THE PHYSIOLOGY OF THE CORTICAL REACTION IN THE EGGS OF PENAEID SHRIMP

A Thesis

Presented to

The Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

JOHN WELTON LYNN
May 1976

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Abstact

Mature penaeid oocytes contain large membrane-bound cortical specializations (rods) in their cortex. The rods are composed of feathery elements consisting of a dense axis with numerous fibrillar projections perpendicular to the axis. Prior to spawning, the cortical rods move to the periphery of the oocyte where membrane fusion takes place between the oolemma and the rod's bounding membrane. At spawning the cortical rods are expelled from the oocyte to form a striking investment layer which rapidly dissipates. During the cortical rod dissipation a hatching membrane forms around the egg. Attempts to retard the cortical reaction, to facilitate in vitro fertilization studies, have included the use of artificial seawaters; (1) lacking the divalent cations Mg^{++}, Ca^{++}, or Ca^{++}-Mg^{++}, (2) containing 1% procaine, (3) containing 10 mM EDTA and (4) containing soybean trypsin inhibitor (3×10^{-4} M). The cortical reaction is inhibited in Mg^{++} free seawater. Upon return to normal seawater the cortical reaction proceeds but the rods do not dissipate. Soybean trypsin inhibitor irreversibly inhibits any stage of the reaction. EDTA also inhibits the reaction.

Cortical rod material was isolated from mature ovary on 60% sucrose or 75% glycerol in a 0.1 M Tris buffered saline at 8000 x g. Isolated cortical rods gave a positive result with a Lowry's test and were dissipated by proteases and alkaline phosphatase. Whole ovaries stained with PAS or alcian blue revealed the presence of mucopolysaccharides in both the investing coat and the cortical rods. The cortical rods do not stain as an acid mucopolysaccharide as does the investing coat; however, an L-cysteine sulfuric acid assay

on isolated cortical rods reveals the presence of 6-deoxyhexoses which are characteristic of neutral mucopolysaccharides. A thiobarbituric acid assay for sialic acids in the isolates was negative. The protein and carbohydrate moeities could not be separated by precipitation with trichloroacetic acid. The above data suggests the cortical rods are either a neutral mucopolysaccharide or glycoprotein whose release and dissipation is dependent on Mg^{++} ions and proteases.

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I.

INTRODUCTION

Egg cortical reactions inititated by sperm penetration or exposure to seawater have long been known in many animal ova. Cortical reactions exist in the eggs of cnidarians (Dewel and Clark 1973), annelids (Pasteels 1965b), arthropods (Hudinaga 1942), molluscs (Humphries 1967), echinoderms (Afzelius 1956 and Lillie 1910), teleosts (Yamamoto 1939 and Kagan 1935), amphibians (Motomura and Kemp 1956), and mammals (Austin and Amoroso 1959, Austin 1956b, Szollosi 1962, also see Austin 1968 for a review).

Dehiscence of the cortical granules at the periphery of a typical ovum into the surrounding media often causes the lifting of an egg investment coat with the resultant formation of a fertilization membrane (Endo 1961). It is widely believed that these cortical reactions are responsible for the prevention of polyspermy by both a chemical and physical block (Epel 1975 for review) and may also establish a microenvironment inside a tough chorionic membrane for the developing embryo (Humphries 1967).

Changes in the cortices of animal ova have been most extensively studied in the echinoderms, annelids, and amphibians. Some of the earliest observations on the surface reactions of sea urchin eggs (Toxopneustes variegatus), were reported by Fol (1879) and reviewed by Wilson and Mathews in 1895. The most notable changes were the formation of an "entrance cone" at the site of sperm entry into the ovum with the subsequent formation of a "new vitelline membrane". While Fol's work did not actually describe the release of cortical granules in the eggs of Toxopneustes variegatus he postulated that the "newly formed vitelline membrane" acted to block

supernumerary sperm.

In descriptions of Nereis eggs by Wilson (1892), two membranes were observed surrounding the ovum which changed at fertilization. Although these changes were not recognized as a cortical reaction, they were reported as changes in the internal structure of the egg during fertilization. Subsequent work on both the eggs of Nereis worms and echinoderms led to the observations that membrane formation was initiated by sperm penetration and could be artificially induced by numerous hyper- or hypo- tonic salt solutions (Loeb 1900, Loeb 1900b, and Fischer 1903).

One of the first accurate descriptions of a cortical reaction was done on the eggs of Nereis by Lillie in 1911. In this work Lillie describes the breakdown of cortical alveolae a short time after insemination with the subsequent formation of a jelly coat around the eggs. Breakdown of the cortical vesicles in the sea urchin or other echinoderm eggs were not observed for some time even though the existence of such a reaction was hypothesized by Lillie in 1911, (see also Lillie 1916).

It was not until Just's observations in 1919 that the elevation of the membrane of echinoderm eggs was described as proceeding in a wave-like fashion from the point of entry of the sperm to the opposite side of the egg. Prior to the elevation of the membrane, Just (1919) described a wave of change that swept through the egg's cortex. The actual cause,however, of the surface changes in the eggs at fertilization was not known until Moser's work (1939) which described the cortical granules in the cortex of the egg. The granules were observed to breakdown in a wave like fashion, 10 to 20 seconds

after insemination. Similar disappearances of cortical platelets in the eggs of Fundulus sp. were reported by Kagan (1935) and in the eggs of Oryzias latipes by Yamamoto (1939).

The occurence, then, of cortical granule breakdown at fertilization or activation was well known by 1940. However, the actual mechanism of release of the cortical granules and their ability to cause the elevation of the fertilization membrane was not fully understood until Endo's 1952 light microscopic work on the sea urchin cortical bodies. Runnstrom et al. (1946 and 1952) had already shown that the material released by the cortical granules was probably involved in the formation and hardening of the elevating membrane (see also Moser 1939). This early work on the sea urchin cortical granules was confirmed by Endo's (1952) observations on the incorporation of the material from the cortical granules into the fertilization membrane. It was not until Endo's (1961a and 1961b) work with the sea urchin egg using the electron microscope that several questions were answered. His observations revealed that the cortical granules released material which lifted the "vitelline membrane" from the egg, became incorporated into this membrane and resulted in the formation of the fertilization membrane. A hyaline layer was also formed by the material released (see also Afzelius 1956).

In 1937 Mazia recognized that calcium played a significant role in the cortical reaction of Arbacia punctulata eggs. The signicance of calcium in cortical reactions of eggs was further documented in both teleosts and other echinoderms by Moser (1939) and Yamamoto (1939). The recent development of divalent cation carrying

antibiotics such as A23187 and X537A have confirmed the involvement of Ca^{++} in the discharge of the cortical granules (Steinhardt 1974, Vacquier 1975). The calcium release is usually from bound stores intracellularly. This release may be involved in membrane fusions (Epel 1975 for a review of activation and cortical granule involvement) and is probably necessary for the activation of proteases released from the cortical granules (Vacquier 1975).

Preparations of isolated cortical granules (Schuel et al. 1969 and 1972) have revealed the presence of β1,3 glucanase (Epel and Weaver 1969, Schuel et al. 1972) and another protease-like enzyme (Vacquier 1975, see also Katsura 1974). Additional studies have illustrated that these enzymes are released during the cortical granule breakdown (Vacquier 1972a, 1972b, 1973, Epel 1975 for a review, Gwatkin 1972). In situ, experiments have shown the presence of acid mucopolysaccharides in cortical granules (Aketa 1962, Ishihara 1968, Monne and Slautterback 1950, and Wolf 1974, Schuel et al. 1974, Monne and Harde 1951, Runnstrom and Immers 1956). These findings have demonstrated that the release of the cortical contents has a three fold function: (1) detachment of the vitelline membrane from the oolemma, (2) elevation and hardening of the fertilization membrane to form a late, long lasting block to polyspermy, and (3) disruption of sperm binding sites on the vitelline membrane (Tegner 1974). Disruption of sperm binding sites has been dramatically shown in a scanning electron microscopy study by Vacquier et al. 1972a. Although the majority of the biochemical work on the cortical granules has been done on sea urchin eggs, there are reports of similar cortical components in amphibians, echiuroids, and mammals.

At present there is very little information concerning the physiology of egg activation in crustaceans. Since decapod crustacean fertilization is often internal, it is difficult to observe reactions of the egg. Hinsch (1971) reported the formation of a membrane around the eggs of Libinia emarginata which might be the result of a cortical reaction. Cheung (1966) has reported membrane formation probably initiated by fertilization around the eggs of Carcinus.

Work on the ovaries and the cortical structure in eggs of animals of the Natantia is very limited. Hudinaga (1942) briefly described a massive cortical reaction in the eggs of Penaeus japonicus and P. monoceros. In both animals regions of "jelly-like substance" were described in the peripheral cytoplasm of the eggs. These peripheral regions were released at spawning and formed a jelly coating around the surface of the egg which remained until the second cleavage division.

King (1948) did some preliminary histochemical investigations on the structure of the ovary and ova of Penaeus setiferus. In these studies, he found that the peripheral bodies in the cortex of the eggs did not stain with lipid specific stains or with ninhydrin reagents which are specific for proteins. No conclusions were made as to the chemical nature of the peripheral bodies.

In a fine structural study of oogenesis in P. aztecus and P. setiferus, Hill (1974) again described large peripheral bodies (cortical rods) around the cortex of mature oocytes. These cortical rods formed in the ooplasm during oogenesis, were membrane bound, and moved to the periphery of the oocyte. Once in the cortical

cytoplasm, the rods' bounding membrane fused with the oolemma, thus forming numerous crypts around the periphery of the oocyte. Each crypt contained a cortical rod which was separated from the external environment by an investing coat. The substructure of the rods was very distinct. Each rod was composed of numerous feathery elements consisting of a dense axis with lateral fibrillar projections giving each element a "bottle brush appearance".

Preliminary reports (Clark et al. 1974, 1975 and Lynn and Clark 1975) have described the morphology of the cortical reaction in the eggs of P. aztecus and P. setiferus. The purpose of this paper is to complete the morphological description of the reaction and introduce information concerning the physiology of the cortical rods and the cortical reaction.

II.

MATERIALS AND METHODS

Procurement of Animals

Live gravid female Penaeus aztecus and P. setiferus were used for the following study. P. aztecus were caught 80-100 miles south of Galveston, Texas at approximately 95° longitude and 28° 15' latitude using commercial trawling equipment. P. setiferus were caught from 1-10 miles off the coast of Galveston, Texas at 95° longitude and 29° 15' latitude. Animals were held in 150 gallon aerated tanks at 15°-20° C. until returned to the laboratory. Animals returned to the laboratory were placed in aerated, inverted five gallon carbouys for spawning. Heaters were placed in the carbouys and the water temperature was slowly brought to 28° C. Spawning females were removed from the carbouys and eggs were collected either directly from the animal in a beaker of seawater or gathered dry from the animal's ovipore with a pipet and then placed in test solutions.

Artificial Seawaters

Spawned eggs were placed in solutions of artificial seawater (Table I) which: (1) lacked the divalent cations Mg^{++}, Ca^{++}, or Ca^{++}-Mg^{++}, (Cavanaugh 1956), (2) contained 10 mM ethylenediamine tetraacetic acid (EDTA), (3) contained 1% procaine (Vacquier 1974), or (4) contained 0.1% soybean trypsin inhibitor (SBTI). Within five to ten minutes after treatment in these solutions, the eggs were returned to normal seawater. Control eggs were placed in solutions of normal seawater filtered through a millipore (0.2μ) filter or held in a solution of artificial seawater (Cavanaugh 1956).

Light Microscopy and Histochemistry

Ovary was used either from wild mature animals or adult animals

TABLE I: SEAWATER SOLUTIONS

MEDIA INTO WHICH EGGS ARE PLACED	CORTICAL ROD EXPULSION	CORTICAL ROD DISSIPATION	EFFECT ON RETURN TO NORMAL SEAWATER
Seawater	Complete	Complete	
MBL Seawater	Complete	Complete	
Ca^{++}-Mg^{++} Free Seawater	Reaction Stopped	Reaction stopped	Rods Extrude But Do Not Dissipate
Ca^{++} Free Seawater	Complete	Complete	
Mg^{++} Free Seawater	Reaction Stopped	Reaction Stopped	Rods Extrude But Do Not Dissipate
Seawater + 1% Procaine	Complete	Complete	
Seawater + 10 mM EDTA	Reaction Stopped	Reaction Stopped	No Effect
Seawater + 0.1% SBTI	Reaction Stopped	Reaction Stopped	No Effect

which had been induced to mature by bilateral eyestalk ablation with a pair of hot haemostats (Duronslet, et al. 1975). Whole mature ovary was dissected from the animals and fixed in 10% phosphate buffered formalin (Gridley 1960), Bouins (Gridley 1960), or Davidson's fixative (Humason 1967) and embedded in paraplast for histological staining. Sections, 5 in thickness, were cut on an American Optical microtome and stained with alcian blue 8GX lot number 74240 at pH 2.0 (Humason 1967), a cold Periodic-Acid Schiffs reagent (Lillie 1965), aldehyde fuchsin (Gridley 1960), or mucicarmine (Gridley 1960). All sections were counterstained with metanil yellow (Gridley 1960), Van Geisons stain (Gridley 1960), or Weigert's haemotoxlin (Gridley 1960).

Observations on live material were made using either a light microscope with a phase condenser or a light microscope equipped with Nomarski phase interference condensers and filters.

Cortical Rod Isolation

Ovaries were dissected from either wild mature animals or from animals induced to mature by eyestalk ablation (Duronslet 1975). Whole ovary was homogenized in a Potter-Elvehjem tissue grinder with a teflon pestle. The tissue was homogenized in a 30% sucrose or a 35% glycerol solution containing 0.5 M NaCl, 0.009 M $CaCl_2$, 0.014 M KCl, 0.015 M $MgCl_2$, and 0.1 M Tris buffer, pH 7.6. The egg brei was centrifuged at 1000 x g for five minutes in 15 ml Corex glass tubes. The supernatant was discarded and the pellet was resuspended in the above described saline solution without sucrose or glycerol and layered over the preceeding saline solution containing 60% sucrose or

70% glycerol. These tubes were then centrifuged at 8000 x g for 60 minutes. The supernatant was again discarded and a pellet consisting mainly of cortical rods was resuspended in the above saline solutions without sucrose or glycerol and recentrifuged at 1000 x g four times to remove the sucrose or glycerol. These rods were either used immediately or freeze-dried and stored at -30° C. for later use.

Biochemical Tests

A qualitative Lowry's total protein determination (Lowry 1951) was run on the isolated cortical rods and spectrophotometrically measured on a Coleman 124 double beam spectrophotometer. A standard was run simultaneously using bovine serum albumin.

Qualitative Molisch α-napthol and anthrone sulfuric acid reactions (Dische 1955) were run to determine the presence of carbohydrates. An L-cysteine sulfuric acid reaction (Dische 1955) was run to determine the presence of hexoses, 6-deoxyhexoses, 2-deoxypentoses, pentoses, hexuronic acids, and heptoses. Solutions of fucose for the 6-deoxyhexoses, glucose for hexose, glucuronic acid for the hexuronic acid, sedoheptulose for the heptoses, and ribose for the pentoses were run simultaneously as standards in the L-cysteine sulfuric acid reaction. These tests were also run spectrophotometrically on a Coleman 124 spectrophotometer.

Tests for the presence of sialic acids were run using the Ehrlich reaction or the thiobarbituric acid assay. The Ehrlich reaction was run according to the methods of Werner and Odin (1952). The thiobarbituric acid assay was run according to the methods of Warren (1959). A standard of N-acetyl neuraminic acid (Sigma) was run simultaneously. All tests were read spectrophotometrically on a Coleman 124

pectrophotometer.

Cortical Rod Disruption

Several solvents and chemical treatments were tested on both fresh and freeze-dried isolated cortical rods and observed on the light microscope and scanned on a Coleman 124 spectrophotometer. All tests were run at 24° C. and observed over a 1½ hour period. Fresh rod preparations were centrifuged into a pellet before suspending in the test solutions and the freeze-dried samples were suspended directly in the solutions. Mercaptoethanol (1% in distilled water), 2 N HCl (diluted with distilled water), 12 N HCl, 2 N NaOH (diluted with distilled water), 8 N NaOH, 18 N H_2SO_4, Triton X-100 (1% in distilled water), chloroform, acetone, methanol, petroleum ether, and phosphate buffer at pH 5.8 were tested for their effects on cortical rod preparations.

