect nature which suggests that the vitelline membrane of the egg of a polychete worm, Pomatoceros triqueter, may be of a similar nature. This author reports that sperm are unable to adhere to the membrane after being treated with oxidized glutathione or iodoacetate. He infers that this treatment blocks SH groups of the sperm. Young and Inman (1938) found that the protein of the casing of salmon eggs is a pseudokeratin. It is insoluble in

all protein solvents but is slowly hydrolyzed by pepsin, and the histidine:lysine:arginine ratio is 1:3:4.

With respect to the action of sucrose, proteins and congo red on the egg membrane, it can be said that this is probably an osmotic phenomenon. Dean and Moore (1947) reported effects, similar to those described in this chapter, of high molecular weight solutes on the fertilization membrane of Dendraster excentricus. Their data show that the concentrations of substances with molecular weights of 4000 and above, required to effect collapse of the membrane, are of the same order of magnitude. With decreasing molecular weights the concentrations required to produce the effect have to be progressively greater. Although their criterion for "unmistakable" collapse is somewhat different than the criterion of threshold action used here, it is of interest that they found the minimal effective concentration of sucrose to be 34,200 mg per liter (100 mM). The required concentration for substances of molecular weight 10,000 to 15,000 (methylcellulose, hydroxyethylcellulose, alginate) was found to be on the order of $2X10^{-2}$ to $2.5X10^{-2}$ mM.

In the present investigation, it has been found that proteins denatured by heating and acidification do not cause collapse or indentation of the egg membrane. By "denaturation" is here meant change in properties of the protein resulting in loss of solubility with consequent appearance of turbidity (or opalescence) in the initially clear solution. It has been found that acidified EML solutions exhibit opalescence or turbidity (depending upon the pH), and also that turbidity develops in EML solutions at alkaline pH when these are heated to 40° and over. Under these conditions, EML is inactivated as shown by failure to cause indentation of the membrane and by failure to dissolve the membrane.

It may be inferred, on the basis of the evidence presented here that

indentation of the egg membrane by EML is an osmotic phenomenon. EML, however, has also a lytic effect on the membrane, which is shown even in concentration as low as that represented by 1 lysin unit.

Sulfhydryl compounds and KCN have been shown to have a lytic effect on the membrane similar to that of EML, and these substances cause indentation and collapse of the membrane in concentrations that are not compatible with their molecular weight (compare, for example, minimum effective concentration of cysteine and sucrose). The osmotic effect, then, in the case of these compounds and EML must be conditioned by their lytic effect on the membrane.

It has been shown that cysteine is not "inactivated" by boiling for 5 minutes. On the other hand, it is ineffective at acid pH at room temperature (as are other SH compounds). In the latter case, it may be assumed that oxidation of the SH group is inhibited.

9. Nitroprusside reaction with EML. Effect of iodoacetate on EML action.

A classical method for the detection of SH groups in proteins is the nitroprusside test. According to Mirsky (1941a) the test is specific for SH groups. This author found a very close correlation between the nitroprusside reaction and ferricyanide titration of protein SH groups. Giroud and Bulliard (1933), on the other hand, found that the nitroprusside reaction gives a positive test with creatinine and acetone, but this does not appear to be of special significance where the test is used for preliminary qualitative determination. A more serious objection to the nitroprusside reaction is that the color developed is ordinarily not stable. For this reason it is not well suited for quantitative use.

Many native proteins do not give a positive nitroprusside reaction (Cohn, 1939). In the case of egg albumin, treatment of the protein with urea cuases the appearance of SH groups. In concentrated urea solutions egg albumin remains soluble (Mirsky, 1941). Other agents with action similar to that of urea are Duponol (a synthetic detergent) and guanadine hydrochloride.

In the present study it was of interest to determine whether a positive nitroprusside reaction could be obtained with "native" EML or with EML treated with urea.

Experiments. Tests were first made with several preparations containing active EML without urea. For the tests, 3 or 4 drops of freshly prepared 5 percent sodium nitroferricyanide (nitroprusside) solution were added to 0.5 ml of the preparation, and then 5 drops of 10 percent NH_4OH. Samples of two EML extracts of living sperm, a freshly prepared extract of frozen-thawed sperm and a 10 percent sus-

pension of fresh sperm were tested. In each case the results were negative. A solution of cysteine in sea water, approximately 10^{-3}M, at pH 8, gave a positive reaction. Precipitation of sea water salts upon addition of NH_4OH did not interfere with the test with cysteine.

An experiment was now performed in which an active EML solution, obtained by alkaline sea water extraction of living sperm, dialyzed against sea water, was saturated with urea and tested with nitroprusside under various conditions. The initial titer of the EML solution was 128. Results of the experiment are presented in Table IXL. The data show that a positive reaction was given only after heating. The strongest reaction was given by a sample heated 45 minutes at 60°, and the color developed was about of the same intensity as that produced by reaction of a $5X10^{-3}$M solution of cysteine. It was noted that heating did not cause the samples containing urea to become turbid.

Determination of lytic activity of samples containing urea, after heating 10 minutes at 60° and without heating were made. Egg membranes were collapsed, but in neither case was there any indication of dissolution in 1 hour. An unheated sample, without urea, effected membrane dissolution in an estimated time of 8 to 15 minutes.

It is of interest, in connection with these results, to refer again to the results of determinations of cystine and cysteine in an acid hydrolysate of dried precipitated material from a dialyzed alkaline sea water extract of living sperm (cf. section 7). With the method that was used (Vassel, 1941), 10 times as much cystine was found. Total cystine plus cysteine represented about 1.7 percent of the total dry weight. Cystine and cysteine sulfur accounted for 30 percent of the total sulfur. These figures must be considered as tentative, since they were obtained in a single determination. Further determinations

TABLE IXL

Nitroprusside reaction with EML, 50% saturated with urea.

	Temp. (°C)	Time at temp.	Nitroprusside reaction
EML, 50% in urea	21-37	2 hr.	-
	60	2 min.	-
	60	15 min.	±
	60	45 min.	++
	95	3 min.	+
Cysteine, ca. $5X10^{-3}M$			++

of cystine and cysteine using different methods should be made. Total sulfur determinations of two preparations gave similar values. It must be remembered that these preparations contain unknown amounts of material other than EML. The amount of cystine and cysteine associated with these substances is not known.

Effect of iodoacetate on EML action

Dickens (1933) showed that halogen acetic acids react with SH compounds, in particular with glutathione and cysteine, forming the corresponding thio-ethers and hydrogen halide. This general type of reaction is of biological significance since in certain enzyme systems, e.g., glyoxylase, reduced glutathione (GSH) acts as a co-enzyme. Activity of glyoxylase is inhibited as a consequence of reaction with halogen acetates.

Barron and Singer (1943) made a systematic investigation of enzyme systems whose activity is inhibited by compounds, the effect of which is to block SH groups (iodoacetamide, chloromercuribenzoic acid, organic arsenicals). These authors found a large number of enzymes which were inactivated by these different compounds and reactivated by GSH. They concluded that these enzymes contain in their protein moiety SH groups essential for their activity, and suggest that glutathione, by maintaining these groups in their reduced form would maintain the enzyme activity of systems possessing essential SH groups.

EML activity is apparently not dependent upon a dialyzable co-enzyme or prosthetic group, since it is not affected by dialysis with sea water. The possibility remained, however, that EML activity might be inhibited by reaction with iodoacetate. Since no evidence of free SH groups in the native protein was obtained with the nitroprusside reaction, it was thought that if such groups are involved in lytic action they

might very well be exposed only at the time of reaction with the egg membrane. It seemed a plausible hypothesis, therefore, that if this were the case, iodoacetate might be able to act at this time with deleterious effect on the lytic action.