Enzyme Treatments

Enzyme digestions were tested on both fresh and freeze-dried cortical rod isolates. All enzyme assays were made at 24°-25° C. Enzymes used were: (1) 0.02% acid phosphatase in 0.15 M sodium acetate buffer, pH 5.0, (2) 0.2% alkaline phosphatase in 0.5 M Tris buffer, pH 8.0, (3) 0.1% trypsin in 0.46 M Tris buffer, pH 8.1, containing 0.0115 M $CaCl_2$, (4) 0.1% protease in 0.46 M Tris buffer, pH 8.1, containing 0.0115 M $CaCl_2$, (5) 0.1% α-chymotrypsin in 0.008 M Tris buffer, pH 7.8, containing 0.1 M $CaCl_2$, (6) 0.02% hyaluronidase in 0.1 M monosodium phosphate buffer, pH 5.3, containing 0.15 M NaCl, (7) 0.1% lipase in 1 M Tris buffer, pH 8.1, (8) 0.1% collagenase in 0.05 M Tris (hydroxymethyl) methyl-2-amino-ethane-sulfonate, pH 7.5,

with 0.36 mM $CaCl_2$, (9) 0.2% aryl sulfatase in 0.2 M sodium acetate, pH 5.0, and (10) 0.1% neuraminadase in 0.1 M sodium acetate, pH 5.0. All enzyme solutions were prepared according to the Worthington Manual (1972) based on the specific activity of each enzyme. Observations were made over a 1-5 hour period on a light microscope. Controls of isolated rods were held in the same buffer system used for each enzyme for the same period of time.

Electron Microscopy

Both normal eggs and isolated cortical rods were fixed for electron microscopy using Karnovsky's paraformaldehyde-glutaraldehyde fixative with 0.1 M $NaPO_4$ buffer, (Karnovsky 1965), pH 7.5, and sucrose added to adjust osmolarity. The samples were postfixed in 1% osmium tetroxide buffered with $NaPO_4$, pH 7.5. After postfixing for 30 minutes, the samples were rapidly dehydrated in a graded acetone series and embedded in a low viscosity epoxy resin (Spurr's 1969). Sections were made with either glass or diamond knives on a Porter Blum MT2-B ultramicrotome. Thick plastic sections were stained with 1% toluidine blue in a .15% sodium borate buffer (Dewel and Clark 1974). Thin sections were stained with saturated methanolic uranyl acetate (Dewel and Clark 1974), and aqueous lead citrate (Veneable and Coggeshell 1965), and examined on a Hitachi HS-8 electron microscope at an accelerating voltage of 50 kv.

III.

RESULTS AND DATA

The Egg Cortical Reaction

A mature, unreacted oocyte removed from the oviduct of a spawning female is spherical and approximately 250 μ in diameter. Light striations are apparent in the peripheral cytoplasm of such oocytes (Figure 1a). These striations represent cortical specializations (cortical rods) which lie in membrane crypts around the cortex of the oocyte. When an oocyte is spawned into seawater these cortical rods are released into the surrounding media. Figures 1a to 1L represent a series of light micrographs illustrating this reaction.

Early stages of the reaction can be stopped for observation as shown in Figures 1b and 1c, by holding a spawning female over a beaker of seawater to collect the eggs and fixed at ten second and twenty second intervals. In these micrographs (1b and 1c) the rods have just begun to emerge from the egg and result in a blebbed appearance around the periphery of the egg. Figures 1d to 1L illustrate one egg undergoing a cortical reaction. The cortical rods are rapidly released from the egg cortex to form a large corona around the egg. Once completely expelled from the egg the rods begin to swell and dissipate (Figures 1g to 1i). To facilitate a complete morphological description, the reaction has been divided into four stages. These stages are the: (1) unreacted stage, (2) early reaction, (3) corona stage, and (4) dissipation stage. The morphology and physiology of the formation of the hatching membrane will not be discussed in this paper.

Unreacted Stage

Figures 2 to 4 illustrate oocytes removed from the oviduct of

Figure 1. A series of phase micrographs showing the cortical reaction of Penaeid oocytes in response to contact with seawater. 1a-fixed unreacted egg from the oviduct of a spawning female; 1b and 1c-fixed eggs in the early reaction stage. Figures 1d to 1L are the same egg. 1d to 1f-corona stage; 1g to 1j-dissipation of the cortical rods; 1k to 1L-formation of the hatching membrane. Arrow-figures 1i to 1L-polar body. All micrographs x 67.

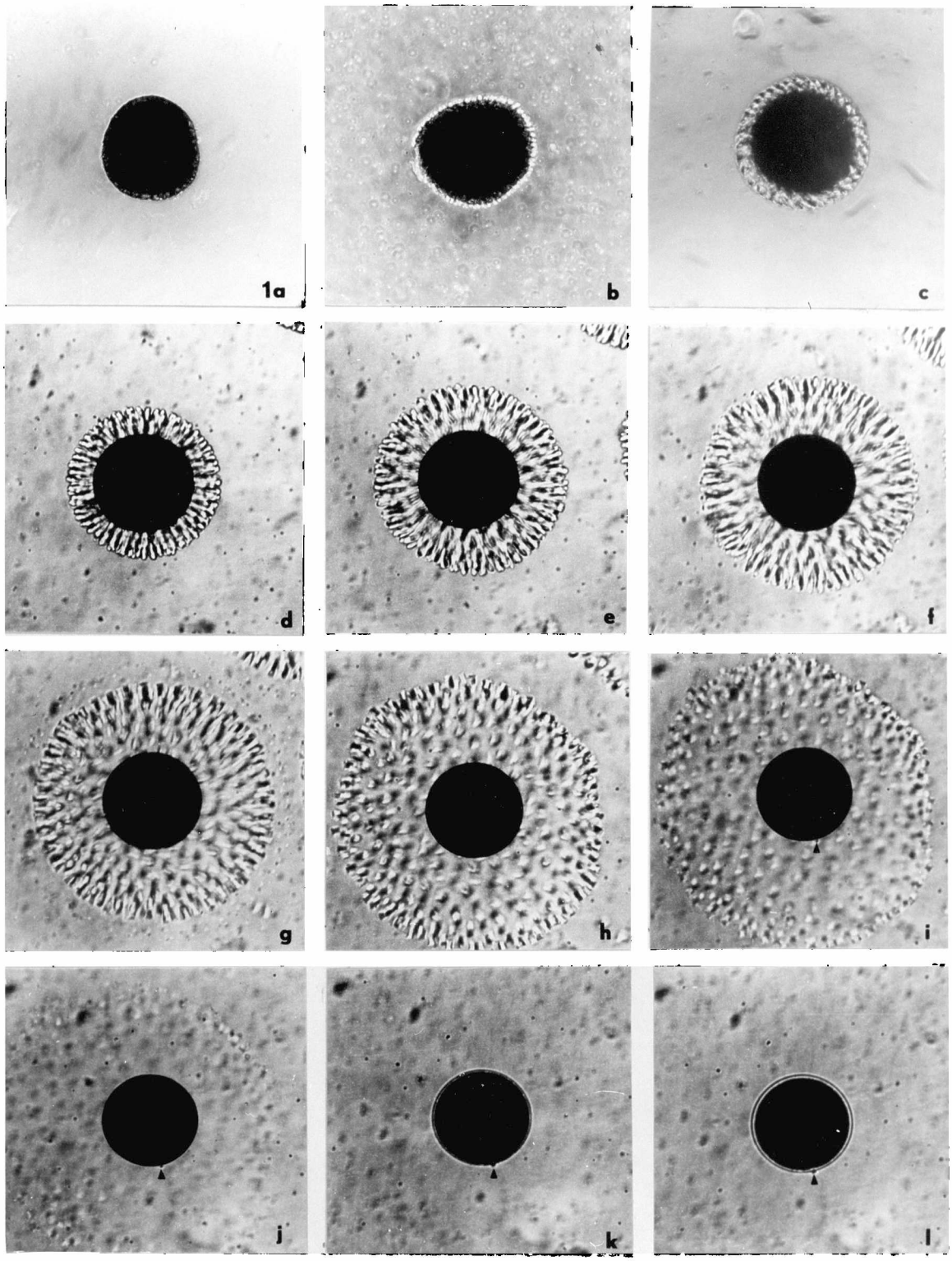
1a
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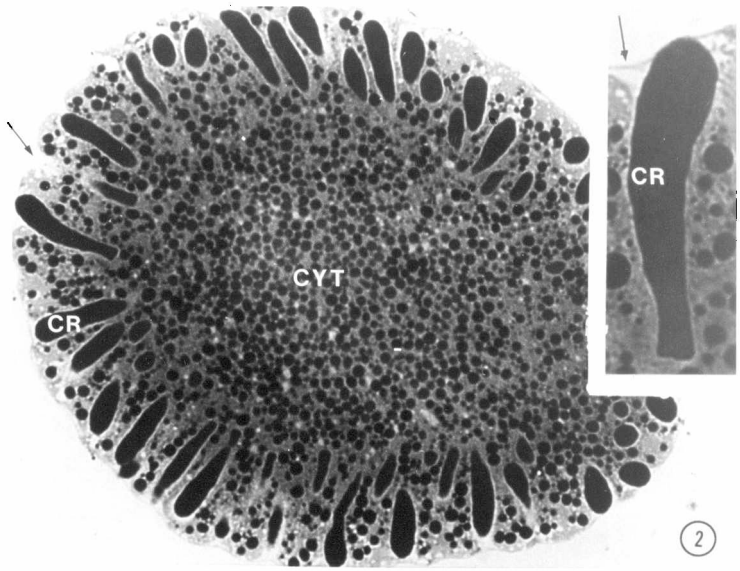
Figure 2. Light micrograph of a thick plastic section of an unreacted oocyte. CR-cortical rod; CYT-cytoplasm; arrow-investment coat. x 380. Insert: cortical rod showing typical club shape. CR-cortical rod; arrow-investment coat. x 1075.

Figure 3. Transmission electron micrograph of a cortical rod in its crypt in the peripheral cytoplasm. CR-cortical rod; CYT-cytoplasm; arrow-investment coat. x 8000. Figure 3a. Insert: substructure of cortical rods showing dense axis with lateral fibrils. x 27,000.

Figure 4. Scanning electron micrograph of an unreacted oocyte. Indentations in the investment coat represent the locations of the cortical rod crypts. CR-cortical rod prematurely exiting its crypt; H-hexagonal array of cortical rod crypts; arrow-sperm on investment caot. x 348.



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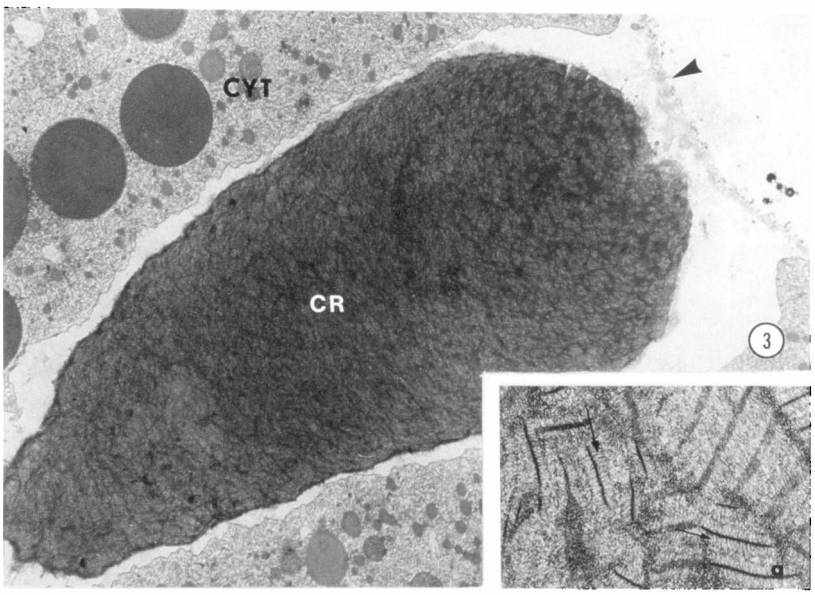

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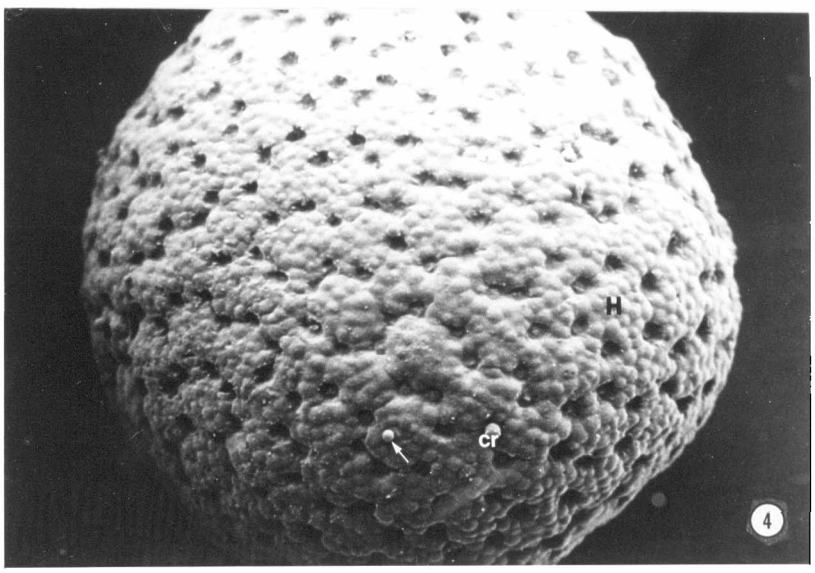

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a spawning female. The oocyte contains large accumulations of yolk platelets within its cytoplasm. In the peripheral cytoplasm, large (40μ) club shaped cortical rods are conspicious. Surrounding the oocytes is a thin investment coat (Figure 2a). The rods lie within crypts which are formed when the rods' bounding membrane fuses with the oolemma (Figure 3). Each rod, as mentioned previously (Hill 1974), is composed of numerous feathery elements (Figure 3a). When viewed with the scanning electron microscope (SEM), hexagonal patterns of cortical rod crypts are revealed as indentations in the investment coat (Figure 4). Occasional sperm are also noted on the surface of the investment coat.

Early Reaction

Eggs in the early stages of the cortical reaction are shown in Figures 5 to 7. Partial release of the cortical rods is apparent at both the light and fine structural level (Figures 5 and 6). No apparent changes are evident in the substructure of the rods. Large blebs in the investment coat caused by the emerging cortical rods are prominent in the SEM (Figure 7). Some loss of integrity of the investment coat is also evident.

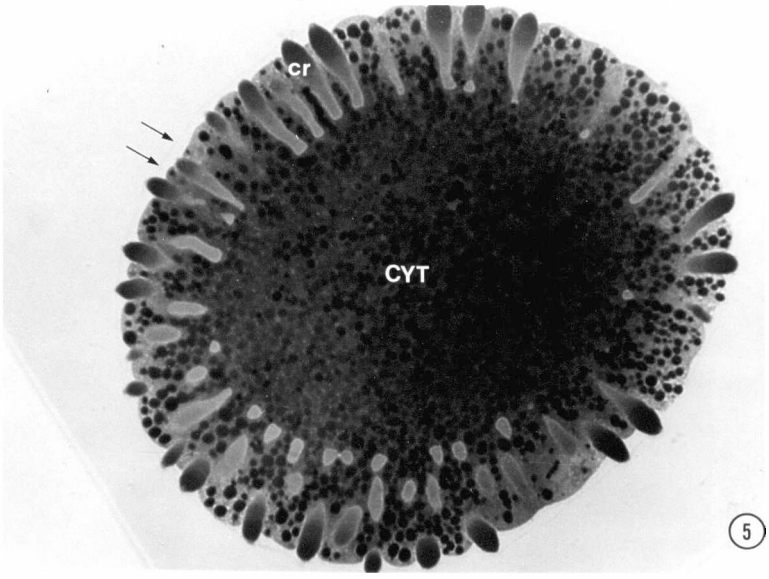
Corona Stage

Eggs whose cortical rods have been almost entirely released are shown in Figures 8 to 10. Still no changes are obvious in the morphology of the cortical rods either at the light of fine structural level. Integrity of the investment coat is almost entirely lost. Small strands of this coat are apparent between several cortical rods as shown in SEM (Figures 10). Small depressions in

Figure 5. Light micrograph of a plastic section showing the early stages of a cortical reaction. CR-cortical rod; CYT-cytoplasm; arrows-investment coat. x 336.

Figure 6. Transmission electron micrograph of cortical rods as they begin to emerge from their crypts. CR-cortical rods; CYT- cytoplasm; arrows-investment coat. x 12,000.

Figure 7. Scanning electron micrograph showing the early stages of a cortical reaction. CR-cortical rod beneath the investment coat; S-sperm; I-investment coat; arrow-area of investment coat beginning to lose its integrity. x 1200.



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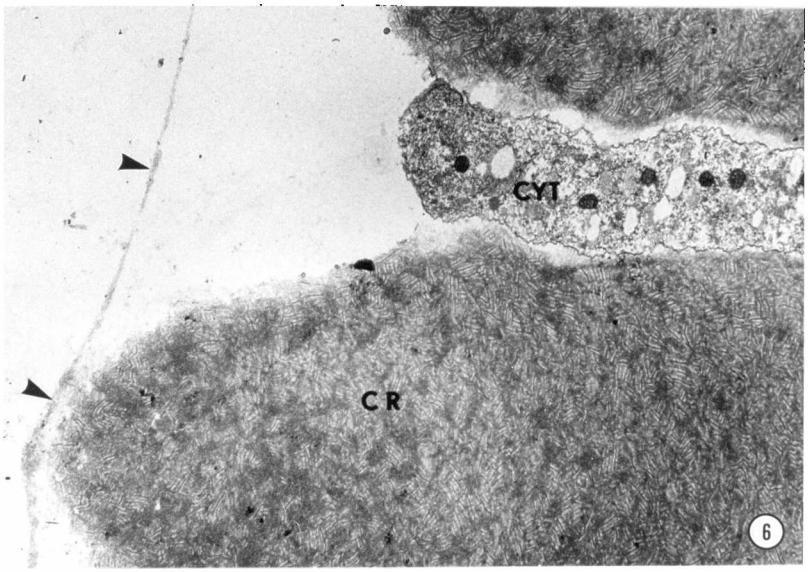

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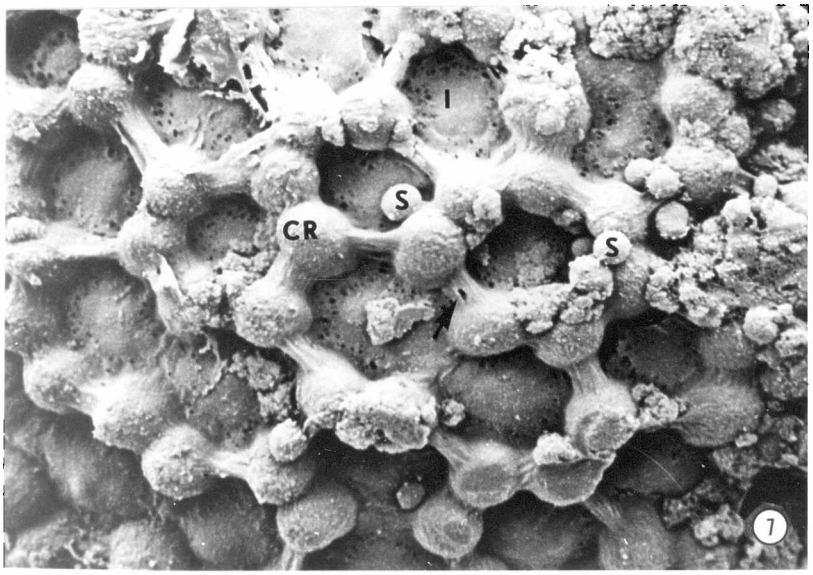

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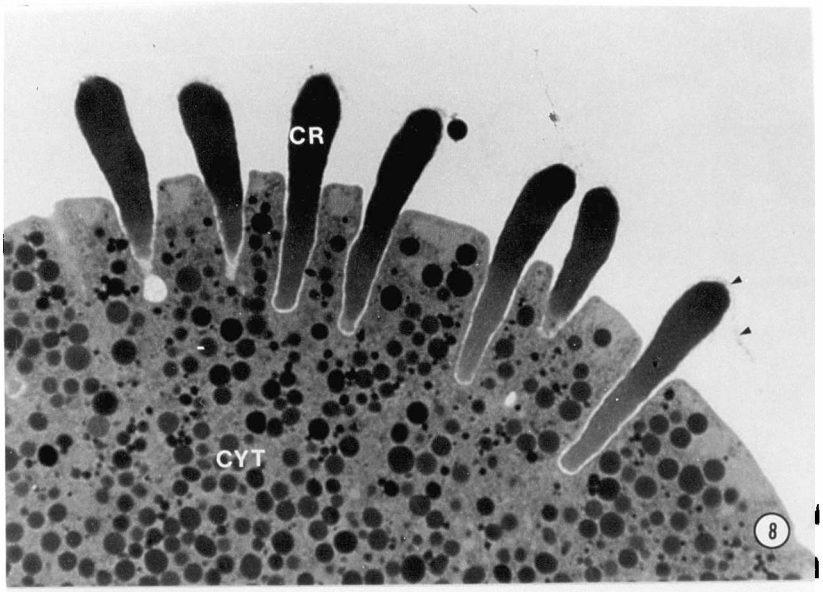
Figure 8. Light micrograph of a thick plastic section showing the corona stage of a reacting Penaeid oocyte. CR-cortical rod; CYT-cytoplasm; arrows-remnants of the investment coat. x 825.

Figure 9. Transmission electron micrograph of a cortical rod in the corona stage. CR-cortical rod; CYT-cytoplasm; x 7000. Figure 9a. Insert: small cortical vesicle dehiscing. x40,000.

Figure 10. Scanning electron micrograph of the corona stage of the reaction. CR-cortical rod; S-sperm; small arrows-depressions in the oolemma; large arrows-vestiges of the investment coat. x 1600.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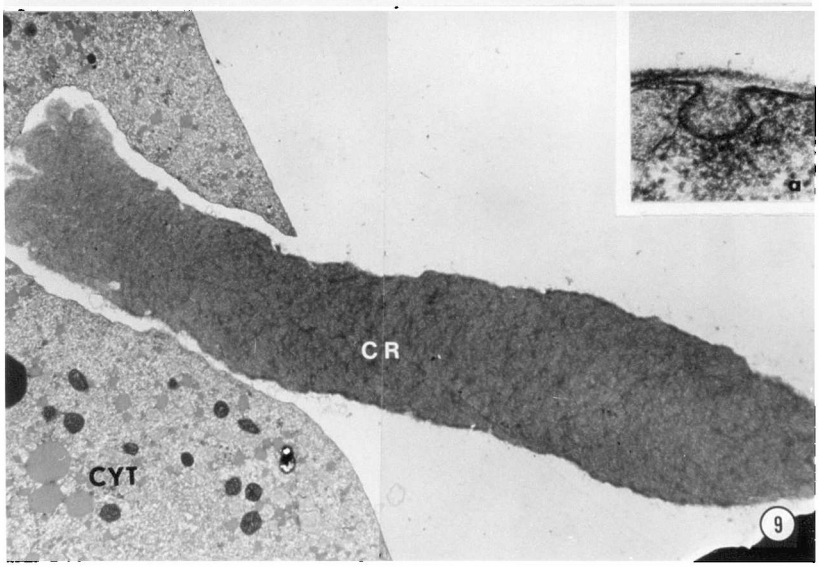
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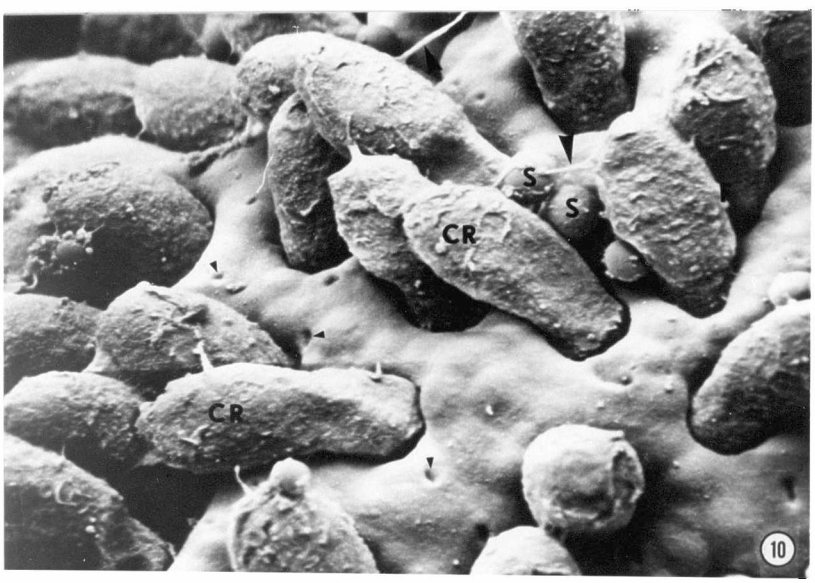
S
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CR
CR
10

the surface of the oolemma are also observable in the SEM. These depressions may correspond to the dehiscence of smaller cortical vesicles (1μ in diameter) apparent at the fine structural level (Figure 9a).

Dissipation Stage

Figures 11 to 13 show the cortical rods fully released from the crypts in the oocyte. At the light and fine structural level swelling and dissipation at both ends of the cortical rods is evident. The investment coat is no longer present. In phase micrographs, a small cap-like portion is visible at one end of the dissipating cortical rods (Figure 12). After dissipation of the cortical rods is complete, a hatching membrane forms around the egg.

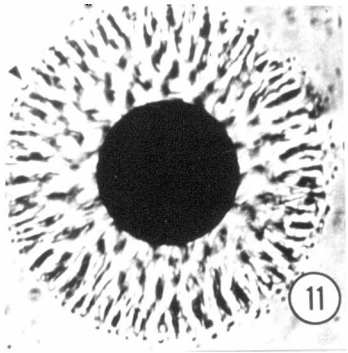
Histochemical Staining

In order to tentatively identify the composition of the investment coat and the cortical rods, various histochemical techniques have been used. Oocytes stained with periodic-acid Schiff's reagent contain rods which exhibit a positive reaction (Figure 14). Production of a demonstrable aldehyde in the PAS reaction is usually due to the presence of polysaccharides at the site of staining. When oocytes are stained with alcian blue, the investment coat exhibits a positive reaction; however, the cortical rods do not (Figure 15). Neither the investment coat nor the cortical rods give a positive reaction to the aldehyde fuchsin or mucicarmine stains; (Figures 16 and 17), thus, eliminating the possibility that these structures contained mucins, elastic fibers, or glycogen. These

Figure 11. Phase micrograph of a live egg. The cortical rods are beginning to dissipate. Arrow-small cap-like portion of rod. x 76.

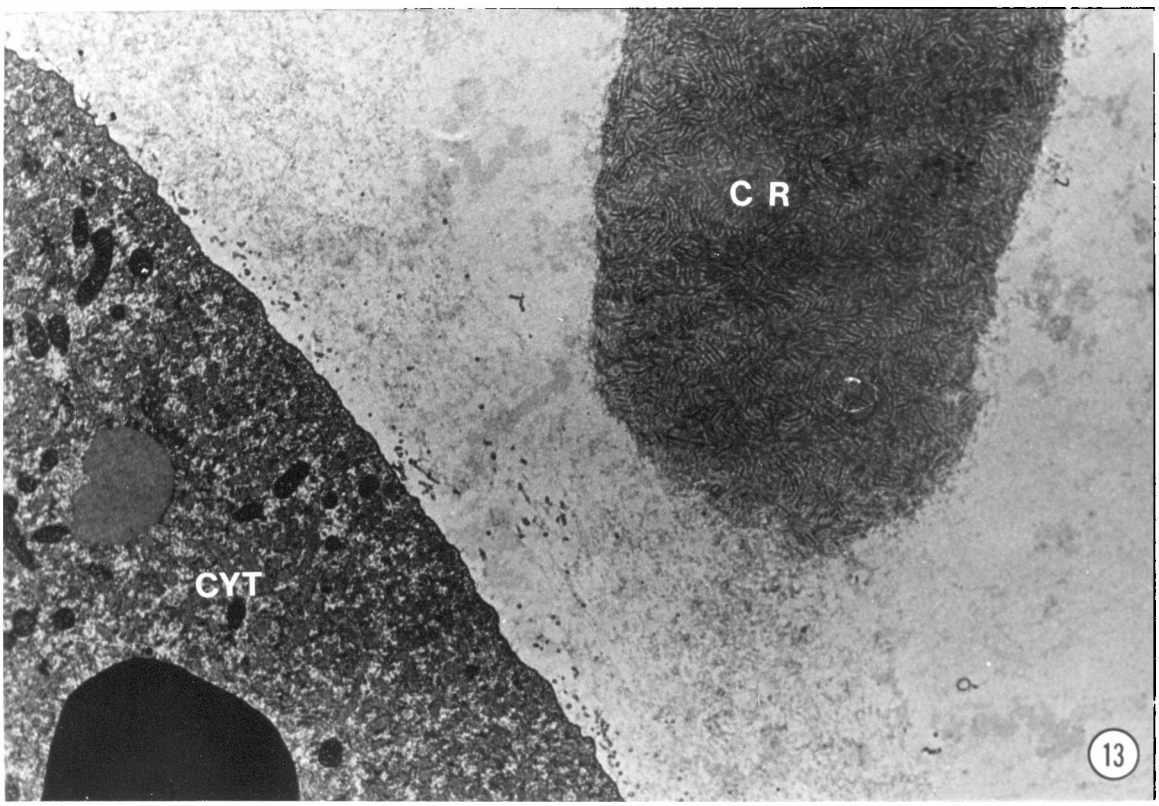
Figure 12. Light micrograph of a thick plastic section of an egg in the stage of cortical rod dissipation. CR-cortical rod; arrow-region of cortical rod dissipation. x 1875.

Figure 13. Transmission electron micrograph of the dissipation stage of the cortical reaction. CR-cortical rod; CYT-cytoplasm. x 12,000.



11

12



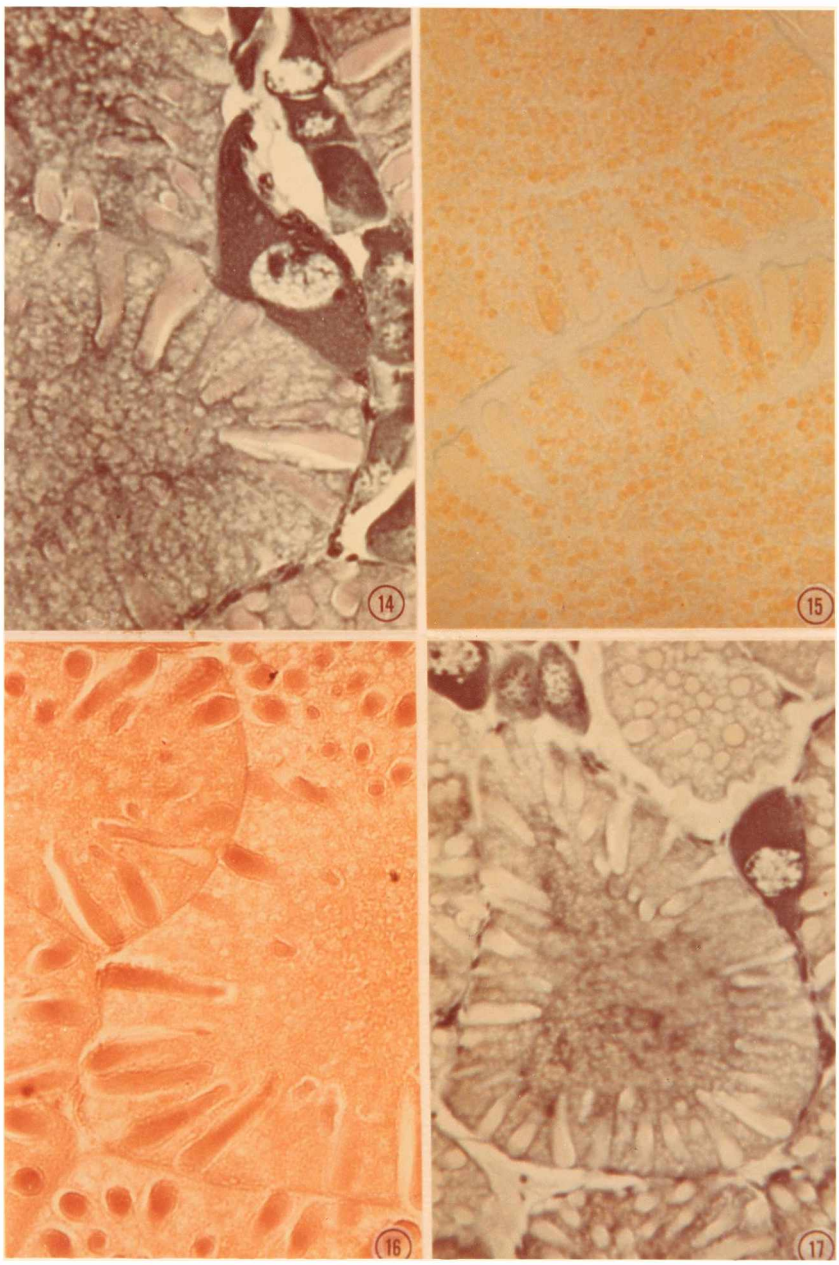
13

Figure 14. Light micrograph of a PAS stain of whole ovary. Counterstained with Weigert's hemotoxlin. x 525.

Figure 15. Light micrograph of alcian blue stain of whole ovary. Counterstained with metanil yellow. Arrow points to investment coat. x 525.

Figure 16. Light micrograph of mucicarmine stain of whole ovary. Counterstained with van Geisons. x 525.

Figure 17. Light micrograph of aldehyde fuchsin stain of whole ovary. Counterstained with Weigert's hemotoxlin and metanil yellow. x 300.


14
15
16
17

results are summarized in Table II and suggest that the rods are a complex polysaccharide or a mucopolysaccharide; while the investment coat is an acid polysaccharide or an acid mucopolysaccharide.

Cortical Rod Isolation

In order to more fully define the nature of the cortical rods an isolation technique has been designed. Unfortunately, attempts to isolate the investment coat have been unsuccessful to date.

A flow chart for the isolation of cortical rods is shown in Table III. A comparison of cortical rods with phase microscopy after the first crude centrifugation and a second purification centrifugation is shown in Figures 18 and 19. Yolk contamination is not apparent at the light level after the second centrifugation. Purified cortical rods exhibited only a small amount of membrane contamination when examined with the electron microscope (Figure 20). No change was seen in the substructure of the isolated cortical rods. The feathery elements described by Hill (1974) are still apparent in rehydrated, freeze-dried rod isolates, Light micrographs of the same rehydrated rods demonstrate that the rods have maintained a distinct club shaped morphology (Figure 19a).