Experiments. Three experiments were performed, all with identical results. The general procedure was the same in each one.

An active extract of frozen-thawed sperm, estimated mean dissolution time 2 to 3 minutes, was used. A 1 normal solution of iodoacetate was prepared by dissolving 1.86 g of iodoacetic acid in sea water, adding NaOH to make the pH 8.1 and making the volume to 10 cc.

For the tests, 9 drops EML solution at pH 8.1 were combined with 1 drop iodoacetate solution just before testing with eggs. Control dishes contained: 9 drops sea water plus 1 drop iodoacetate solution; 9 drops 0.5M cysteine solution in sea water, pH 8, plus 1 drop sea water; 9 drops 0.5M cysteine solution plus 1 drop iodoacetate solution. Each mixture was thus approximately 0.1N in iodoacetate. A comparable set was also run in which the EML solution was first diluted 100 fold with sea water.

Results are shown in Table VIII L. In no case was EML activity noticeably inhibited by the presence of iodoacetate. It is seen from the table that cysteine was completely inhibited under these conditions.

Discussion. The evidence presented in this chapter shows that "native" EML solutions do not give a positive nitroprusside reaction but that after saturation with urea and heating at 60° a positive test may be obtained. Iodoacetate has been shown not to inhibit the action of EML in causing lysis of the egg membrane.

These data suggest two possibilities. (1) Dissolution of the egg membrane by EML is not mediated by SH groups; the mechanism may be total-

TABLE VIIIL

Effect of iodoacetate on action of EML and cysteine. See text for details of protocol.

Sample	pH	Mean DT (min.)	Percent increase in membrane diameter in 1 hr.
EML - sea water	8	2-3	
EML - iodoacetate	8	2-3	
Sea-water - iodoacetate	8	-	-
0.5M cysteine - sea water	8	8-15	
0.5M cysteine - iodoacetate	8	-	-
EML (100-fold dil'n) - iodo-acetate	8	-	35
EML (100-fold dil'n) - sea water	8	-	40

ly different from that suggested by experiments with SH compounds. (2) Dissolution of the egg membrane by EML may be mediated by SH groups but at the site of action these are not susceptible to blocking by reaction with iodoacetate. It should be pointed out in connection with the second possibility that determination of cystine and cysteine in an acid hydrolysate does not necessarily disclose their relative amounts in the unhydrolyzed or native protein.

10. Role of the egg membrane lysin in fertilization

The demonstration that in mammals dispersal of the cumulus oöphorus by hyaluronidase is not an essential prerequisite to fertilization brings up the question of the extent of dissolution of the keyhole limpet egg membrane that is necessary before fertilization can be accomplished in this species. If mammalian sperm may be assumed to be individually equipped with sufficient hyaluronidase to enable a single spermatozoön to penetrate the cumulus layer, it appears reasonable to suppose, a priori, that keyhole limpet sperm may be similarly individually equipped with sufficient egg membrane lysin. As shown in figure 30, the egg membrane may be completely dissolved in the presence of a large number of sperm. No evidence was available, however, to show whether a small number of sperm, insufficient to effect complete membrane dissolution, might nevertheless be able to cause fertilization to occur.

In order to test this, some experiments were performed at the Marine Laboratory where conditions are favorable for this kind of work.

Eggs were obtained from a ripe female and washed in several changes of fresh, filtered sea water. Sperm was kept dry until just before insemination was to be performed, then suspensions of various concentrations were prepared and eggs added. At the time the eggs were placed in the sperm suspensions the membrane was fully raised. The possibility of parthenogenesis was controlled by placing a number of eggs, equivalent to that placed in the sperm suspensions, in a similar amount of sea water under otherwise identical conditions.

The concentrations of the sperm suspensions used were approximately 0.5, 0.1, 0.01 and 0.001 percent on the basis of cc dry sperm per 100 cc of sea water. Into each suspension 8 to 12 eggs were pipetted.

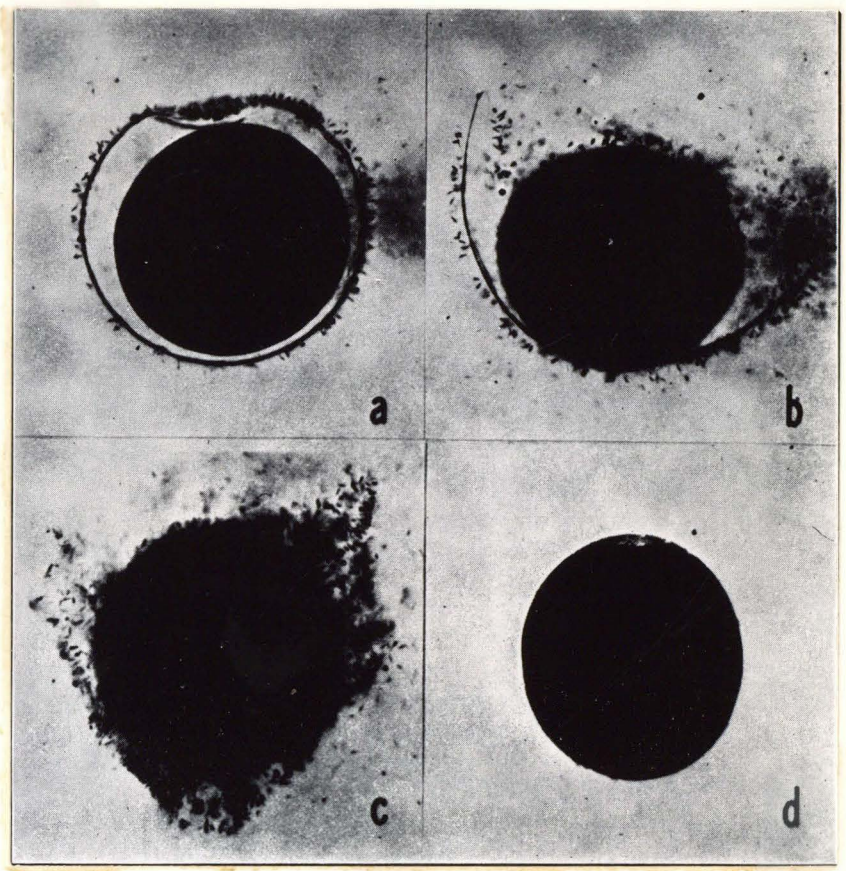

Figure 30. Dissolution of egg membrane in a dense sperm suspension. From Tyler, 1939.

The criterion of fertilization was cleavage.

The results of this experiment are best illustrated by the photographs shown in figures 31 and 32. These show that eggs were fertilized without complete dissolution of the membrane. In some cases there was no visible change in the membrane (figure 31). The egg shown in figure 31 was fertilized in 0.01 percent sperm suspension. No cleaved eggs were found in 0.001 percent sperm suspension or in sea water without sperm. In 0.5 percent suspension 50 percent of the eggs were cleaved and the membrane of all eggs in this dish was partially or completely dissolved (figure 33). In 0.1 percent sperm suspension 25 percent of the eggs cleaved and in 0.01 percent suspension 12.5 percent.

Attempts were made to observe individual sperm penetrating the membrane. It was thought that sperm might be able to enter through the micropyle. As shown in figure 34 the micropyle in eggs with the jelly hull intact is occluded by a plug of material. This may be attached by a strand running through the jelly layer and joined distally to a fragment of follicle tissue. In a number of prolonged periods of observation no sperm have been seen to enter the micropyle or to penetrate the membrane at any other place. It was seen that individual spermatozoa can each make its own little dent in the membrane (see figure 32).

One possibility, in addition to continuing observations of the kind made heretofor, offers a little promise. It was recalled from observations made with fixed eggs that the egg is generally located eccentrically within the membrane. A portion of the egg surface (the lower surface when viewed from above) is thus apparently in contact with a part of the inner surface of the membrane. It was thought that in this region there might be a mutual interaction between the egg surface and the membrane which might facilitate sperm entry. In order to test this

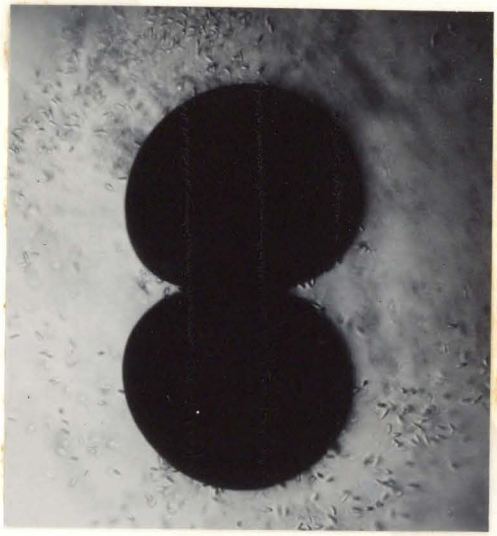

Figure 31. Cleaved egg with intact membrane.

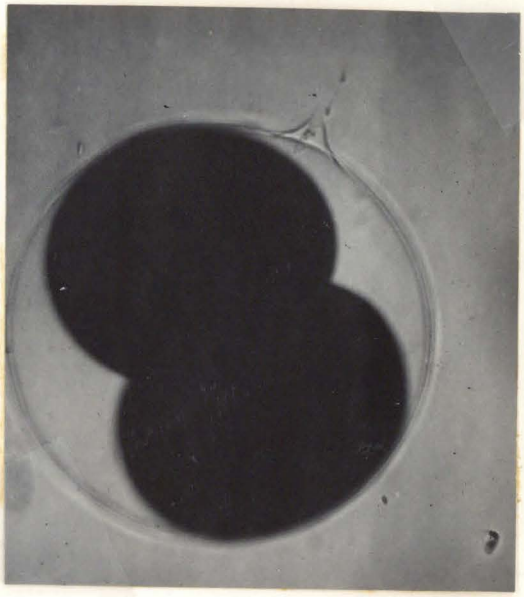

Figure 32. Cleaved egg with intact membrane.

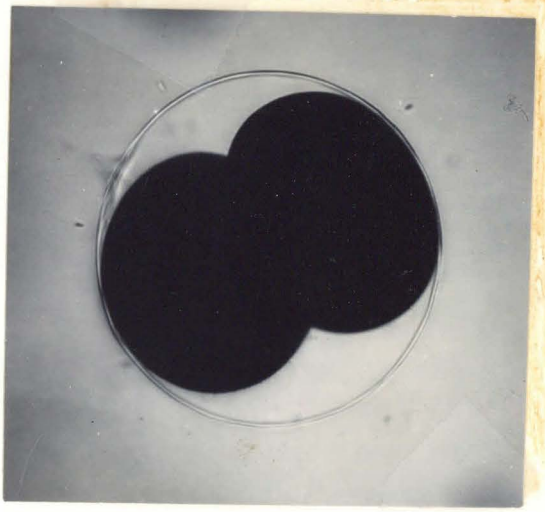

Figure 33. Cleaved egg with membrane dissolved.

Figure 34. Same as fig. 31. Focus on micropyle.

possibility the following experiment was done.

A vaseline slide was prepared, leaving a gap in the vaseline on one side. A drop containing a number of fresh eggs was placed inside the vaseline border and a cover slip pressed into place over the drop. The slide was placed on the stage of a microscope and the tube tilted to a horizontal position so that the stage and slide were now in a vertical plane. The open side of the chamber was at the top, and through the opening a drop of dilute sperm suspension was added.

Examination of the eggs showed that the lower surface of each was indeed apparently in contact with the inner surface of the membrane. Observation was continued after addition of sperm, and individual spermatozoa were seen to come into contact with the membrane in the region where it seemed to be in contact with the egg. None of these sperm were seen to penetrate the membrane and none of the eggs in the suspension were fertilized. It needs to be added, however, that the eggs of this particular lot were poor, and possibly unfertilizable (under-ripe). It is not inconceivable that failure of fertilization was due to failure of the egg to react to stimulus of the sperm in the region under observation. Further observations of this kind, with ripe, fertilizable eggs are required.

Discussion. The experiments reported in this section show that complete dissolution of the egg membrane of the keyhole limpet is not necessary for the egg to be fertilized. It has been shown, as a matter of fact, that fertilization can occur without any obvious change in the appearance of the membrane. From this it would appear, therefore, that individual spermatozoa of this species are able to penetrate the membrane in some manner without the assistance of additional EML pro-

vided by a large number of sperm.

The manner in which a single spermatozoon may penetrate the membrane has not been determined. Lack of favorable material and of time have made it impossible to carry out adequate observations.

It is suggested that penetration of the membrane by a spermatozoön may occur in the region of the membrane where its inner surface appears to be in contact with the egg surface. It is possible that the egg may in some way participate in the process. A method is described whereby it should be possible to observe sperm penetration if it occurs in this region.

EGG MEMBRANE LYSINS OF OTHER MARINE INVERTEBRATES

11. Egg membrane lysin of echinoderms. Effect of cysteine on sea-urchin eggs.

In connection with some experiments in which sea-urchin (Lytechinus pictus) eggs were treated with Megathura EML, it was thought to be of interest to see if extracts of Lytechinus sperm might have any effect on the Megathura egg membrane. It was found, not only that extracts of Lytechinus sperm contain a lytic factor capable of dissolving the egg membrane of Megathura, but that such an agent can also be demonstrated in extracts of sperm of two other species of sea-urchin, Strongylocentrotus purpuratus and S. franciscanus. The experiments in which it was shown that sperm extracts of these echinoids contain an egg membrane lysin are described in this ~~chapter~~ section.

Experiments. A 15 to 20 per cent suspension of Lytechinus sperm in sea water at about pH 8 was left overnight in the refrigerator. In the morning the suspension was centrifuged in the cold and the supernatant filtered through washed asbestos. The pH of the filtrate was about 7.5. It was tested for antifertilizin with homologous eggs and found to contain only a trace (about 2/3 of treated eggs stuck to the dish with no visible precipitation membranes formed). It was then tested on Megathura eggs and found to have no effect on the membrane.

The sperm residue had been resuspended in sea water to approximately the original volume. The suspension was placed in the freezer. Two days later it was removed and thawed at room temperature. The clear, foamy supernatant at pH 7.2 was tested with Megathura eggs. Five eggs were used in the test and the membrane of each egg was completely dissolved in less than 15 minutes. The extract was also found to have good antifertilizin activity.

A portion of the extract was now heated at 60° for 2 minutes. When tested with Megathura eggs the membranes were not affected in a period of 50 minutes. Eggs treated with the unheated extract again were denuded of the membrane in 10 to 15 minutes. The heated extract showed approximately the same degree of antifertilizin activity as the unheated portion.

In another experiment Megathura eggs were placed in a 10 percent suspension of active Lytechinus sperm. No effect on the membrane was observed in 10 to 15 minutes.