Solvent Extracts of Isolated Cortical Rods

Cortical rod isolates were treated with several solvents to find a method of artificial dissipation. The suspensions were examined with phase microscopy and the solutes run spectrophotometrically (Table IV). Rod dissipation was obtained in solutions of 2 N NaOH, 8 N NaOH, and 18 N H_2SO_4. Absorption maxima were obtained

TABLE II: HISTOCHEMICAL STAINS USED WITH WHOLE OVARY

STAIN	STAINS FOR	COMPONENTS STAINED
Alcian Blue	Acid Mucopolysaccharides	Investment Coat
Aldehyde Fuchsin	Elastic Fibers	None
Mucicarmine	Mucus	None
PAS	1,2 glycols (polysaccharide)	Cortical Rods, Investment Coat

TABLE III

CORTICAL ROD ISOLATION PROCEDURE

WHOLE MATURE OVARY

↓ Homogenize in Tris buffer + 30% sucrose or 35% glycerol

EGG BREI

↓

CENTRIFUGE
1000 x g 15 Minutes

↓ Discard Supernatant

RESUSPEND PELLET IN TRIS* BUFFER

↓ Layer on 60% sucrose or 70% glycerol

CENTRIFUGE
8000 x g 60 Minutes

↓ Discard Supernatant

RESUSPEND PELLET IN TRIS* BUFFER

↓

CENTRIFUGE TO PELLET

Repeat Four Times

↓

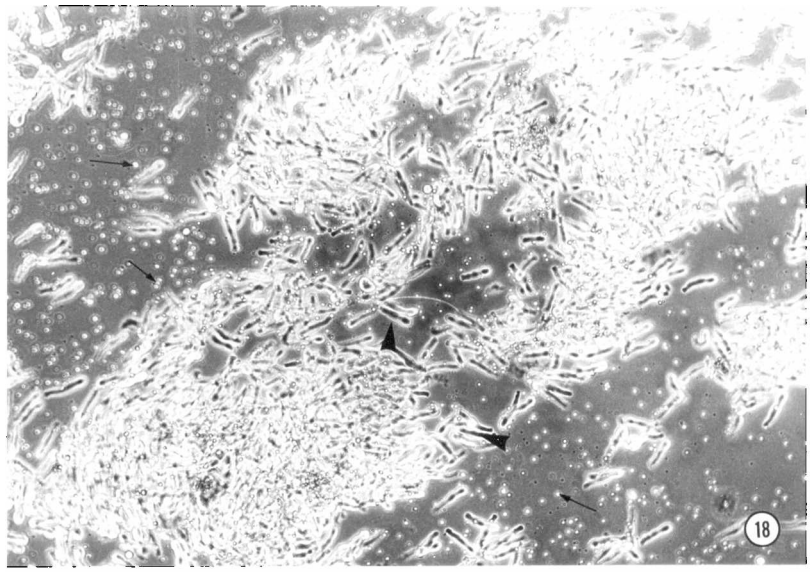
ISOLATED PURIFIED RODS
(Use Immediately or Freeze Dry Pellet)

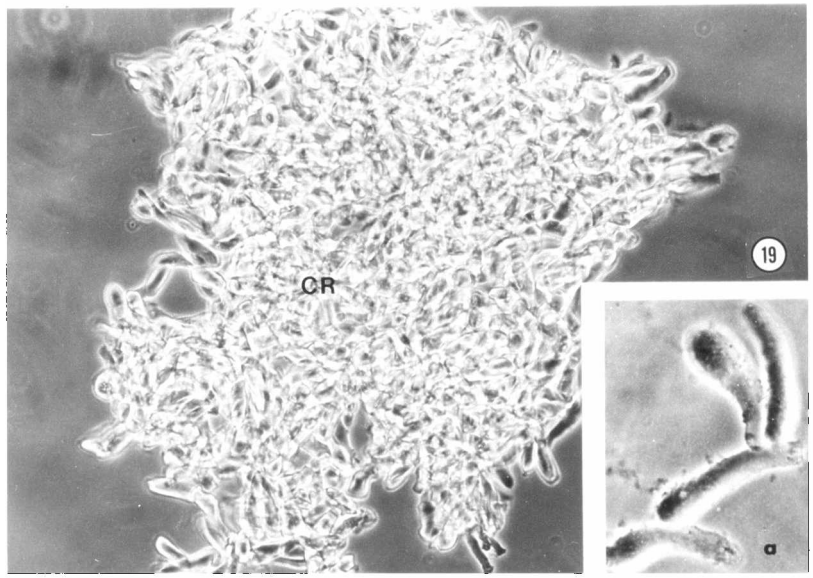
*Tris Buffer: 0.5 M NaCl, 0.009 M $CaCl_2$, 0.014 M KCl, 0.015 M $MgCl_2$, and 0.1 M Tris, pH 7.6.

Figure 18. Phase micrograph of a preliminary isolation of cortical rods. Small arrows-cytoplasmic particles; large arrows-individual cortical rods. x 100.

Figure 19. Phase micrograph of a purified preparation of cortical rods. CR-cortical rods; x 150. Figure 19a. Insert: phase micrograph of rehydrated, purified cortical rods showing their club shape. x 500.

Figure 20. Transmission electron micrograph of purified isolated cortical rods. CR-cortical rods; arrows-cytoplasmic contamination. x 3250.


18


CR
19
a

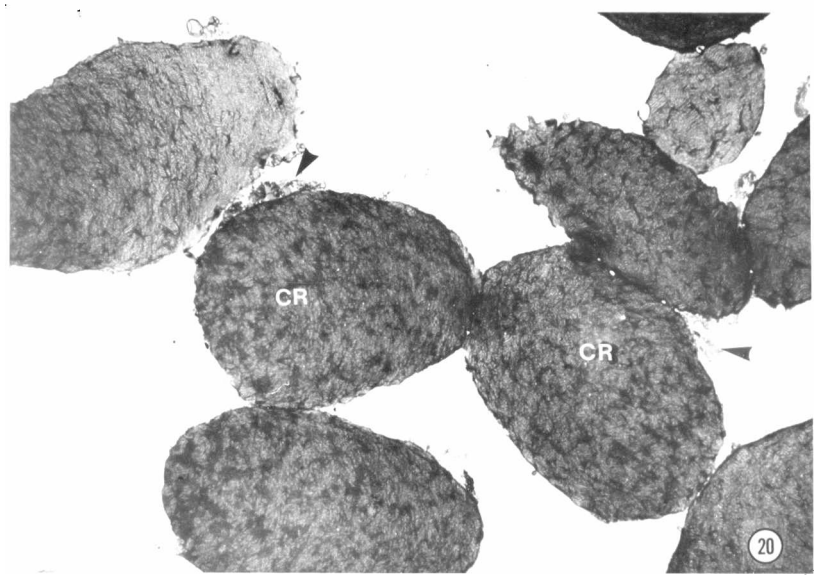

CR
CR
20

TABLE IV: SOLVENT EXTRACTS OF CORTICAL ROD ISOLATES

SOLVENT	ROD DISSIPATION	ABSORPTION PEAKS OF SOLUTES
8 N NaOH	Complete	260 nm, 225 nm
2 N NaOH	Complete	270 nm, 220 nm
18 N H_2SO_4	Complete	465 nm, 370 nm 320 nm, 250 nm
Chloroform	Partial*	270 nm
12 N HCl	Partial*	280 nm, 220 nm
2 N HCl	Partial*	280 nm, 220 nm
PO_4 Buffer, pH 5.8	Partial*	265 nm, 250 nm
Acetone	None	None
Methanol	None	None
Petroleum Ether	None	None
Mercaptoethanol	None	None
Triton X-100	None	None

* Swells and becomes flocculent

at 270 nm and 220 nm with 2 N NaOH, 260 nm and 225 nm with 8 N NaOH, and at 530 nm, 465 nm, 370 nm, 320 nm, and 250 nm with H_2SO_4. Readings at 220 nm to 280 nm usually suggest the presence of nucleic acids and aromatic amino acids. Absorption peaks associated with H_2SO_4 hydrolysis are probably caused by the formation of furfurals.

Isolates treated with a phosphate buffer at pH 5.8, chloroform, 2 N HCl, and 12 N HCl were not completely dissipated, but did give absorption maxima when read spectrophotometrically. Absorption peaks were at 265 nm and 250 nm for phosphate buffer treated isolates, 270 nm for chloroform, and 280 nm and 220 nm for both treatments with HCl. Absorption peaks observed in these extracts are probably produced by amino acids, nucleic acids, or carbohydrates.

Solutions which neither affected the morphology of the cortical rod isolates nor produced significant spectrophotometric peaks included methanol, Triton X-100, acetone, petroleum ether, or mercaptoethanol.

Biochemical Assays

Assays for protein and carbohydrate components were run and the results of the tests are presented in Table V. The Lowry's total protein determination was positive, but quantitation of the assay was not possible due to interference from carbohydrates (Bonitati 1969). A slight but steady decrease in the readings at 750 nm was inconsistent with the bovine serum albumin standard. Precipitation of the protein components using trichloroacetic acid and resolubilization in 1.5 N NaOH was ineffective in separating the interferring groups from the protein.

Figure 21. Graph of continous spectrophotometric readings of solvent treatments of isolated cortical rods. Abscissa-wavelength readings in nanometers. Ordinate-relative absorption of the extracts.

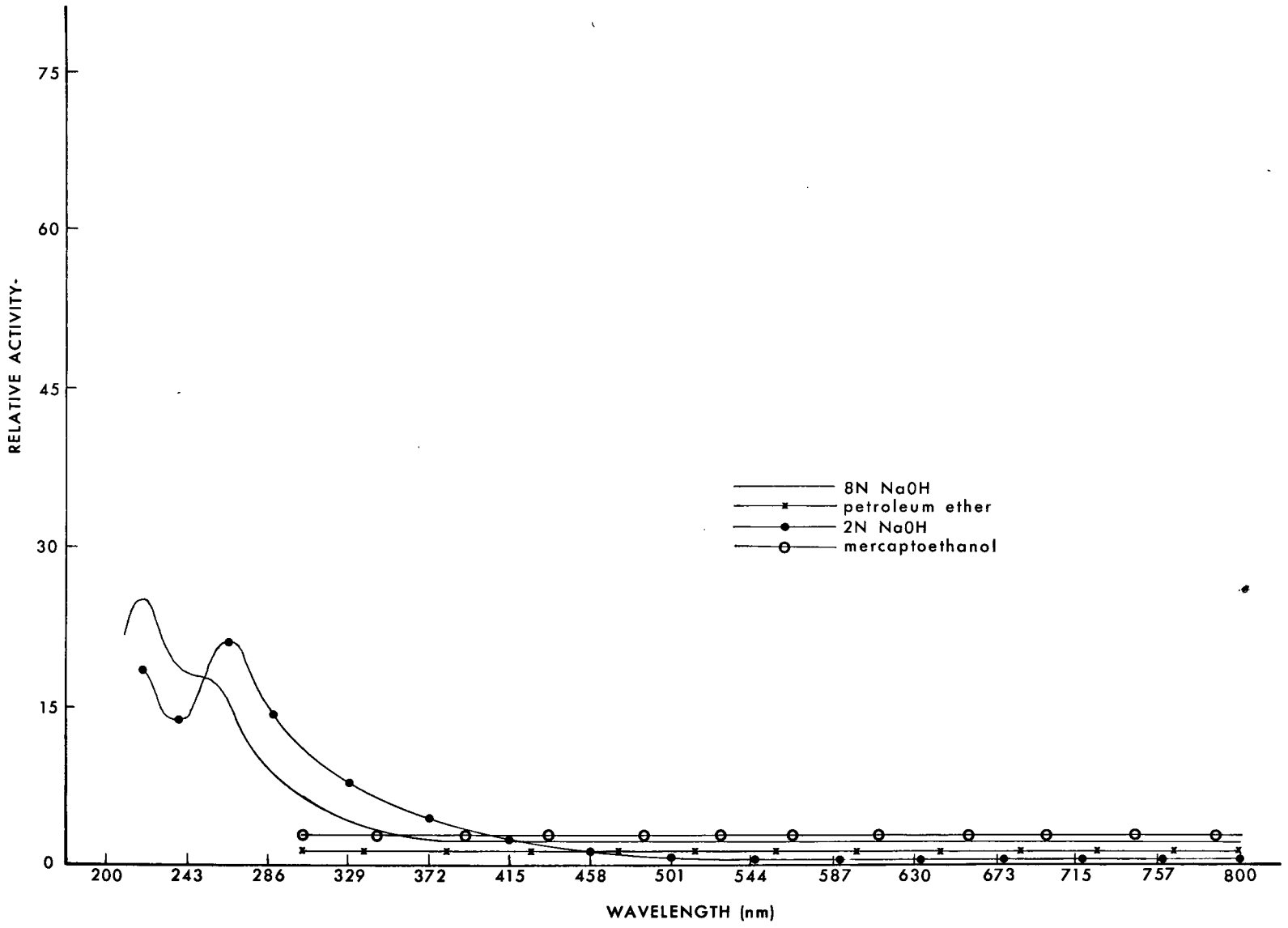

RELATIVE ACTIVITY-
75
60
45
30
15
0
200
243
286
329
372
415
458
501
544
587
630
673
715
757
800
WAVELENGTH (nm)
8N NaOH
petroleum ether
2N NaOH
mercaptoethanol

Figure 22. Graph of continous spectrophotometric readings of solvent treatments of isolated cortical rods. Abscissa-wavelength readings in nanometers. Ordinate-relative absorption of the extracts.

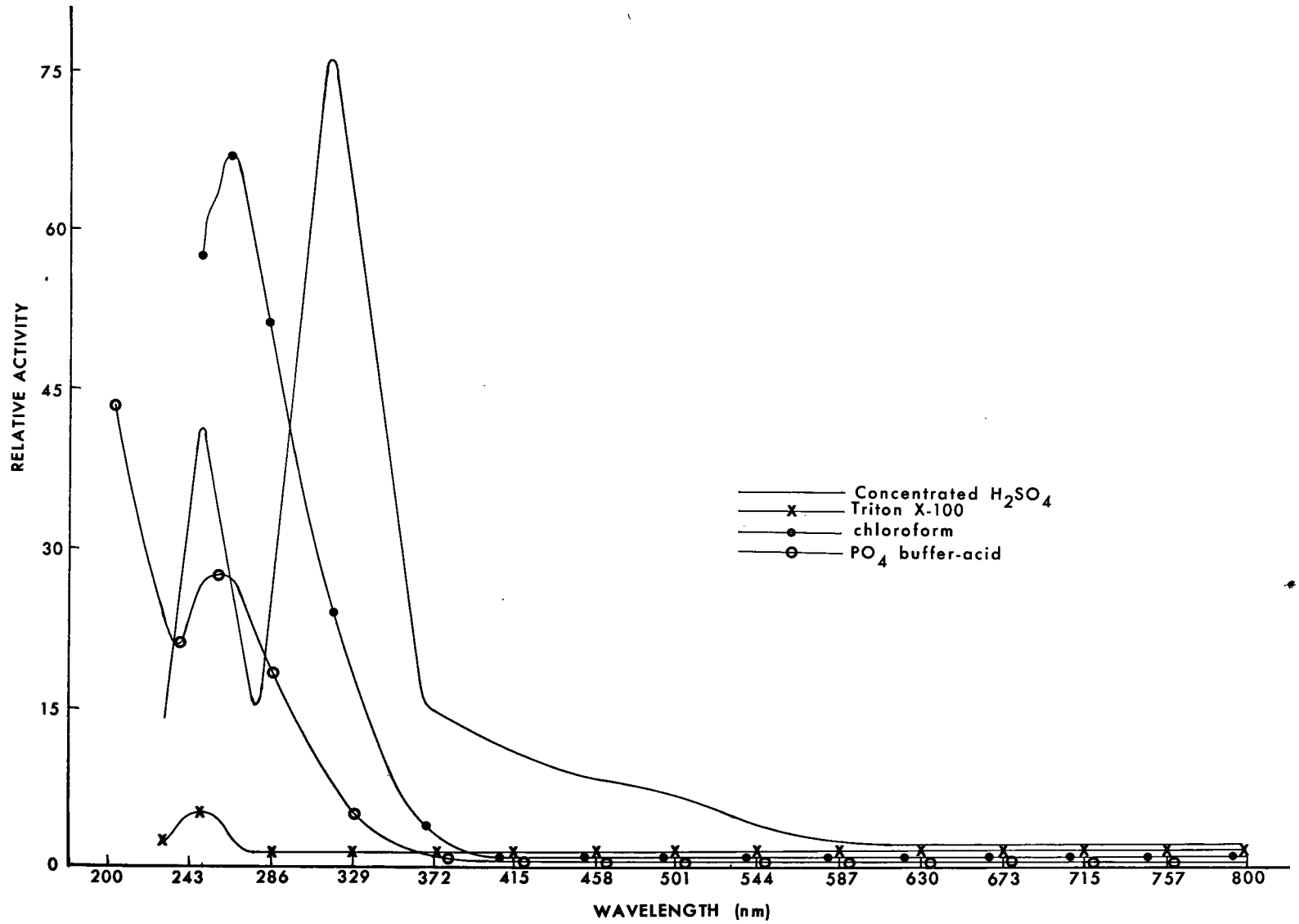

RELATIVE ACTIVITY
75
60
45
30
15
0
200
243
286
329
372
415
458
501
544
587
630
673
715
757
800
WAVELENGTH (nm)
Concentrated H_2SO_4
Triton X-100
chloroform
PO_4 buffer-acid

Figure 23. Graph of continous spectrophotometric readings of solvent treatments of isolated cortical rods. Abscissa-wavelength readings in nanometers. Ordinate-relative absorption of the extracts.