Similar experiments were performed using suspensions of S. purpuratus and S. franciscanus sperm. The results of all of the tests are summarized in Table VII L. From the table it is seen that EML activity of extracts of frozen-thawed S. purpuratus sperm was weaker (from comparable sperm suspensions) than that of either Lytechinus or S. franciscanus sperm. In each case activity was abolished by heating.

Results similar to those found with frozen-thawed sperm extracts were obtained with extracts prepared from lyophilized sperm of Lytechinus and S. purpuratus. It was found desirable to blend the suspensions briefly on the Waring blender using a chilled stainless steel head and then to freeze and thaw the suspensions. For comparison, lyophilized Megathura sperm was treated in the same way. The resulting extract was found to contain active EML. Extracts of lyophilized S. purpuratus sperm were definitely less active than comparable extracts of Lytechinus sperm.

In Table VII L it is noted whether fresh or fixed eggs were used in the various tests. It is of interest that there was no apparent difference in the reaction.

Finally, it should be mentioned that no EML activity could be de-

TABLE VII L

Effect of sperm, seminal fluid and sperm extracts of 3 species of echinoid on the egg membrane of fixed and fresh (living) eggs of Megathura.

Species	Preparation	Effect on Megathura egg membrane	Eggs
Lytechinus pictus	10% sperm susp.	-	fresh and fixed
	seminal fluid	-	fresh and fixed
	extract of frozen-thawed sperm	++++*	fresh and fixed
	Ht'd 2 min at 60°	-	fixed
S. purpuratus	10% sperm susp.	-	fresh and fixed
	seminal fluid	-	fresh and fixed
	extract of frozen-thawed sperm	+++	fresh and fixed
	Ht'd 2 min. at 60°	-	fixed
	extract of lyophilized sperm	+++	fresh
S. franciscanus	extract of frozen-thawed sperm	++++	fresh
	Ht'd 2 min. at 60°	-	fresh

*This means membranes dissolved; +++ = Membranes dissolved more slowly than indicated by ++++.

tected in seminal fluid of S. purpuratus or in concentrated suspensions of living sperm. These observations parallel those with Lytechinus seminal fluid and sperm suspensions.

Reciprocal tests. Effect of Megathura EML on sea-urchin eggs.

There are, unfortunately, scarcely any worthwhile data concerning the effect of Megathura EML on unfertilized sea-urchin eggs. A number of experiments was performed in attempts to obtain evidence concerning possible effects on the vitelline membrane or the developing fertilization membrane, but the results were not sufficiently clear-cut to warrant presentation.

On the other hand, it does seem reasonably clear that EML does not affect the fully developed fertilization membrane nor does it seem to have any action on the jelly hull or on the precipitation membrane formed by interaction of fertilizin and antifertilizin.

Effect of cysteine on sea-urchin eggs

Experiments in which the effect of solutions containing cysteine were tested on unfertilized and fertilized sea-urchin eggs yielded reasonably clear-cut results.

Unfertilized, washed eggs of Strongylocentrotus purpuratus were used in these experiments. The jelly coat was left intact. Only eggs from lots showing 100 percent fertilization membrane elevation were used. The experiments were performed in a constant temperature room at 18°.

Careful attention was paid to cleaning all glassware used in the tests. Dishes, pipets, etc., were washed thoroughly with a powerful detergent in hot water then rinsed 12 to 24 hours in running tap water. After a final rinse in distilled water, glassware was dried in air.

Cysteine solutions were prepared with sea water and the pH was adjusted to 8.0. The solutions were made up just before use.

For the tests, about 0.5 ml of solution was placed in a salt cellar. Eggs were pipetted from a stock suspension, a single drop, containing 50 to 75 eggs, being placed in each test solution. Eggs in each dish were inseminated with 1 drop of a 0.1 percent sperm suspension.

Results. It was found that 0.01M cysteine solutions had but slight effect on the eggs but that 0.1M solutions had pronounced effects. Results of tests with 0.1M solutions are shown in Table VI L.

The data show that when eggs were treated for periods of from 10 seconds to one minute prior to insemination and then inseminated in the cysteine solutions, 90 to 100 percent of the eggs raised fertilization membranes. These, however, were thin and appeared fragile in comparison with fertilization membranes raised by control eggs. Within 5 minutes after the fertilization membranes were raised they had disappeared in all of the treated eggs. Disappearance of the membranes appeared to be due to dissolution. When exposed to cysteine for 3 to 5 minutes before insemination and inseminated in the solution, only about 10 percent of the eggs were observed to raise very thin membranes and these all disappeared within 15 to 30 seconds. The hyaline layer appeared to form normally in the treated eggs. With eggs treated for 10 minutes prior to insemination and inseminated in the solution, less than 5 percent showed a very faint "blistery" membrane which disappeared almost at once.

When eggs were treated for 20 minutes in 0.1M cysteine and then washed in several changes of sea water before insemination "tight" fertilization membranes appeared on about 50 percent of the eggs after insemination. Controls handled the same way showed 100 percent normal fertilization membrane elevation. After 5 minute treatment followed by washing before insemination, all of the treated eggs raised apparently normal fertilization membranes.

TABLE VI L

Effect of cystein on fertilization membrane of sea-urchin eggs (S. purpuratus).

		Time of treatment with 0.1M cysteine prior to insem. (min.)	Time after insem. placed in 0.1M cysteine (min.)	Percent fert. memb. elev.	Time f.m. persists
		10		0(5% show small blisters)	-
Unfertilized eggs	Eggs left in cysteine after insem.	5		10	15 sec.
		3		10	30 sec.
		1		90	5 min.
		1/6		100	5 min.
	Eggs washed prior to insem.	20		50 tight	1½ hrs.*
		5		100	1½ hrs.
Fertilized eggs	Eggs tr't'd in cysteine after insemination		1/6	100	8-10 m.
			1/2	100	8-10 m.
			1	100	8-10 m.
			2	100	8-10 m.
			5	100	1 hr.
	All controls (fert. and unfert.)			100	1½ hrs.

*Duration of observations.

When eggs were inseminated in sea water, and cysteine solution added at different times after insemination it was found that if the cysteine was added within about 2 minutes the fertilization membrane appeared in 100 percent of the eggs but seemed thinner than normal and was dissolved within 8 to 10 minutes. If, however, cysteine was not added until 5 minutes after insemination the membranes were not dissolved up to 1 hour after fertilization. Many of these membranes did seem somewhat "soft", inasmuch as they were less regular in outline than membranes of control eggs.

Discussion. Results somewhat similar to those reported here were obtained by Runnström, Monné and Broman (1943) in experiments in which they observed the action of 0.1 percent cysteine solutions ("neutralized") on fertilization membranes of Echinocardium eggs. These authors found that jelly-coated eggs, after treatment in cysteine and insemination in the solution, raised fertilization membranes more rapidly than controls but that membranes of the treated eggs, though they appeared larger than normal ones, did not disappear. Up to several hours after fertilization, however, the membranes of treated eggs could be shaken off by an amount of shaking which did not shake off normal membranes. Runnström, et al., suggest that hardening of the fertilization membrane is delayed by cysteine. The larger size of the membranes elevated by cysteine-treated eggs was attributed, however, to softening of the jelly by the cysteine. When jellyless eggs of either Psammechinus or Echinocardium were treated in 0.1 percent cysteine solutions at pH 7.8 to 8.0, normal membrane elevation was not prevented after insemination in the cysteine solution. Sooner or later, however, the membranes faded away during the first hour after fertilization. No collapsed membranes could be demonstrated on the surface of the eggs.