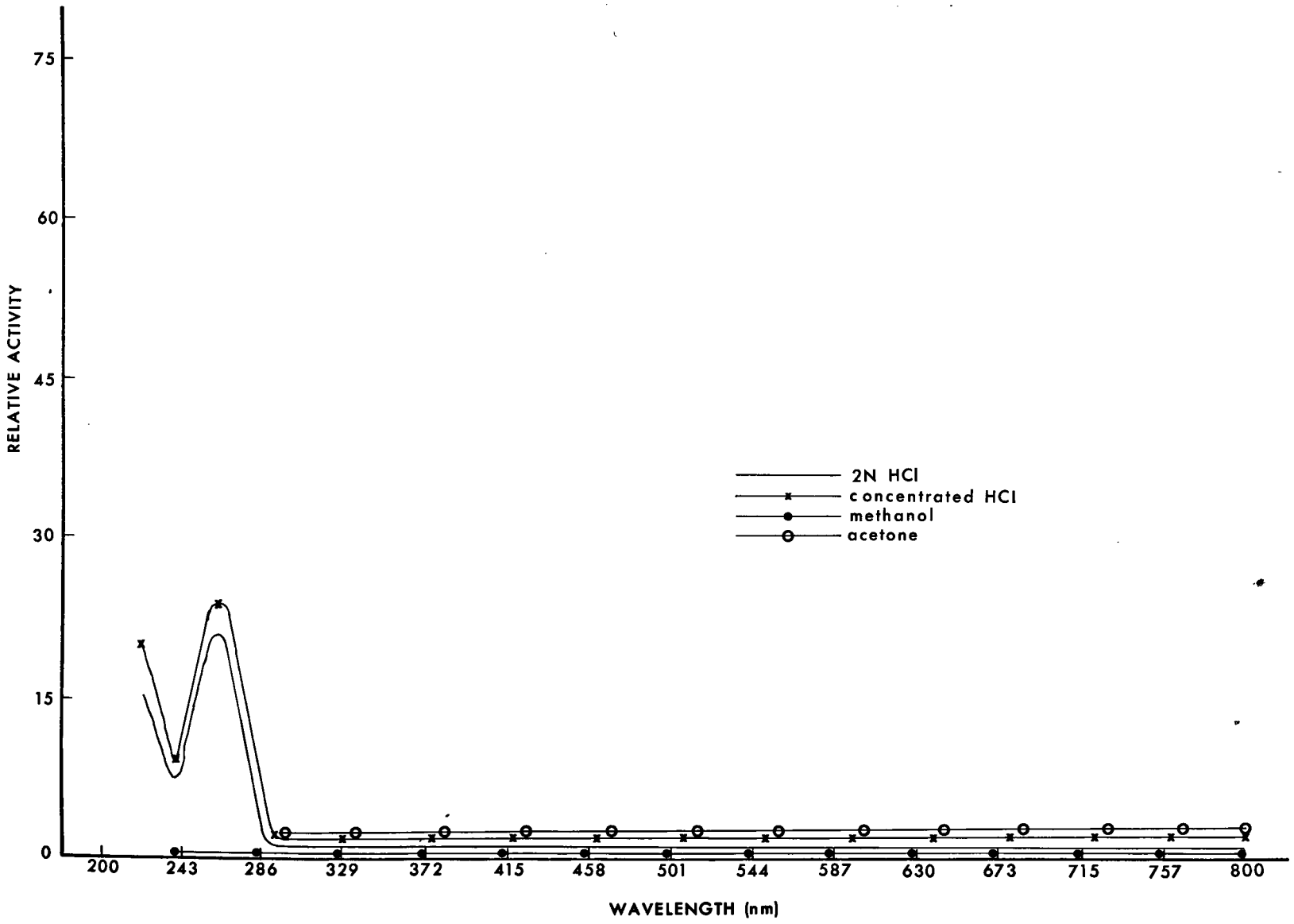

75
60
45
30
15
0
RELATIVE ACTIVITY
2N HCl
concentrated HCl
methanol
acetone
200
243
286
329
372
415
458
501
544
587
630
673
715
757
800
WAVELENGTH (nm)

TABLE V: BIOCHEMICAL ASSAYS OF CORTICAL ROD ISOLATES

TEST	SUBSTANCE REACTING	RESULTS
Lowry's	Protein	+
Molisch alpha Napthol Reaction	Carbohydrates	+
Anthrone-Sulfuric Acid Reaction	Hexoses, Pentoses, 6-Deoxyhexoses, Hexuronic Acid, Heptoses	+
L-Cysteine Sulfuric Acid Reaction	Pentoses	-
	Hexuronic Acid	-
	Hexoses	+
	Heptoses	+
	6-Deoxyhexoses	+
	2-Deoxypentoses	-
Ehrlich Reaction	Neuraminic Acid	-
Thiobarbituric Acid Assay	Neuraminic Acid	-

The Molisch α-napthol test and the anthrone sulfuric acid assay were used as preliminary tests for the presence of carbohydrates. Both tests were positive, but neither was sensitive enough to distinguish between the possible types of carbohydrates. Thus, specific groups of carbohydrates present in the isolates were determined using the L-cysteine sulfuric acid assay. Figures 24 to 27 show the results of the sample and standards run. An initial peak in the sample was observed at 396 nm indicating a 6-deoxyhexose and possibly overlapping absorption due to hexoses or pentoses. Upon addition of water and a twelve hour waiting period, "peaks" were observed at 510 nm, 460 nm, and 411 nm. Involvement of heptoses in the sample is suggested by the peaks at 510 nm and 460 nm. Since the addition of water destroys the absorption due to hexoses in the standards, the peak at 411 nm is probably due to a shift in the absorption of 6-deoxyhexoses. The presence of hexoses is suggested, however, by the dedrease in the initial readings at 396 nm after the addition of water. Involvement of 2-deoxypentoses is unlikely since no initial peak at 460 nm was observed as in the standard. Pentose involvement is also unlikely since a spontaneous decrease in the readings at 375 nm to 390 nm does not occur as predicted by Dische (1955). Contamination due to sucrose from the cortical rods isolation is ruled out as a source of hexoses since the same results for the assay are obtained using rods isolated over glycerol. A glycerol standard gave no peaks with the reaction.

Assays run for the presence of sialic acids were negative. The activity peak for sialic acids in the direct Ehrlich reaction is 530 nm. No peaks were observed for the sample at this wavelength. The

Figure 24. Graph of continous spectrophotometric readings of an L-cysteine sulfuric acid reaction on the isolated cortical rods. After twelve hours, water was added to the assay and read again. Abscissa-wavelength readings in nanometers. Ordinate-absorption of the reaction.

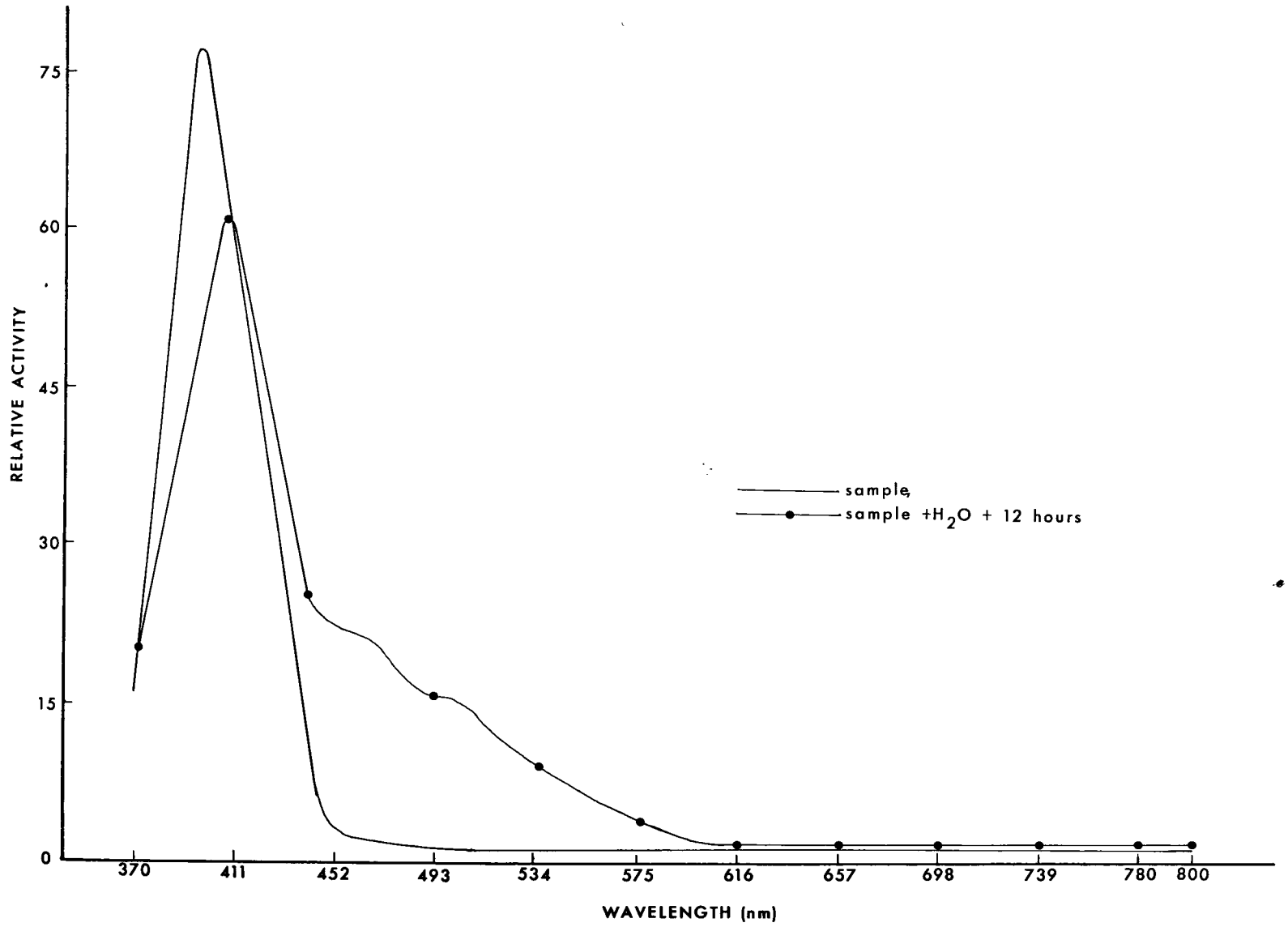

RELATIVE ACTIVITY
75
60
45
30
15
0
370
411
452
493
534
575
616
657
698
739
780
800
WAVELENGTH (nm)
sample
sample +H2O + 12 hours

Figure 25. Graph of continous spectrophotometric readings of L-cysteine sulfuric acid reactions of standards of glucuronic acid, glucose, fucose, and sedoheptulose. Abscissa-wavelength readings in nanometers. Ordinate-absorption of the reactions.

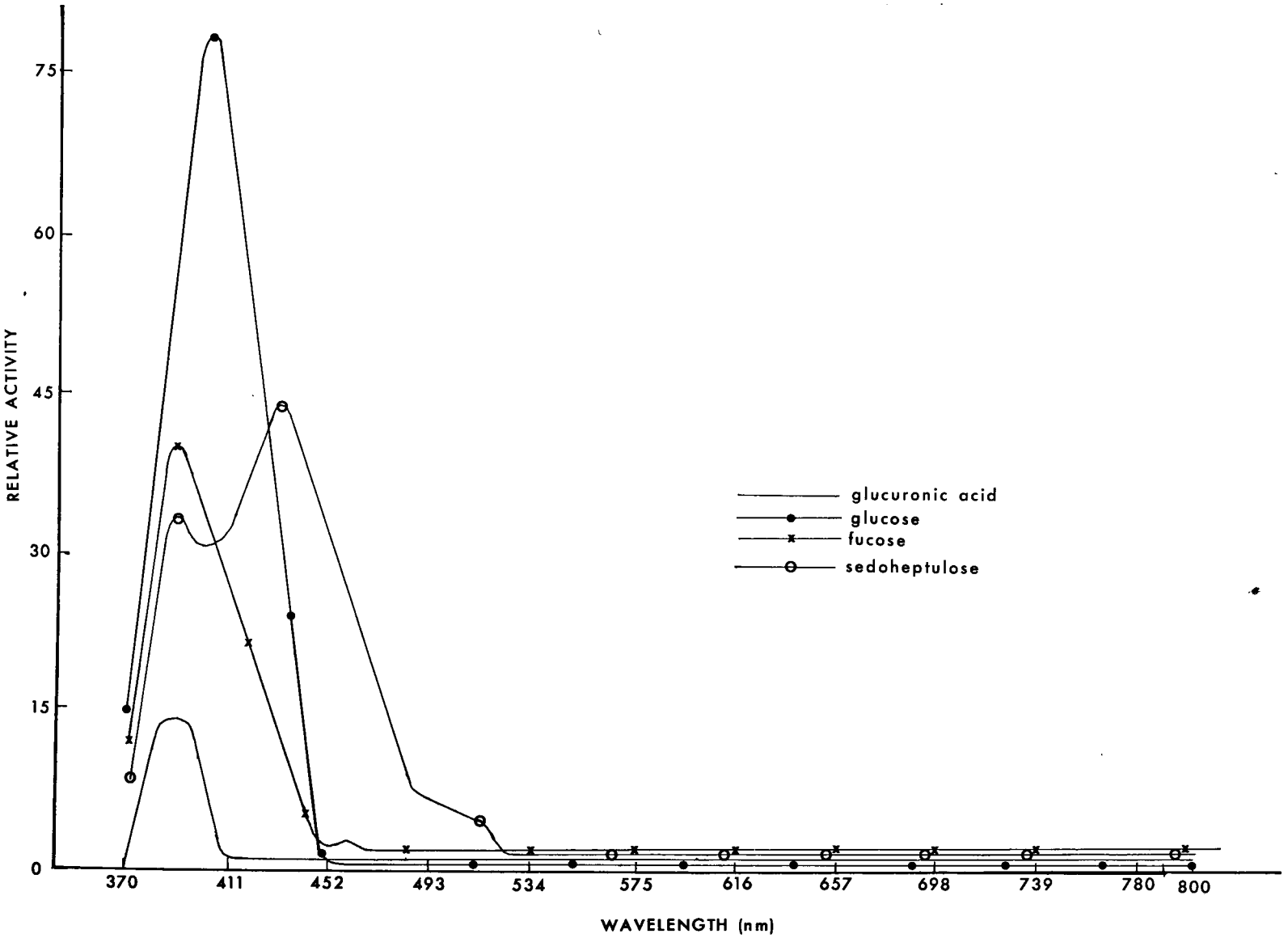

RELATIVE ACTIVITY
75
60
45
30
15
0
370
411
452
493
534
575
616
657
698
739
780
800
WAVELENGTH (nm)
glucuronic acid
glucose
fucose
sedoheptulose

Figure 26. Graph of continous spectrophotometric readings of L-cysteine sulfuric acid reactions of ribose, 2-deoxy-D-ribose, and of sedoheptulose after a twelve hour waiting period and 2-deoxy-D-ribose after a twenty-four hour waiting period. Abscissa-wavelength readings in nanometers. Ordinate-absorption of the reaction.

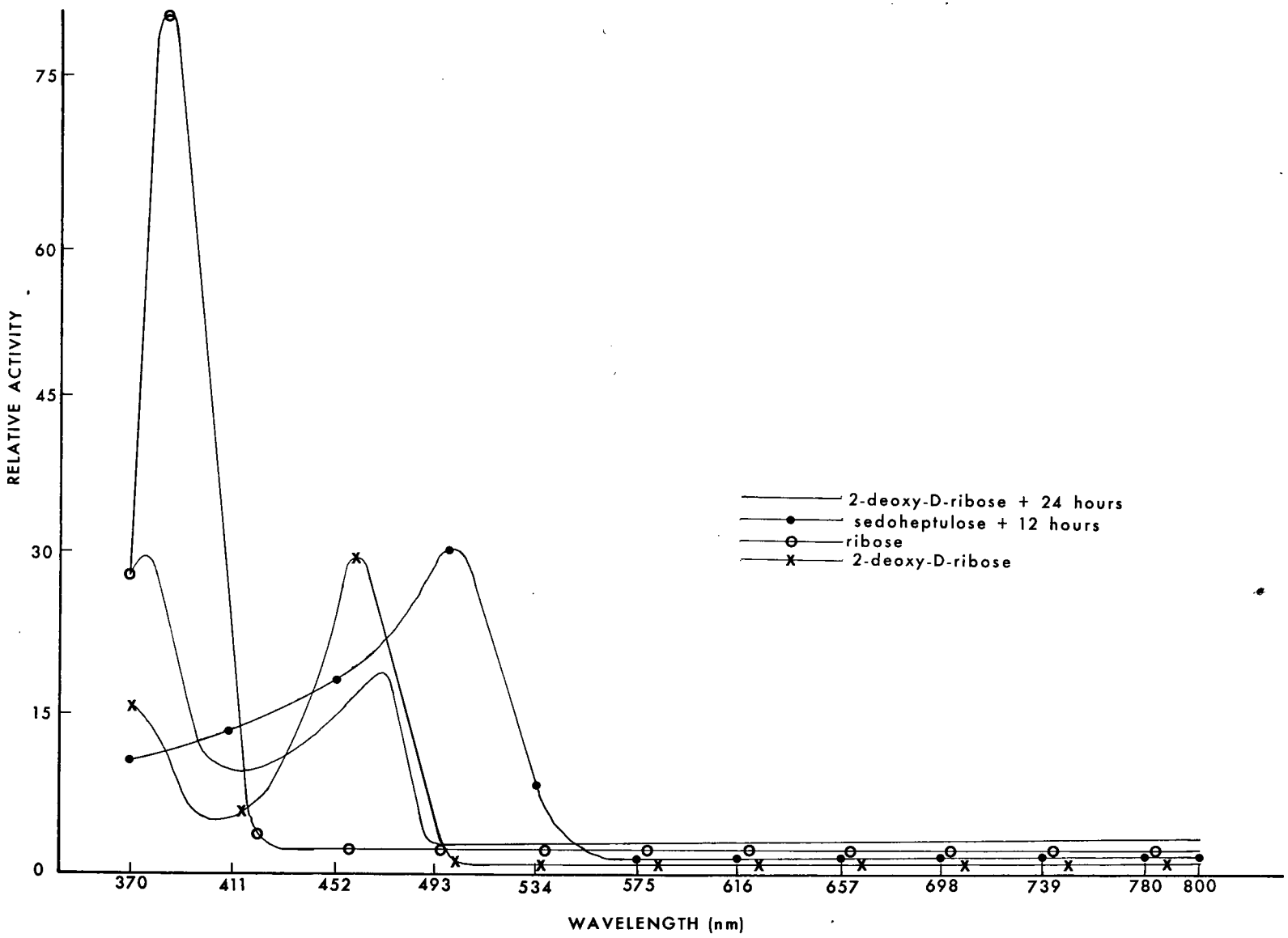
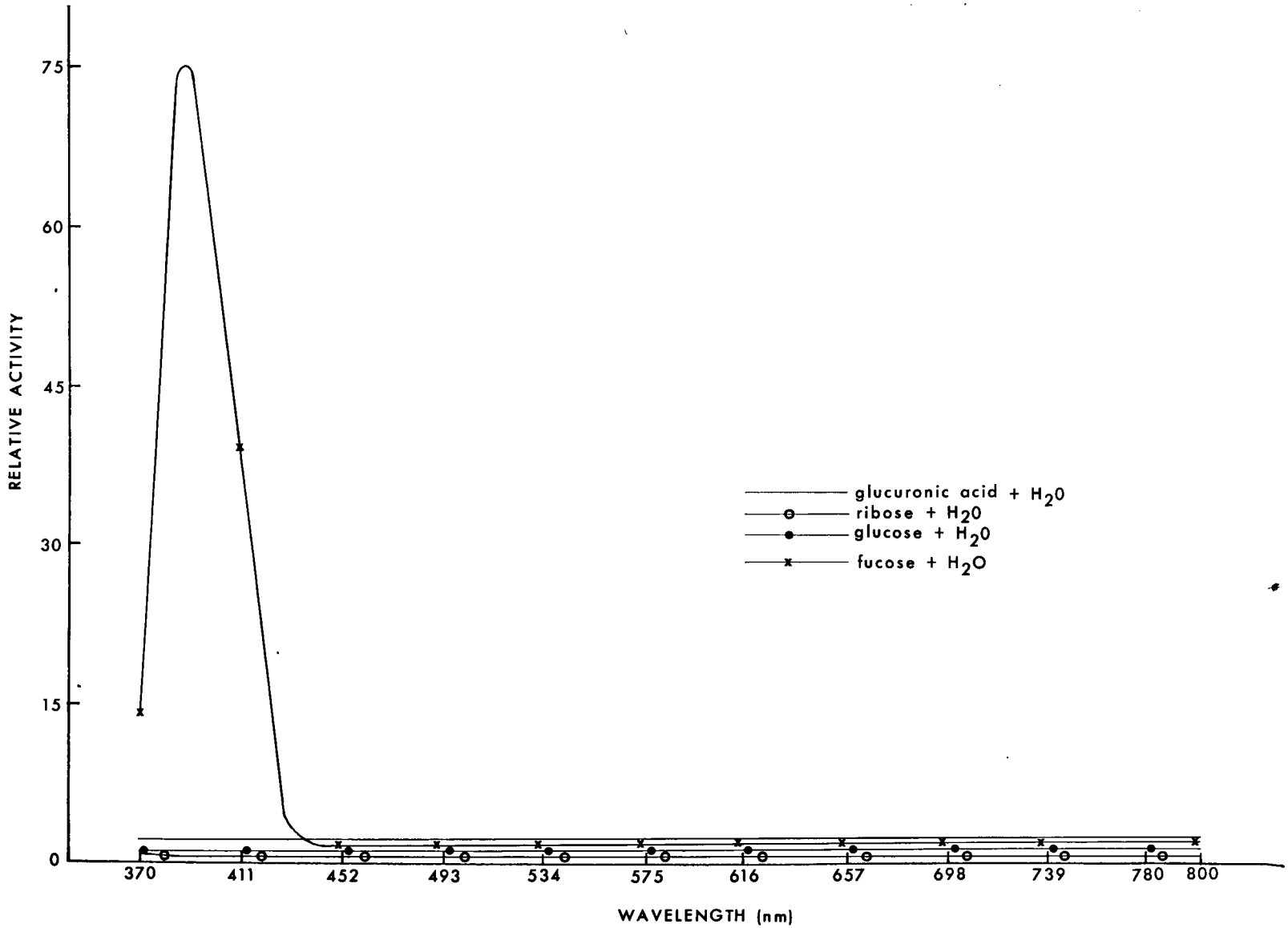

RELATIVE ACTIVITY
75
60
45
30
15
0
370
411
452
493
534
575
616
657
698
739
780
800
WAVELENGTH (nm)
2-deoxy-D-ribose + 24 hours
sedoheptulose + 12 hours
ribose
2-deoxy-D-ribose

Figure 27. Graph of continous spectrophotometric readings of L-cysteine sulfuric acid reactios of glucuronic acid, ribose, glucose, and fucose after water had been added to the assays. Abscissa-wavelength readings in nanometers. Ordinate-absorption of the reaction.


RELATIVE ACTIVITY
75
60
45
30
15
0
370
411
452
493
534
575
616
657
698
739
780
800
WAVELENGTH (nm)
glucuronic acid + H20
ribose + H20
glucose + H20
fucose + H2O

spectrophotometric readings of the thiobarbituric acid assay were also negative (Figure 28). Since the standards in the thiobarbituric acid assay were run at the 0.5μ mole level, insensitivity to the sialic acids is precluded.

Control of the Cortical Reaction

The last portion of this paper deals with the control of the cortical reaction. In these experiments a single animal was usually used for each set of experiments since the animals were capable of spawning large numbers of eggs (100,000 to 250,000). Microscopic observations were made on eggs spawned into normal seawater or artificial seawaters lacking the divalent cations Mg^{++}, Ca^{++}, Ca^{++}-Mg^{++}, or containing 10 mM EDTA, 1% procaine, or 0.1% soybean trypsin inhibitor (SBTI) and then returned to normal seawater (Table I). The cortical rod expulsion was completely inhibited in Mg^{++} free or Ca^{++}-Mg^{++} free seawater. Ca^{++} free seawater was slightly effective in slowing the cortical reaction but did not stop the reaction. Procaine, which inhibits membrane bound Ca^{++}, also slightly inhibited the cortical reaction. When completely inhibited eggs were returned to normal seawater, the rods were expelled from the eggs, but did not dissipate and no hatching membrane was formed (Figure 29). In all other respects the eggs returned to normal seawater from the Ca^{++}-Mg^{++} free or Mg^{++} free seawater looked normal. If the container of eggs was gently shaken at this stage the cortical rods were detached from the eggs. However, this detachment did not promote the dissipation of the cortical rods or the formation of a hatching membrane. Eggs which were spawned into seawater containing 0.1%

Figure 28. Graph of continous spectrophotometric readings of the Ehrlich reaction for neuraminic acid in both isolated cortical rods and a standard of N-acetyl neuraminic acid and of the thiobarbituric acid assay of isolated cortical rods and a standard of N-acetyl neuraminic acid. Abscissa-wavelength readings in nanometers. Ordinate-absorption of the reaction.

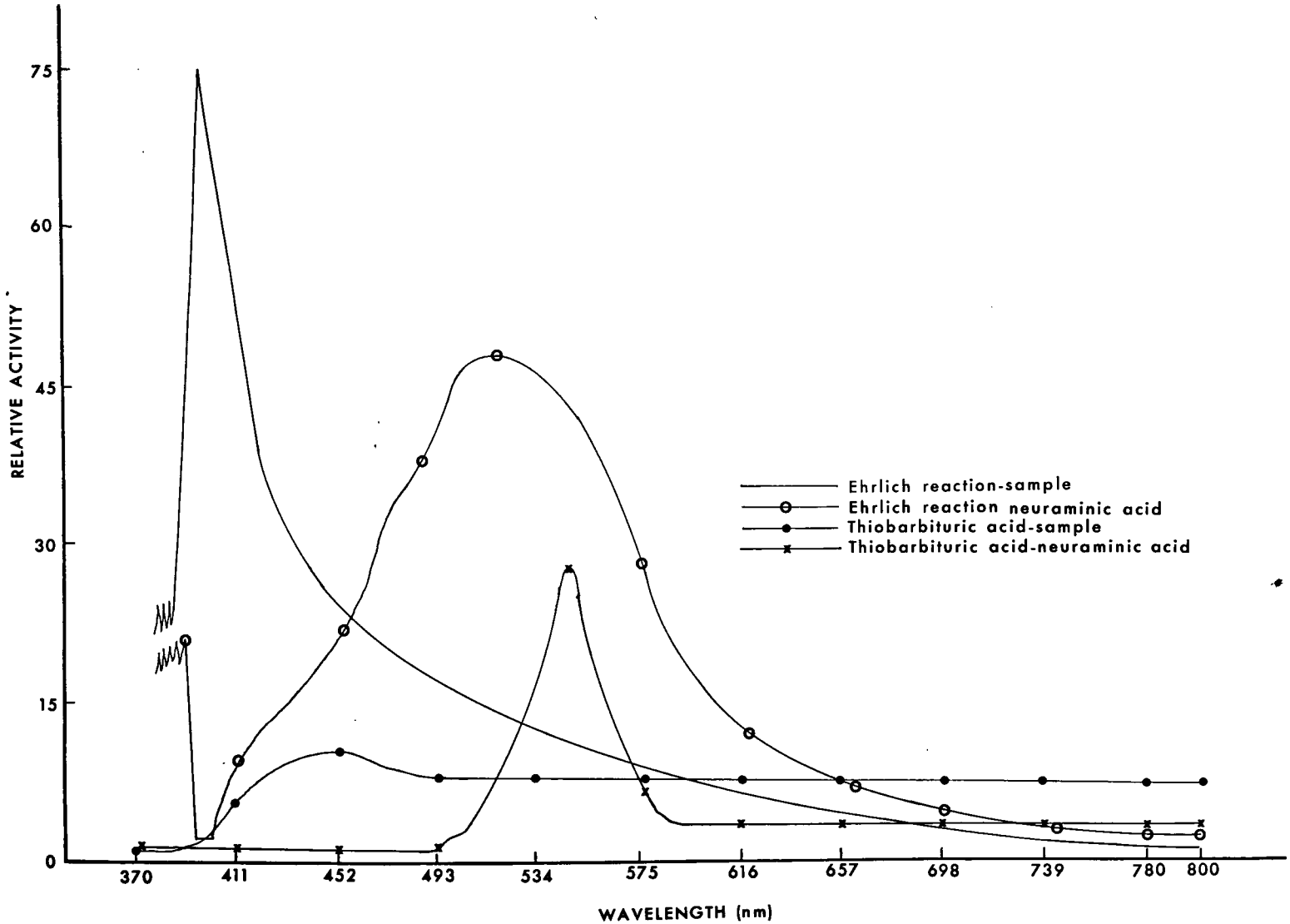
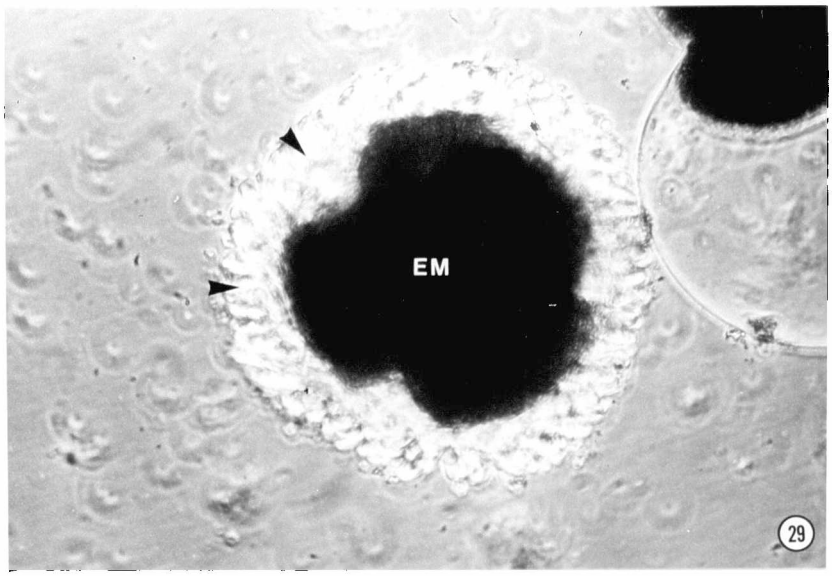
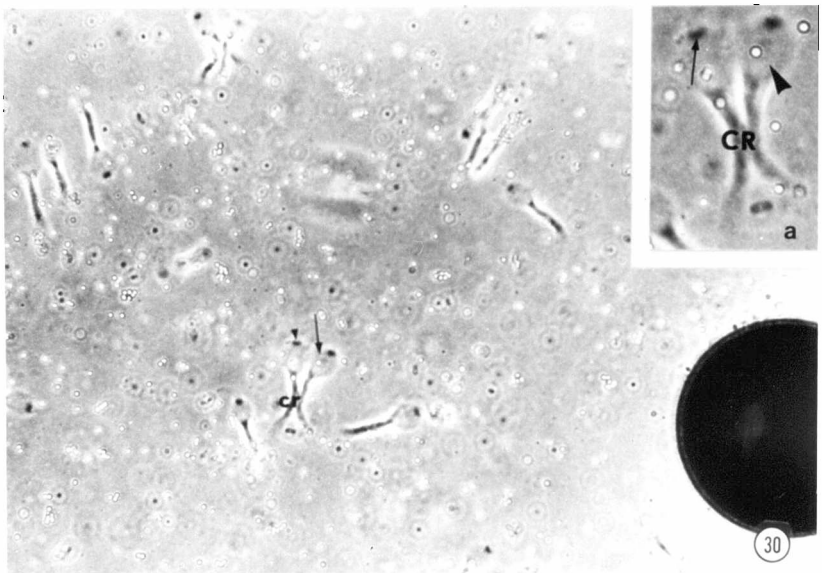

RELATIVE ACTIVITY
75
60
45
30
15
0
370
411
452
493
534
575
616
657
698
739
780
800
WAVELENGTH (nm)
Ehrlich reaction-sample
Ehrlich reaction neuraminic acid
Thiobarbituric acid-sample
Thiobarbituric acid-neuraminic acid

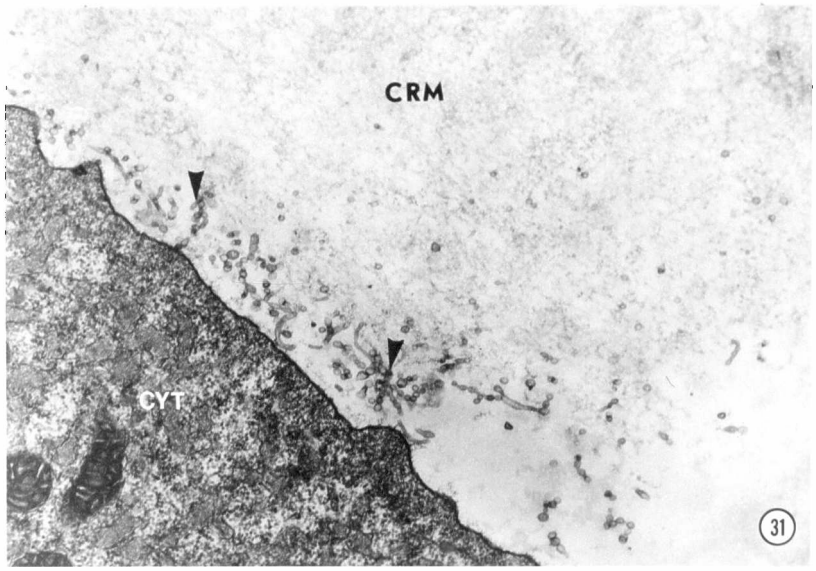
Figure 29. Phase micrograph of a Penaeid embryo at approximately the 8 cell stage. The egg was spawned into Mg^{++} free seawater to stop the cortical reaction, fertilized, and returned to normal seawater to reinitiate the reaction. The cortical rods were expelled but remain around the embryo. No hatching membrane is present. EM-embryo; arrows-cortical rods. x 170.