The Swedish authors auggest that with jellyless eggs the fertilization membranes "obviously dissolve under the action of cysteine". When the jelly is present, however, it is asserted that this supports the membranes so that dissolution occurs only under the influence of mechanical force, which removes the jelly and accelerates the breakdown of the fertilization membranes.

Results of the present study are in harmony with those reported by Runnström, Monné and Broman except that in the present experiments it has been found that fertilization membranes of jelly coated eggs are rapidly dissolved when they are inseminated in cysteine solutions. This is undoubtedly due to the higher concentration of cysteine employed in the present experiments (0.1M cysteine = 12.1 g. per liter; 0.1 percent = 1 g. per liter).

12. Egg membrane lysin of other marine molluscs. Cross reactions.

The presence of an egg membrane lysin in extracts of abalone sperm was reported by Tyler (1939) and confirmed, in related species, by von Medem (1942). More recently, Berg (1949) has reported the presence of an egg membrane lysin in extracts of mussel sperm. In each case, the demonstration of the presence of egg membrane lysin was made with homologous eggs. According to Tyler (personal communication) extracts of Astrea (top shell) sperm contain an egg membrane lysin.

The present author is able to confirm the presence of an egg membrane lysin in extracts of sperm of abalone (Haliotis cracherodii) and mussel (Mytilus californianus). Astrea sperm extracts, have not been tested.

Except for the cross reaction experiments to be described below, no experiments have been undertaken with EML of any of these molluscs. It is worthy of note, however, that it was found, quite accidentally, that the abalone egg membrane is rapidly dissolved in sea water at pH 3. This is in striking contrast to the resistance of keyhole limpet egg membrane to acid. It is also of interest that the Mytilus EML is resistant to heat. It is not inactivated to a noticeable extent by 5 minutes heating at 60°. According to Berg (1949) this lysin is resistant even to boiling for 10 minutes. These points may be kept in mind in considering the results of cross reactions .

Cross reactions

For the cross reaction tests, extracts of frozen-thawed sperm of Megathura crenulata, Haliotis cracherodii, Mytilus californianus, Lytechinus pictus, Strongylocentrotus purpuratus and S. franciscanus were used. Extracts of lyophilized sperm of Megathura, Haliotis, Mytilus and S. purpuratus were also tested, and some tests with seminal fluid (supernatant

from concentrated sperm suspensions) of Megathura, Haliotis, Mytilus, Lythechinus and S. purpuratus were made. The various extracts were prepared from sperm suspensions of comparable concentration and were adjusted to a pH between 7.5 and 8 for the tests. The extracts were tested on eggs of Megathura, Haliotis and Mytilus.

In these tests the criterion of complete or partial dissolution of the egg membrane was used as a basis for defining a positive reaction. Only complete or nearly complete dissolution was considered "definitely" positive; partial dissolution, i.e., obvious thinning and swelling, "softening" and "spreading out", was considered probably positive. In presenting the results, 4-plus means definitely positive, and 3-plus means probably positive.

Indentation or collapse of the membrane, not followed or accompanied by thinning, swelling or dissolution, were not considered positive reactions, since these are non-specific. It was of interest, however, to include these in the scoring system, so 2-plus means the membrane was collapsed within 5 minutes but no further action was observed, and 1-plus means the membrane was indented but not collapsed within this period. A minus sign means that no change of any kind was observed in the membrane within the period of observation.

The results of the tests are summarized in Table VL. These are representative of a number of different tests made at different times with various preparations. In the table, blank squares indicate that the particular reactions were not observed. A question mark indicates that tests were not completely unambiguous. It is seen that all question marks are in connection with eggs of Mytilus. No fully ripe females of this species were available at the times of testing. Large numbers of immature eggs were usually obtained, with only occasional mature

TABLE V L

Reaction of sperm extracts and seminal fluid of various molluscs and echinoids with egg membranes of 3 species of molluscs.

Sperm extract	Eggs		
	Megathura	Haliotis	Mutilus
Megathura			
f.t. or lyoph.*	++++	+	+++
sem. fl. **	++++	-	-?
Haliotis			
f.t. or lyoph	+++	++++	-?
sem. fl.	-	+++	
Mytilus			
f.t. or lyoph	++++	+++	++++
sem. fl.	++	+	?
Lytechinus			
f.t.	++++	-	
sem. fl.	-	-	
S. purp.			
f.t. or lyoph.	+++ to ++++	-	-?
sem. fl.	-	-	
S. fran.			
f.t.	++++		-

*F.t. = frozen-thawed extract.

++Sem. fl. = seminal fluid.

ones. In some cases no mature eggs were found in a suspension. Although a well-defined membrane is present about the oocytes it is possible that it may differ in some way from that of mature eggs.

The cross reaction tests show some interesting features. These will be pointed out in the following discussion.

Discussion. With respect first to cross reactions between the different species of molluscs, it was found that cross reaction occurs between sperm extracts (frozen-thawed or lyophilized) of Mytilus and egg membranes of both Megathura and Haliotis. Mytilus seminal fluid, on the other hand, does not give a positive reaction with either of these species. Reaction of the seminal fluid with homologous eggs was not definitely established. Haliotis sperm extracts (frozen-thawed or lyophilized) reacted positively with Megathura egg membranes. Reaction with Mytilus membranes is not certain. Seminal fluid of Haliotis sperm does not give a positive reaction with Megathura egg membranes, but does react weakly with the membrane of homologous eggs. Megathura frozen-thawed extracts and extracts of lyophilized sperm do not give a positive reaction with Haliotis eggs, but do seem to act on Mytilus egg membranes. Megathura seminal fluid did not have a positive action on Haliotis or Mytilus eggs.

Two conclusions can be drawn from these results. The first is that in those cases where tested, seminal fluid, i.e., supernatant fluid from concentrated sperm suspensions, does not give a positive reaction with heterologous eggs, whereas the corresponding extracts of frozen-thawed or lyophilized sperm may do so. Living sea-urchin sperm were also found to be incapable of lysing the keyhole limpet egg membrane. This is probably attributable to the fact that the concentration of active material in the seminal fluid is much less than in extracts prepared by methods

which cause disintegration of the sperm.

The second conclusion is that the lytic agents are highly non species specific. This is particularly well illustrated by the occurrence in sea-urchin sperm extracts of lysins capable of dissolving the egg membrane of at least one of the molluscs (Megathura). A degree of specificity is shown by Megathura EML, since it does not react with the Haliotis egg membrane. In this case it may be significant that the membrane of the abalone egg is dissolved by low concentration of acid in the medium. This undoubtedly reflects a basic difference in the molecular architecture of this membrane. The Mytilus egg membrane, on the other hand, is attacked by Megathura EML, and in this case the membrane is not affected by acid as strong as 0.1 normal. Thus, there may be more similarity in the molecular structure of this membrane to that of the keyhole limpet egg. Of interest in this respect, however, is the fact that Haliotis sperm extract shows a positive reaction with the Megathura egg membrane. This is difficult to reconcile with failure of the reciprocal reaction and the demonstrable difference in nature of the egg membranes of the two animals. It is possible at the present time only to make a guess as to a possible explanation of this situation.

It might be supposed that Haliotis sperm extracts contain at least 2 distinct egg membrane lysins, one of which reacts with the abalone type of membrane and the other with the Megathura type. This would be analogous to the existence in the serum of various animals of natural heteroagglutinins (Tyler, 1946; Tyler and Metz, 1945). Megathura sperm extracts, in contrast, might contain different lysins, one of which acts on the Mytilus membrane, one on the homologous membrane but none of which act on the Haliotis membrane. On the other hand it is possible that the Mytilus type of membrane is similar enough in its structure to that of

Megathura so that the same agent is able to react with both but not the abalone membrane.