Figure 30. Phase micrograph of cortical rod dissipation inhibited by the addition of SBTI. A hatching membrane is just beginning to form around the egg. CR-cortical rods; arrowhead-cap-like portion of a dissipating cortical rod; arrow-region of cortical rod dissipation. x 110. Figure 30a. Insert: inhibited cortical rod dissipation. CR-cortical rod; small arrow-cap-like portion of a dissipating cortical rod; large arrow-region of cortical rod dissipation. x 300.

Figure 31. Transmission electron micrograph showing membrane vesiculation. CRM-material from a dissipating cortical rod; arrows-membrane vesiculation. x 16,000.


EM
29


CR
a
30


CRM
CYT
31

SBTI also had their cortical reaction completely stopped. Return to normal seawater did not reverse any part of the reaction. If 0.1% SBTI was added to seawater containing eggs at any stage during rod expulsion or dissipation, the reaction was immediately stopped and return to normal seawater did not reverse the reaction. Dissipating cortical rods, stopped by the addition of SBTI, are shown in Figure 30. Although the cortical rod dissipation was stopped, a hatching membrane was still able to form with non-dissipated rods around the eggs. Seawater containing 10 mM EDTA, a chelating agent, was also effective in stopping the entire cortical reaction of Penaeid shrimp eggs. Expulsion and dissipation of the cortical rods was completely stopped. Return to normal seawater did not reverse the effects of the EDTA. The millimolar concentration of the EDTA is just slightly higher than that of the Ca^{++} and Mg^{++} ions in seawater.

Enzyme Dissolution

Efforts to promote the dissolution of cortical rods involved the use of enzymes, the results of which are summarized in Table VI. Only protease, trypsin, and alkaline phosphatase were effective in causing the dissipation of the cortical rods. Loss of rod morphology at the light level was preceded by a period of swelling. Dissipation was complete with the exception of a small cap-like portion at one end of the rods. A similar cap-like portion is observed in the natural dissipation of cortical rods as mentioned previously. Dissipation of isolated cortical rod preparations by alkaline phosphatase was not due to protease contamination in the enzyme preparation

TABLE VI: ENZYMIC TREATMENTS OF CORTICAL ROD ISOLATES

ENZYME PREPARATIONS	CONCENTRATION	EFFECT ON ISOLATED RODS
Alkaline Phosphatase	0.2%	++
Alkaline Phosphatase + SBTI	0.2%	++
Acid Phosphatase	0.02%	--
Protease	0.1%	++
Trypsin	0.1%	++
alpha-Chymotrypsin	0.1%	+
aryl-Sulfatase	0.2%	--
Hyaluronidase	0.02%	--
Collagenase	0.1%	--
Neuraminadase	0.1%	--
Lipase	0.1%	--

++ Dissipated + Swelling, No Dissipation -- No Effect

since a 0.02% solution of SBTI added to the assay only caused a slowing of the dissipation process. The same degree of dissipation was achieved as in the enzyme preparation not treated with SBTI. After five hours of treatment with chymotrypsin, swelling of the rods was evident, but dissolution was not observed. Cortical rods which were held in the buffers used with the enzyme assays maintained a normal morphology and did not exhibit a noticeable swelling or dissipation.

It should be noted that neither lipase, sulfatase, or neuraminidase were effective in causing swelling or dissipation of the cortical rods.

IV.

DISCUSSION

The cortical reactions in the eggs of P. setiferous and P. aztecus are unique in several ways. These unusual features are: (1) enormous cortical specializations (rods) in the peripheral cytoplasm; (2) rapid expulsion and dissipation of these cortical rods on contact with seawater; (3) the Mg^{++} dependence of the reaction; (4) the dehiscence of smaller cortical bodies associated with the expulsion of the cortical rods; and, (5) decrease in egg size after activation. Only a few examples of such a massive and rapid cortical release as seen in the Penaeid shrimp are reported in the literature.

A similar reaction, morphologically, to the one displayed in the eggs of Penaeid shrimp occurs in the eggs of Nereis limbata (Lillie 1911, Wilson 1892, Fallon and Austin 1964 and 1967, Novikoff 1939a and 1939b and Costello 1948). A jelly-like material is contained as fibrous whorls in large "alveolar vesicles" in a broad band of cytoplasm at the surface of the eggs. The alveolar material is extruded at fertilization and forms a layer around the egg and remains there throughout the early development of the zygote. The material in the cortical alveolae has a similar substructure to the cortical rods in Penaeus sp. in that both are fibrous in appearance. However, the feathery substructure of the Penaeid egg cortical rods is lacking in the alveolar material of N. Limbata.

Teleost eggs also undergo a massive cortical reaction comparable in magnitude to Penaeid egg reactions (Yamamoto 1939). The teleost egg retains the extruded jelly-like material beneath a vitelline membrane. This is in contrast to the rapid dispersal of

the substance released from Penaeid eggs.

The cortical rod material of Penaeid eggs differs chemically from the jelly-like substances released from teleost and annelid eggs, and from jellies surrounding the eggs of sea urchins and amphibians. The material involved in the cortical reaction of the eggs of Nereis limbata has been tentatively analyzed and found to contain acid mucopolysaccharides with sulfate esters (Raven 1961 and Costello 1949). Cortical alveolae of teleost eggs have also been shown to contain an acid and in some instances, a neutral mucopolysaccharide (Raven 1961). Several workers have also shown the presence of acid mucopolysaccharides in the cortical granules of other animal ova (Aketa 1962, Monne and Harde 1951, Runnstrom and Immers 1956, Monne and Slautterback 1950, Ishihara 1968 and Schuel et al. 1974).

Sulfated mucopolysaccharides have been demonstrated in the egg jellies of both sea urchins (Lorenzi and Hedrick 1973, and Monne and Slautterback 1950) and amphibians (Freeman 1968, Humphries 1966, Katagiri 1973, Lee 1967, Minganti 1955, and Gusseck and Hedrick 1971). Portions of the jelly of amphibian eggs have also been reported to contain neutral mucopolysaccharides (Lee 1967, Freeman 1968, and Humphries 1966). Additionally, several types of sialic acids have been reported in the egg jellies of sea urchins (Hotta et al. 1970a and 1970b, also Isaka 1968), and amphibians (Lee 1967, Freeman 1968, and Katagiri 1973).

Although there are some similarities between the cortical rod material from the eggs of P. setiferous and P. aztecus and other types of egg jellies, significant differences suggest that the cortical rods are not a typical egg jelly, if in fact a jelly at all.

These differences are: (1) rapid dispersal of the cortical rod material; (2) the inability to digest the cortical rods with sulfatases, as in sea urchin and amphibian egg jelly; (3) the lack of sialic acid in the cortical rods; and, (4) the absence of acid mucopolysaccharides in the cortical rods.

The present data suggests that the cortical rods are either a neutral mucopolysaccharide or a glycoprotein. These data are: (1) the presence of both protein and carbohydrates in the cortical rods as shown by biochemical assays; (2) the inability to separate the protein from the carbohydrates using trichloroacetic acid precipitation; (3) the presence of 6-deoxyhexose, a common component of neutral mucopolysaccharides (Dische 1955), as demonstrated in the L-cysteine sulfuric acid reaction, and (4) the inability to histochemically stain the cortical rods for an acid mucopolysaccharide using alcian blue. It should be pointed out that acid mucopolysaccharides were demonstrated in the investment coat using the latter method.

The Mg^{++} dependent release of the cortical rods from the shrimp oocyte is interesting since it is quite unlike the Ca^{++} dependent reactions reported in other animal ova to date (Epel 1975, Paul 1975, Schroeder and Strickland 1974, Steinhardt 1974, Vacquier 1975, and Yamamoto 1939b). Since Mazia'a work (1937) on sea urchin eggs, the release of bound intracellular Ca^{++} has been implicated in cortical reactions and egg activation. Recent use of divalent cation carrying ionophores to parthenogenetically activate eggs has supported the role of Ca^{++} in the activation of egg cortical reactions (Epel 1975, Steinhardt 1974, and Vacquier 1975). The role of Ca^{++}, in the cortical

reaction, has been related to the activation of proteolytic enzymes released from the cortical granules of sea urchin eggs (Vacquier 1975). The release of enzymes from cortical granules has been demonstrated by several workers on different animal ova (Epel and Weaver 1969, Gwatkin 1972, Katsura and Tominaga 1974, Paul 1975, Schuel et al. 1973, Vacquier 1975, and Runnstrom and Immers 1956). The enzymes released are beleived to have two possible roles: (1) the breakdown of cortical granules (Schuel et al. 1973) and (2) the formation of a block to polyspermy (Vacquier 1973, Tegner 1974, Schuel et al. 1973, Epel et al. 1975, and Longo and Schuel 1973). Polyspermy is prevented by detachment of the supernumerary sperm, and by detachment of the vitelline "membrane" from the oolemma.

Unlike the Ca^{++} dependence of other cortical reactions, involvement of Mg^{++} ions has been established in the shrimp oocyte cortical reactions by the use of artificial seawaters. The Mg^{++} dependency of the shrimp ova cortical reaction initially suggested the involvement of microtubules whose contractile abilities are dependent on Mg^{++} ions. It appeared that microtubules might be active in the expulsion of the cortical rods since their contractile properties would aid in moving the rods from the crypt. However, microtubules have not been found associated with the cortical rod crypts to account for such a dependency on Mg^{++} ions.

Reports of other workers have suggested that enzymes released from cortical granules in sea urchins are activated by a divalent cation (Ca^{++}) released intracellularly at fertilization (Vacquier 1975, and Epel 1975). In the present system Mg^{++} may have a similar function; that is, it may either activate an enzyme or act as a cofactor for an

nzyme released upon egg contact with seawater (Dixon and Webb 1964). The ability of SBTI to completely and irreversibly stop the cortical reaction in Penaeid oocytes suggests the presence of a trypsin-like protease which is responsible for the expulsion of the cortical rods. Inhibition of the cortical rod expulsion by EDTA is probably mediated through the chelation of Mg^{++} ions bound to an active enzyme involved in the cortical reaction. Although some enzymes may be inhibited by direct binding of EDTA to the enzyme, many are probably inhibited by the chelation of Mg^{++} or Ca^{++} also (Loftfield 1973, Webb 1963, and Aldridge and Reiner 1972). Mg^{++} has been shown to be a necessary cofactor or activator in several enzyme systems (Dixon and Webb 1964). The role of Mg^{++}, then, may be as an activator for a trypsin-like enzyme released during the cortical reaction in the shrimp ova. The release of such a protease-like enzyme may be from smaller cortical bodies which dehisce in the cortex of the ovum (Insert, Figure 9) during cortical rod extrusion. Release of the protease-like enzymes from similar cortical bodies (granules) has been shown in the eggs of sea urchins (Epel 1975, Schuel et al. 1973, Epel and Weaver 1969- and Vacquier 1975).

There are several possibilities for the role of such a trypsin-like enzyme in the expulsion of the cortical rods. One is that the trypsin-like enzyme attacks loose proteinacious bonds between the cortical rods and their crypts. During this process, membrane permeability may be increased resulting in a positive osmotic pressure in the ooplasm which may push the rods through the investing coat. A second possibility for the role of the enzyme would be the disruption of the investment coat. Removal of the investment coat would

then allow the cortical rods to exit from the crypts, again probably due to increased osmotic pressure within the egg.

During the process of rod expulsion, a corresponding decrease in the size of the egg occurs. This decrease requires the rapid removal of excess plasmalemma in the cortical rod crypts. Membrane vesiculation may account for the removal of the excess oolemma to accommodate the decrease in the size of the eggs. A size decrease in the ova of the anthozoan Bunodosoma sp., attributed to a process of membrane vesiculation during the cortical reaction (Dewel and Clark 1974), has been described in a recent study and Wilson has described a decrease in the size of ova during their activation in several species.

Once the cortical rods have been expelled from the eggs, rod dissipation occurs rapidly. This reaction is again inhibited by SBTI suggesting that dissipation is also due to a trypsin-like enzyme. However, the dissipation of the rods is probably the result of a second enzyme released from the egg or investment coat. If the same enzyme were responsible for expulsion and dissipation, the reaction would go to completion when the eggs are returned to normal seawater from Mg^{++} free seawater. There are two possible explanations for this lack of dissipation on return to normal seawater. These are: (1) the release of the dissipating enzyme is not Mg^{++} dependent and the released enzyme dissipates, (2) the enzyme is released but is short lived and broken down before the cortical rods can be released.

Several possible roles for the cortical reaction in Penaeid oocytes exist. These are: (1) a block to polyspermy; (2) the formation of an egg jelly to act as a protective layer; (3) a chemical

defense mechanism; and , (4) as an aid in the formation of the hatching membrane.

The removal of supernumerary sperm from the surface of many animal ova has been demonstrated to be the result of a cortical reaction at the time of fertilization (Epel 1975 for a review, and Paul 1975). Many sperm, attached to the investment coat, are lifted off the surface of the ova during the expulsion of the rods. However, the removal of supernumerary sperm from the shrimp ova does not appear to be the primary function of the cortical rod release since the reaction is initiated by contact with seawater and not by sperm penetration. There are two additional arguments against the role of the Penaeid ova cortical reaction as a block to polyspermy. These are: (1) sperm are non-motile and are applied to the ova by the female shrimp during spawning (Clark et al. 1974); and (2) physiological polyspermy has been reported in many arthropods and is a prerequisite for successful fertilization (Austin 1965 for a review, Brown and Knouse 1967, and Hinsch 1971). Although pronuclear fusion has not been observed with the Penaeid system, the large number of non-motile sperm attached to the ova suggests that polyspermy may be present.

The formation of a protective layer against mechanical damage may also be a possible role. Such a layer may function like the egg jelly or investing layers around many ova as protection against an unstable environment (e.g. shallow pools, etc. Berril 1971). Hudinaga (1942) indicated that cortical rod material remained around the ova of P. japonicus and P. monoceros until the second cleavage division. By the second division a protective coat (hatching membrane) has

formed. In contrast, our studies indicate that the cortical rod material rapidly dissipates and is entirely absent prior to the formation of the hatching membrane, resulting in a short period when the egg is unprotected by an investment layer. Further, as discussed previously, the cortical rod material differs significantly from typical egg jellies and the contents of cortical granules found in other animal ova. Thsu the role of the cortical rods as a precursor for egg jelly is questionable.

The development of a chemical defense barrier against microorganisms is a third possible role for the cortical rods. Although morphologically the rods are rapidly broken up, the chemical constituents may remain around the ova for a long period of time. In this form, the chemical constituents could act as a bacterialcidal agent (Arnold, personal communication). The possibility that the rods act in such a fashion is presently being tested for.

Lastly, the dispersal of the cortical rods may aid in the formation of the hatching membrane. As the cortical rods break down, some of their constituents may be used in the formation of the hatching membrane. At the present time we have evidence which both supports and questions this hypothesis. For example, eggs treated in Mg^{++} free seawater and subsequently returned to normal seawater appear unable to produce a hatching membrane; however, eggs whose cortical rod dissipation is inhibited with SBTI are unable to produce a hatching membrane.

At present, several lines of investigation are in process to determine the exact role of the cortical rods in the development of shrimp ova. Other experiments have been initiated to isolate and

and identify the factors responsible for the extrusion and dissipation of the cortical rods.

V.

SUMMARY

The cortical reaction of the eggs from Penaeus aztecus and Penaeus setiferous has been discussed both morphologically and physiologically. The findings of this investigation can be summarized as follows:

(1) The cortical response in Penaeid oocytes is a massive release of cortical specializations (rods) into the surrounding media and the release is initiated by contact with seawater.

(2) The cortical rods released during the reaction are rapidly dissipated in the surrounding media and do not appear to remain associated with the eggs.

(3) A method for the isolation of the cortical rods from mature ovary has been designed.

(4) Whole mature ovary stained with alcian blue 86X and the Periodic-acid Schiffs (PAS) indicated that the rods were a polysaccharide whereas the investment coat was an acid mucopolysaccharide.