The same line of reasoning might also be applicable in the case of Mytilus sperm extract. This reacts with both Megathura and Haliotis egg membranes and might thus be assumed to contain at least 3 different lysins, each with a characteristic specificity.

If sperm extracts of each of these molluscs contain "heterolysins" which are responsible for the observed cross reactions, the data would fall in line with the general concept of heteroagglutinins. The presence in extracts of sea-urchin sperm of lysin(s) capable of reacting with the egg membrane of at least one mollusc (Megathura) presents a situation that is not dissimilar to that of lobster serum. This has been shown by Tyler and Metz (1945) to possess heteroagglutinins which are capable of agglutinating the spermatozoa or blood cells of a large number of animals belonging to different groups on the order of taxonomic classes.

An observation of significance with respect to this problem is that heated Mytilus sperm extract reacts with apparently undiminished effect on Megathura egg membranes (extract heated at 60° for 5 minutes). According to Berg (1949) this is also true in the case of its reaction with homologous egg membranes. In this respect, therefore, "hetero"(?) lysin of Mytilus sperm extract is different from the heat labile Megathura EML.

In contrast to the cross-reactivity of the frozen-thawed extracts, the living sperm are rather specific in their membrane-dissolving action, as noted in section 11.

GENERAL DISCUSSION

The "Mucin Clot" reaction

The finding that fertilizin co-precipitates with proteins in acid solution is consistent with what is known concerning its chemical constitution. It has been shown (seeTyler, 1948 a,b) that fertilizin is a high molecular weight substance of highly acidic properties belonging to that class of amino-acid and sugar-containing compounds that is termed glycoprotein or mucopolysaccharide. Certain acidic polysaccharides such as hyaluronic acid (Myer and Palmer, 1936) and chondroitin sulfate(Meyer and Palmer, 1937) have been found to have the capacity of co-precipitating with protein in acid solution, and this may well be a general property of such substances, as Meyer (1945) has intimated. Neither the present work nor that of other workers identifies the particular chemical groups of the type of bonding that is involved in this reaction. Some suggestions concerning the precipitation mechanism were made in Part III, section 3 of this thesis, and are examined further below.

Of particular interest is the reversibility of the reaction. When the pH is raised above 5.6 the precipitate dissolves and the fertilizin is recovered with unaltered titer. It is safe to conclude that the reaction does not involve alteration of the specific combining groups of the fertilizin. Co-precipitation with protein in acid solution is employed routinely in the isolation of hyaluronic acid from native fluids (Meyer, 1947). It appears, therefore, that for fertilizin, too, the precipitation reaction provides an additional tool in purification.

Study of the effects of pH on the reaction involving dilute fertilizin solutions permits certain suggestions concerning the mechanism of precipitation. Since precipitation occurs at a pH at which both the fertilizin and protein are anionic, as well as at a pH at which the protein

~~are anionic, as well as at a pH at which the protein~~ is positively charged, electrostatic and Van der Waal's forces may be involved, as is postulated for analogous reactions of proteins with other organic and inorganic anions (see Teresi and Luck, 1948). Dissociation of these complexes at a pH higher than the protein isoelectric point (5.6 in the case of fertilizin) has been inferred to be caused by electrostatic repulsion overcoming Van der Waal's forces of attraction.

Of further interest in the case of the fertilizin-protein combination, however, is the fact that the amount of precipitate (turbidity) produced with dilute fertilizin solutions attains a maximum at a pH somewhat below the isoelectric point of serum albumin (4.8) and then this decreases rapidly beginning at about pH 4.2 to 4.4, depending upon the amount of fertilizin in the system. Dissociation of the co-precipitate at a pH where the protein is positively charged is most readily understood on the assumption that the fertilizin becomes less anionic in character. Data from electrophoresis experiments (Tyler, 1948 b), however, do not support such an assumption since these show that fertilizin migrates to the anode with practically constant velocity from pH 8.6 to 2. The electrophoresis data may be open to revision, since the value at the lowest pH is based on only one determination (Tyler, personal communication). In this connection it may be noted that amino acid residues occur in fertilizin and, unless all of the free groups are combined in ester or other linkages with other constituents of the molecule, these may enter into the reaction with protein.

It is of interest that co-precipitation of hyaluronic acid and protein is affected similarly by the pH. Thus, for example, Dorfman and Ott (1948) determined that a maximum turbidity is attained at a pH of about 3.8. Somewhat earlier, Seastone (1939) found that turbidity pro-

duced by reaction of hyaluronic acid and protein disappears almost entirely above pH 6 and below pH 3. Hyaluronic acid does not contain amino acid residues, hence some mechanism other than interaction of ionizable groups of amino acids with the protein must account for the phenomenon in this case. This may also be true for fertilizin. It is apparent that considerably more experimental data must be obtained before this phenomenon can be described in other than speculative terms.

Co-precipitation of protein and fertilizin at about pH 4.5 and with small amounts of fertilizin, with which precipitation occurs as a turbidity, is favored by minimal ionic strength of the medium. The effect of ionic strength is probably not due to a specific anion, since both Cl^- and Ac^- cause reduction of the precipitation in about the same degree. Whether this is a "salting-in" effect on the fertilizin-protein complex, or whether it may be due to reaction of the constituents separately with the added salts cannot be decided on the basis of the present data. A similar effect has been observed in the case of co-precipitation of hyaluronic acid and protein by Dorfman and Ott (1948), who, however, attempt no interpretation of the phenomenon.

The type of precipitate that forms upon interaction of hyaluronic acid with protein in acid solution is considered to depend upon the state of polymerization, or perhaps the purity of the hyaluronate (McClean, 1943; Meyer, 1947). With native hyaluronic acid, i.e., synovial fluid, etc., a fibrous mucin clot is formed. After incubation with hyaluronidase the quantity of the clot is reduced and the character of the precipitate changes from a fibrous to a flocculent precipitate, until finally the solutions remain clear. Purified hyaluronate, on the other hand, precipitates with protein in acid solution in flocculent

form. The difference, according to Meyer (1947) might be due to the fact that native hyaluronic acid may exist in combination with protein. This might account, then, both for the higher viscosity of the native substance and the character of the precipitate that is formed by co-precipitation with added protein in acid solution. More recently, however, Meyer (1948) has reported a method for the preparation of highly viscous sodium hyaluroniate (presumably free from protein). The character of the precipitate formed by this material with acidified protein solution was not noted. McClean (1943) found that the clotting power of hyaluronic acid is destroyed by hyaluronidase before any appreciable fall in viscosity occurs, and suggests that destruction of the clotting power is an early stage in the degradation of the polysaccharide.

In dilute solutions it is not easy to distinguish between a "clot" and a flocculent precipitate. Whether these actually represent different degrees of polymerization of the hyaluronic acid or may depend upon the concentration of this compound in the solution is not clear. In the case of fertilizin, the character of the precipitate seems to depend mainly upon the concentration. Thus, a viscous solution of "egg water" will precipitate in a heavy, clot-like form, but as it is diluted the precipitate is correspondingly decreased in size, and at high dilution may appear flocculent or, if sufficiently diluted, only a turbidity may be produced. It should be noted, too, that when fertilizin is treated in such a way as to convert it into the so-called univalent form it may still give the clot type of precipitate. The general question of the relation of type of precipitate to state of aggregation does not, then, appear to be resolved by the investigations of Meyer and McClean and may well warrant further investigation.

Apparent dissolution of the gelatinous coat by hyaluronidase and sea-urchin sperm extracts

The results reported in Part III of this thesis refute earlier claims that hyaluronidase or hyaluronidase-like agents occur in sea-urchin sperm extracts. It has been shown that neither hyaluronidase nor sea-urchin sperm extracts are effective in reducing either the clot formed with concentrated fertilizin solutions or the turbidity formed with dilute fertilizin solutions ~~or the turbidity formed with dilute fertilizin solutions~~ when these are mixed, after incubation with the various extracts, with acidified protein solutions. Additonally, direct observations of sea-urchin eggs treated in these extracts,including hyaluronidase, revealed no effect on the egg jelly that could not be attributed to the presence of antifertilizin.

The reported presence in sea-urchin sperm extracts of jelly-dissolving agents (Hartmann and Schartau, 1939; Hartmann, Schartau and Wallenfels, 1940; Ruffo and Monroy, 1946; Monroy and Ruffo, 1947) may possibly be attributable to one of two, or perhaps both, circumstances. The first of these is the fact that the jelly hull of sea-urchin eggs normally swells on standing in sea-water and undergoes some degree of dissolution. In different lots of eggs, varying percentages of jelly-less eggs may be found, and unless an experiment includes adequate controls the results may have no significance. The second point is that reaction of antifertilizin in sea-urchin sperm extracts with fertilizin of the egg results in the formation of a precipitation membrane on the jelly which may contract to the egg surface. With active antifertilizin solutions contraction to the egg surface may occur very rapidly and unless observations are made immediately after exposure of the eggs to an extract containing antifertilizin the precipitation membrane may not be

seen. As a consequence, since the jelly is apparently gone, and the precipitation membrane is not normally visible on the surface of the egg, the result may be attributed to dissolution of the jelly by a lytic agent of the sperm extract.

Lysins of sea-urchin sperm

While a jelly-dissolving agent is evidently lacking, there are two distinct lytic agents that can be extracted from the sperm of sea-urchins. The first of these to be described was the so-called egg surface lysin, a methanol-soluble substance which acts on the surface of the egg. The presence of egg surface lysin in methanol extracts of sea-urchin sperm was demonstrated by Runnström, Lindvall and Tiselius (1944), and activity similar to that attributed by these authors to this lysin has been obtained in the course of the present work in lipoid extracts of sea-urchin sperm. In a series of publications, Runnström and his group (see literature review for references) have shown that the action of egg membrane lysin can be duplicated to a considerable degree by bee venom and synthetic detergents in appropriate concentration. It is claimed that the lysin exerts a liquefying action on the egg cortex as a consequence of possessing lecithinase-like or detergent-like properties, and because of this action it is of central importance in activation of the egg and in establishing the block to polyspermy. It is of interest that material with similar activity is also obtained from fish sperm.

In the present work it has been shown that egg surface lysin activity is associated with water-soluble constituents of total lipid and phospholipid fractions of sea-urchin sperm extracts. Runnström claims that egg surface lysin is also present in supernatant fluid obtained from concentrated suspension of living sperm. In the present study, egg surface lysin activity was obtained from a concentrated suspension of

living sperm in only one experiment of several that were tried. In this case the egg surface lysin was found to be accompanied by antifertilizin. It has been reported by Runnström, Monne and Wicklund (1946) that "antifertilizin" may affect the egg surface in a manner that appears to be, in some respects, similar to that which is considered to be characteristic of the effect of egg surface lysin. Thus, for example, these workers found in one experiment that, after treatment in antifertilizin solution the eggs retained a smooth surface upon exposure to hypertonic medium. In other experiments various degrees of wrinkling were observed when treated eggs were exposed to hypertonic sea water. In view of these results, the evidence concerning the presence of egg surface lysin in seminal fluid is believed to be inadequate, and its availability under physiological conditions is held to be unproven.

The second lytic agent of sea-urchin sperm to be demonstrated is an egg membrane lysin, reported in the present paper. This acts on the egg membrane of the giant keyhole limpet. Its presence has been demonstrated in aqueous extracts of frozen-thawed and lyophilized sperm, but not, to the present time, in seminal fluid or heavy suspensions of living sperm. Specific effects of this agent on the vitelline membrane of the sea-urchin egg have not as yet been observed. The agent is heat-labile and in this respect resembles the egg membrane lysin of keyhole limpet sperm.

Egg membrane lysin of keyhole limpet sperm

The egg membrane lysin of the giant keyhole limpet is probably of protein nature, as was shown by Tyler (1939) and by evidence presented in the present paper. The lysin is a labile substance, easily inactivated to a considerable degree by procedures which result in precipitation of constituents of the sperm extracts. Thus, it has been found that salting out and isoelectric precipitation result in the loss of much of the

initial activity. Purification of the lysin is made difficult for this reason.

The apparent ease with which the egg membrane lysin is inactivated (denatured?) indicates that in active condition it exists in a state of relatively unstable equilibrium, which is easily destroyed by the various inactivating treatments, including changes in pH and electrolyte concentration. Maintenance of the equilibrium condition may depend upon retaining a specific molecular configuration, involving various charged groups of the lysin molecule. Treatment, then, that leads to change in the configuration or distribution of charges may result in inactivation.

One phase of the lytic reaction appears to be due to an osmotic effect of the lysin. This is the indentation of the membrane which is usually seen to occur in the early stages of lysis. Non-lytic colloids, such as various proteins and the dye congo red may cause indentation and collapse of the membrane. Smaller molecular weight substances such as sucrose may also have this effect when present in sufficient concentration. Ions, however, do not cause indentation. Of interest here is the fact that denatured proteins do not cause indentation. "Denaturation" is used here to mean a change in properties resulting in loss of solubility, which is manifested by the appearance of turbidity in initially clear solutions. Denatured lysin fails to cause indentation, as well as failing to dissolve the egg membrane. It is thought from this that inactivation is associated with loss of solubility.

Various tests with simple substances that show activity and that imitate EML action suggest that lysis of the egg membrane may result from the action of particular atomic groups. The most active of the simple compounds tested were those containing sulfhydryl groups. Of these, the most effective seemed to be cysteine. It is of interest in this con-

nection that glutathione and thioglycolate are both somewhat less effective, mole for mole, than cysteine despite the fact that all three of these compounds contain one SH group per mole. None of these substances, however, approach in effectiveness the egg membrane lysin, when comparison is made on a weight per volume basis. The latter substance shows activity in a concentration of less than 2 mg per liter, whereas the minimal effective concentration of cysteine, for example, is on the order of 120 mg per liter (minimal effective concentration with respect to causing measurable swelling of the membrane). This difference may be attributable to specific configruation of the lysin molecule (complementary configuration), to the presence of other active groups, or to both of these factors. It was found that an acid hydrolysate of dried material from an active lytic preparation yielded cystine and cysteine in small amount (accounting for about 30 percent of the total sulfur). Cystine was found in about 10 times the amound of cysteine. This, however, does not necessarily mean that the same relation holds in the native molecule.

It was found that the nitroprusside reaction was negative with undenatured lytic material, indicating that SH groups are not exposed. With material denatured by saturation with urea and accompanied by heating, a positive nitroprusside reaction was obtained. It is of interest that membrane lysis is not inhibited by iodoacetate. This may mean that at the site of action, SH groups (if these participate in the reaction) of EML must in some way be protected from reaction with iodoacetate.

The occurrence in extracts of sea-urchin sperm and in extracts of the sperm of other molluscs, of lytic agents that are capable of dissolving the keyhole limpet egg membrane, suggests two possibilities with respect to the nature of the substances exhibiting this action. The first is that they are similar in their composition and molecular configuration

to the keyhole limpet lysin. In the case of the abalone and mussel, this does not necessarily imply that the lysin which acts on the keyhole limpet egg membrane is the same as that which is responsible for lysis of the homologous egg membrane. The second possibility is that the specificity of the reaction is broad and these substances may be similar only in certain general features. The only point of evidence at this time which may have some significance in this connection is the fact that the relatively heat-stable mussel lysin acts on the keyhole limpet egg membrane. The reciprocal reactions, where they occur, are relatively weaker, but the same kind of reasoning may be applied to these.

No positive cross-reactions were observed with seminal fluid or with living sperm in cases where these were tested. This is of interest since it suggests that under physiological conditions corss reaction of lysins may not occur, and this may be a factor in maintaining the specificity of fertilization.

Conclusion

It is perhaps clear that the various events of fertilization (approach, attachment and penetration of the sperm, activation of the egg, etc.) involve a complex series of reactions. There are evidently a number of different substances concerned in these events and it is quite probable that no single substance has a central role. Of the various substances that have been extracted and may be extractable from eggs and sperm it can be said at the present time that each may enter into the reaction chain that culminates in the initiation of development. A complete picture of the sequence of interactions is lacking, and its attainment is the goal of present and future research in this field.

SUMMARY

1. It has been found that sea-urchin fertilizin co-precipitates with protein in acid solution. Precipitation of fertilizin with serum albumin occurs in the pH range of about 3 to 5. Depending upon the concentration of fertilizin (with serum albumin in excess) the reaction may result in a heavy clot-like precipitate, the so-called "mucin clot reaction", a more flocculent type of precipitate or a turbidity. The latter is produced by mixtures containing as little as 3 micrograms of fertilizin per ml. With dilute fertilizin solutions, which produce only a turbidity, the reaction has been termed the "turbidity reaction".

2. It has been found that the amount of turbidity produced by a dilute solution of fertilizin is dependent upon the pH, salt concentration and albumin concentration, as well as upon the concentration of fertilizin.

3. The mucin clot and turbidity reactions are reversible. With the former, the precipitate is dissolved above pH 5.6 and the fertilizin is recovered with undiminished titer. With the latter, maximal turbidity is produced at about pH 4.2 to 4.5, depending upon the concentration of fertilizin (with albumin in excess) and the amount of turbidity decreases rapidly above and below this range.

4. Co-precipitation of fertilizin and serum albumin occurs on the alkaline side as well as the acid side of the isoelectric point of the protein. It is suggested that this is analogous to combination of certain other organic and inorganic anions with protein. Further experimental data appear to be required to provide an adequate explanation of the phenomenon of reversibility of the turbidity reaction at low pH.

5. Fertilizin which has been made univalent continues to give the mucin clot reaction. The type of precipitate is similar to that produced

by comparable solutions of normal, multivalent fertilizin. For this reason the type of precipitate formed cannot be directly related to the degree of aggregation of the fertilizin.

6. Sea-urchin sperm extracts and hyaluronidase were found to be ineffective in preventing the mucin clot reaction of fertilizin or in reducing the amount of turbidity formed in the turbidity reaction.

7. Sea-urchin sperm extracts and hyaluronidase have been shown to have no lytic effect on the jelly hull of sea-urchin eggs. The apparent dissolution of the jelly, reported by other workers, can be attributed to the presence in sperm extracts of antifertilizin, which causes a precipitation membrane to form on the jelly surface. Contraction of this to the egg surface accounts for the apparent disappearance of the jelly material.

8. The presence in lipoid extracts of sea-urchin sperm of an egg-surface lysin, described by Runnström is confirmed. This is shown to be obtainable in water-soluble constituents of the lipoid extracts. The manner of action of this agent has been studied. Living sperm do not liberate the substance and evidence for a central role of egg surface lysin in fertilization is held to be inadequate.

9. The egg membrane lysin of the giant keyhole limpet has been characterized to a certain extent. The evidence shows that it is probably of protein nature. Lytic activity of sperm extracts is destroyed by mild heating, by proteolytic enzymes, heavy metals, irradiation with ultraviolet light and mild shaking. Treatment which results in precipitation of material from sperm extracts, such as salting out and removal of electrolytes, removes lytic activity from the solution but only a small fraction can be recovered from the precipitate. Reprecipitation of active material recovered from such a precipitate results in further loss of

activity.

10. A method for estimating the lytic activity of weak and moderately active extracts has been developed. This depends upon the appearance of indentations in the egg membrane in the presence of EML in low concentration. It has been shown that the number of indentations that appear in an egg membrane exposed to lytic solutions is inversely related to the dilution of the extract. For assay purposes, the apparent titer is defined as that dilution of an extract which results in the appearance of a single indentation, not exceeding certain limits, in the membrane of at least two out of three eggs in five minutes. It has been useful to define as "one lysin unit" the concentration of EML in the dilution of an extract giving the titer. The total nitrogen associated with one lysin unit of a number of different preparations was found to vary not more than about two-fold, where the titer had been determined using two-fold dilutions of the extracts.

11. It has been shown that the lysin is released from living sperm in mildly alkaline sea water. Under these conditions antifertilizin apparently is not released from the sperm. As a consequence of being able to obtain extracts showing lytic activity, but demonstrably free of antifertilizin activity it can be concluded that the presence of antifertilizin is not required for egg membrane lysis.

12. The lysin is released from living sperm independently of the respiratory rate and relatively independently of temperature from 1° to 30°. It is suggested that with otherwise comparable sperm suspensions, the pH of the medium is the main factor in influencing the release of EML. Release of lysin from living sperm at pH 7 to 8 is not accompanied by extensive visible damage to the sperm.

13. It has been shown that EML is effective in a concentration of

less than 2 micrograms per ml dry weight.

14. The present experiments show that action of the lysin is probably not catalytic in nature, though it is sensitive to pH, salt concentration and temperature.

15. EML is associated with non-dialyzable constituents of extracts obtained from living sperm. Such extracts may contain from 60 to 85 percent dialyzable nitrogen. Dialyzed extracts do not show an absorption maximum in the ultra-violet, hence it is concluded that nucleic acid is not associated with lytic activity. Material precipitated from dialyzed extracts and dried contains about 10 percent nitrogen, 1.5 percent sulfur and about 4 percent alcohol-ether soluble substances. Of the total sulfur, about 30 percent may be accounted for by combined cystine and cysteine. In one acid hydrolysate of dried precipitated material it was found that cystine was present in about 10 times the amount of cysteine.

16. Certain sulfydryl compounds and dilute alkali can dissolve the egg membrane. The former are effective in low concentration. It is suggested that the egg membrane may be of keratin-like nature. Lytic action of EML may depend upon the presence of certain atomic groups, such as SH, in the molecule. The greater effectiveness of EML as compared with simple compounds is attributed, in part, to specific configuration of the molecule.

17. Extracts of sperm of three species of sea-urchin, abalone and mussel, obtained by freezing and thawing and by extraction of lyophilized sperm, have been found to be capable of dissolving the keyhole limpet egg membrane. Cross-reactions were not obtained with seminal fluid nor with living sperm.

18. It is suggested that the presence of "heterolysins" in the sperm of molluscs and echinoderms may account for the observed cross-

reactions.

19. Complete dissolution of the egg membrane of the keyhole limpet is not necessary for fertilization in this species.

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