(5) Biochemical tests on the isolated cortical rods show the presence of a protein component and a complex association of carbohydrates.

(6) Dissipation of the isolated cortical rods has been demonstrated using alkaline phosphatase, protease, trypsin, and to a limited degree, a chymotrypsin.

(7) The cortical reaction is a Mg^{++} dependent reaction in contrast to the Ca^{++} dependent cortical reaction found in other animal ova.

(8) In addition, the cortical reaction in Penaeid oocytes is mediated through a trypsin-like enzyme or enzymes.

(9) The role of the cortical rods is discussed as a possible block to polyspermy, the formation of a protective layer, and as a possible bacterialcidal agent.

(10) A second cortical reaction consisting of smaller cortical vesicles is described and occurs in the later stages of cortical rod expulsion.

VI.

BIBLIOGRAPHY

REFERENCES

Afzelius, B. A. 1956. The ultrastructure of the cortical granules and their products in the sea urchin egg as studied with the electron microscope. Exptl. Cell Res. 10:257-285.

Aketa, Kenji. 1962. Studies on the production of the fertilization acid in sea urchin eggs; cytochemical examination on the possible role of acid mucopolysaccharide components in the acid production. Embryologia. 7:223-227.

Aldridge, W.N. and Elsa Reiner. 1972. Enzyme Inhibitors as Substrates. American Elsevier Publishing Co., Inc. New York. p. 368.

Austin, C.R. 1965. Fertilization. Prentice-Hall, Inc. Englewood Cliffs, New Jersey.

Austin, C.R. and E.C. Amoroso. 1959. The mammalian egg. Endeavor. 18:130-143.

Austin, C.R. 1968. Ultrastructure of Fertilization. Holt, Rinehart, and Winston, International. New York.

Bangle, Raymond, Jr. 1954. Gomori's Paraldehyde-Fuchsin stain. I. Physio-chemical and staining properties of the dye. J. Histochem. and Cytochem. 2:291-299.

Berril, N.J. 1971. Dev. Biol. McGraw-Hill Book Co. New York. 225-227.

Bonitati, J., W.B. Elliot, and P.G. Miles. 1969. Interference by carbohydrate and other substances in the estimation of protein with the Folin-Ciocalteu reagent. Analytical Biochem. 31:399-404.

Brown, George G. 1973. Effects of sperm concentration, sperm aging and other variables on fertilization in the horse-shoe crab, Limulus polyphemus L. Biol. Bull. 144:462-470.

Carrol, E.J., Jr. and D. Epel. 1975. Elevation and hardening of the fertilization membrane in sea urchin eggs. Exptl. Cell Res. 90:429-432.

Cavanaugh, Gail M. ed. 1956. Formulae and Methods V. of the Marine Biological Laboratory Chemical Room. Woods Hole, Massachusetts. pp. 55-56 and 83-84.

Cheung, T.S. 1966. The development of egg membranes and egg attachment in the shore crab, Carcinus maenas and some related decapods. J. Marine Biol. Ass. United Kingdom. 46:373.

Chou, Shao-Chia and A. Goldstein. 1960. Chromogenic groupings in the Lowry protein determination. Biochem. J. 75:109-115.

Clark, Wallis H., Jr., J.W. Lynn, and A.I. Yudin. 1975. The cortical reaction in the egg of the Penaeid shrimp. International Symposium on Reproductive Physiology of Invertebrates. 1:21.

Clark, W.H., Jr., H. Persyn, and A.I. Yudin. 1974. A microscopic analysis of the cortical specializations and their activation in the egg of the prawn, Penaeus sp. Amer. Zool. 14:1251.

Clark, W.H.,Jr., P. Talbot, R.A. Neal, C.R. Mock, and B.R. Salser. In vitro fertilization with non-motile spermatozoa of the brown shrimp Penaeus aztecus. Marine Biol. 22:353-354.

Costello, Donald P. 1949. The relations of the plasma membrane, vitelline membrane and jelly in the egg of Nereis limbata. J. Gen. Phys. 32:351-366.

Dewel, W.C. and W.H. Clark, Jr. 1974. A fine structural investigation of surface specializations and the cortical reaction in eggs of the Cnidarian Bunodosoma cavernata. J. Cell Biol. 60:78-91.

Dische, Z. 1955. New color reactions for determination of sugars in polysaccharides. In: Methods of Biochemical Analysis, Vol. II, David Glick, ed. Interscience Publications, New York. pp. 313-358.

Dixon, M. and E.C. Webb. 1964. Enzymes. Academic Press Inc. New York. p. 1245.

Duronslett, M.J., A.I. Yudin, R.S. Wheeler, and W.H. Clark, Jr. 1975. Light and fine structural studies of natural and artificially induced egg growth of Penaeid shrimp. Proceedings of the Sixth Annual Workshop, World Mariculture Society, In Press.

Endo, Yoshiyuki. 1952. The role of the cortical granules in the formation of the fertilization membrane in eggs from Japanese sea urchins. Exptl. Cell Res. 3:406-418.

Endo, Y. 1961a. Changes in the cortical layer of sea urchin eggs at fertilization as studied with the electron microscope. Exptl. Cell Res. 25:383-397.

Endo, Y. 1961b. The role of the cortical granules in the formation of the fertilization membrane in the eggs of sea urchins II. Exptl. Cell Res. 25:518-528.

Epel, David. 1975. The program of and mechanisms of fertilization in the echinoderm egg. Amer. Zool. 15:507-522.

Epel, D., A.M. Weaver, A.V. Muchmore, and R.T. Schimke. 1969. β-1,3 glucanase of sea urchin eggs: release from particles at fertilization. Science. 163:294-296.

Epel, D., A.M. Weaver, and D. Mazia. 1970. Methods of removal of the vitelline membrane of sea urchin eggs. I and II. Exptl. Cell Res. 61:64-68.

Fallon, John F. and C.R. Austin. 1967. Fine structure of gametes of Nereis limbata before and after interaction. J. Exptl. Zool. 166:225-242.

Fischer, Martin H. 1903. Artificial parthenogenesis in Nereis. Amer. J. of Physiol. 9:100-109.

Freeman, Sallie B. 1968. A study of the jelly envelopes surrounding the egg of the amphibian Xenopus laevis. Biol. Bull. 135:501-513.

Gridley, M.F. 1960. Manual of Histological and Special Staining Techniques. second ed. McGraw-Hill Book Co., Inc. New York.

Gusseck, D.J. and J.L. Hedrick. 1971. Disulfide bonds in Xenopus laevis jelly coat and a molecular hypothesis of fertilization. Dev. Biol. 25:337-347.

Gwatkin, R.B.L., D.T. Williams, J.F. Hartman, and M. Kniazuk. 1973. The zona reaction of hamster and mouse eggs: production in vitro by a trypsin-like protease from cortical granules. J. Reprod. Fert. 32:259-265.

Hendler, Richard W. 1971. Biological membrane ultrastructure. Membrane Ultrastructure. 51:66-96.

Hill, Frederick L. 1974. A fine structural investigation of oogenesis in the brown shrimp Penaeus aztecus. Master's Thesis. University of Houston. Houston, Texas.

Hinsch, Gertrude W. 1971. Penetration of the oocyte envelopes by spermatozoa in the spider crab. J. Ultrastruc. Res. 35:86-97.

Hotta, K., H. Kamazaki, and M. Kurokawa. 1970a. Isolation and properties of a new type of sialopolysaccharide protein complex from the jelly coat of sea urchin eggs. J. Biol. Chem. 245: 5434-5440.

Hotta, K. adn M. Kurokawa. 1970b. Isolation and identification of two sialic acids from the jelly coat of sea urchins eggs. J. Biol. Chem. 245:6307-6311.

Hudinaga, Motosaku. 1942. Reproduction, development, and rearing of Penaeus japonicus Bate. Jap. J. Zool. 10:305-393.

Humason, G.L. 1967. Animal Tissue Techniques. second edition. W.H. Freeman and Co. San Francisco.

Humphries, A.A., Jr. 1966. Observations of the deposition, structure, and cytochemistry of the jelly envelopes of the egg of the newt, Triturus viridescens. Dev. Biol. 13:214-230.

Humphreys, Walter J. 1967. The fine structure of cortical granules in eggs and gastrulae of Mytilus edulis. J. Ultrastruc. Res. 17: 314-326.

Isaka, S., and M. Akino. 1968. Sperm isoagglutination and sialic acid content of the jelly coat of sea urchin eggs. Exptl. Cell Res. 54:247-249.

Ishihara, K. 1968. An analysis of acid polysaccharides produced at fertilization in sea urchins. Exptl. Cell Res. 51:473-484.

Just, E.E. 1919. The fertilization reaction in Echinarachinus parma. Biol. Bull. 36:1-10.

Kagan, B.M. 1935. The fertilization period of the eggs of Fundulus heteroclitus and some associated phenomena. Biol. Bull. 69:185-201.

Karnovsky, M.J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. 27:137a.

Katagiri, Chiaki. 1973. Chemical analysis of toad egg jelly in relation to its sperm capacitation activity. Dev. Growth and Differ. 15:81-92.

Katsura, S. and A. Tominaga. 1974. Peroxidatic activity of catalase in the cortical granules of sea urchins eggs. Dev. Biol. 40: 292-297.

King, J.E. 1948. A study of the reproductive organs of the common marine shrimp, Penaeus setiferous (Linnaeus). Biol. Bull. 94: 244-262.

Lee, Peter A. 1967. Studies of frog oviducal jelly secretion: chemical analysis of secretory product. J. Exptl. Zool. 166:99-106.

Lillie, Frank R. 1911. Studies of fertilization in Nereis. I. The cortical changes in the egg. II. Partial fertilization. J. of Morph. 22:361-393.

Lillie, F.R. 1916. The history of the fertilization problem. Science. 43:39-53.

Lillie, R.D. 1965. Histopathological Technic and Practical Histochemistry. McGraw-Hill Book Co. New York.

Loeb, J. 1900. Further experiments of artificial parthenogenesis and the nature of the process of fertilization. Amer. J. Physiol. 4:178.

Loeb, J. 1900. On the artificial production of normal larvae from the unfertilized eggs of the sea urchin (Arbacia). Amer. J. Physiol. 3:434.

Loftfield, R.B. 1973. Inhibitors of Amino Acid Activation. in Metabolic Inhibitors, vol. II. Hochster, R.M., M. Kates, and J.H. Quastel, eds. Academic Press. New York.

Longo, Frank J. and E. Anderson. 1970. A cytological study of the relation of the cortical reaction to subsequent events of fertilization in urethane treated eggs of the sea urchin, Arbacia punctulata. J. Cell Biol. 47:646-665.

Longo, F.J. and H. Schuel. 1973. An ultrastructural examination of polyspermy induced by soybean trypsin inhibitor in the sea urchin Arbacia punctulata. Dev. Biol. 34:187-199.

Longo, F.J., H. Schuel, amd W.L. Wilson. 1974. Mechanism of soybean trypsin inhibitor induced polyspermy as determined by an analysis of refertilized sea urchin eggs. Dev. Biol. 41:193-201.

Loranzi, Mabel and J.L. Hedrick. 1973. On the macromolecular composition of the jelly coat from Strongylocentrotus purpuratus eggs. Exptl. Cell Res. 79:417-422.

Lowry, Oliver H. et al. 1951 Protein measurements with the folin phenol reagent. J. Biol. Chem. 193:265-272.

Lynn, John W. and W.H. Clark, Jr. 1975. A Mg^{++} dependent cortical reaction in the egg of Penaeid shrimp. J. Cell Biol. 67:251a.

Mazia, Daniel. 1937. The release of calcium in Arbacia eggs of fertilization. J. Cell. and Comp. Physiol. 10:291-304.

Mehl, J.W. and M.M. Swann. 1961. Acid and base production at fertilization in the sea urchin. Exptl. Cell Res. 22:233-245.

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

Monne, Ludwick and David B. Slautterback. 1950. Differential staining of various polysaccharides in sea urchin eggs. Exptl. Cell Res. 1:477-491.

Moser, F. 1939. Studies on a cortical layer response in the Arbacia egg. II. Response to chemical and physical agents. J. of Exptl. Zool. 80:447-471.

Moser, F. 1939. Studies of a cortical layer response to stimulating agents in the Arbacia egg. J. of Exptl. Zool. 80:423-445.

Novikoff, Alex B. 1939a. Changes at the surface of Nereis limbata eggs after insemination. J. of Exptl. Biol. 16:403-408.

Novikoff, A.B. 1939a. Surface changes in unfertilized and fertilized eggs of Sabellaria vulgaris. J. of Exptl. Zool. 82:217-237.

Pasteels, Jean J. 1965b. Aspects structuraux de la fecondation vus au microscope electronique. Archive Biol. 76:463-509.

Paul, M. 1975. The polyspermy block in eggs of Urechis caupo. Exptl. Cell Res. 90:137-142.

Raven, C.P. 1961. Oogenesis. The Storage of Developmental Information. Pergamon Press. London.

Rehbun, Lionel I. 1962. Electron microscope studies on the vitelline membrane of the surf clam, Spisula solidissima. J. Ultrastruc. Res. 6:107-122.

Runnstrom, John. 1952. The cell surface in relation to fertilization. S.E.B. Symposia. 6:39-88.

Runnstrom, J. and G. Kriszat. 1952. The cortical propagation of the activation impulse in the sea urchin egg. Exptl. Cell Res. 3: 419-426.

Runnstrom, J., L. Monne, and E. Wicklund. 1946. Studies of the surface layers and the formation of the fertilization membrane in sea urchin eggs. J. Colloid Science. 1:421-452.

Runnstrom, John. 1949. The mechanism of fertilization in Metazoa. Adv. in Enzymology. 9:241-327.

Runnstrom, J. and J. Immers. 1956. The role of mucopolysaccharides in the fertilization of the sea urchin egg. Exptl. Cell Res. 10:354-363.

Schroeder, T.E. and D.L. Strickland. 1974. Ionophore A23187, calcium and contractility in frog eggs. Exptl. Cell Res. 83:139-142.

Schuel, H., W.L. Wilson, R.S. Bressler, J.W. Kelly, and J.R. Wilson. 1972. Purification of cortical granules from unfertilized sea urchin egg homogenates by zonal centrifugation. Dev. Biol. 29:307-320.

Schuel, Herbert, W.L. Wilson, J.R. Wilson, and R. Schuel. 1969. Separation of intact cortical granules from homogenate of unfertilized sea urchin eggs by zonal centrifugation. J. Histo. and Cytochem. 17:703-713.

Schuel, H., J.W. Kelly, E.R. Berger, and W.L. Wilson. 1974. Sulfated acid mucopolysaccharides in the cortical granules of eggs. Exptl. Cell Res. 88:24-30.

Schuel, H., W.L. Wilson, K. Chen, and L. Lorand. 1973. A trypsin-like proteinase localized in cortical granules isolated from unfertilized sea urchin eggs by zonal centrifugation. Role of the enzyme in fertilization. Dev. Biol. 34:175-186.

Schuel, H., W.L. Wilson, F.J. Longo, K. Chen, and L. Lorand. 1973. Discharge of secretory granules: enzymatic mechanism. J.C.B. 59:307a.

Spurr, A.R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruc. Res. 26:31-43.

Steinhardt, R.A. and David Epel. 1974. Activation of sea urchin eggs by a calcium ionophore. Proc. Nat. Acad. Sci. 71:1915-1919.

Steinhardt, Richard A., S. Shen, and D. Mazia. 1972. Membrane potential, membrane resistance and an energy requirement for the development of potassium conductance in the fertilization reaction of echinoderm eggs. Exptl. Cell Res. 72:195-203.

Steinhardt, R.A., D. Epel, E.J. Carrol, and R. Yanagimache. 1974. Is calcium ionophore a universal activator for unfertilized eggs? Nature. 252:41-43.

Tegner, M.J. 1974. Protease plays a three part role in the block against polyspermy in sea urchin. Amer. Zool. 13:1316.

Vacquier, V.D. 1975. Calcium activation of esteroproteolytic activity obtained from sea urchin egg cortical granules. Exptl. Cell Res. 90:454-456.

Vacquier, Victor D. 1975. The isolation of intact cortical granules from sea urchin eggs: calcium ions trigger granule discharge. Dev. Biol. 43:62-74.

Vacquier, V.D., and David Epel. 1972a. Sea urchin eggs release protease activity at fertilization. Nature. 237:34-37.

Vacquier, V.D., Mia Tegner, and D. Epel. 1972b. Protease activity establishes the block against polyspermy in sea urchin eggs. Nature. 240:352-353.

Vacquier, V.D., D. Epel, and L.A. Douglas. 1973. Protease released from sea urchin eggs at fertilization alters the vitelline layer and aids in prevernting polyspermy. Exptl. Cell Res. 80:111-119.

Veneable, J.H. and R. Coffeshell. 1965. A simpilfied lead citrate stain for use in electron microscopy. J. Cell Biol. 25:407.

Warren, Leonard. 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. 234:332-338.

Webb, J. Leyden. 1963. Enzyme and Metabolic Inhibitors. vol. I. Academic Press. New York.

Werner, Ivar and Lars Odin. 1952. On the presence of sialic acids in certian glycoproteins and in gangliosides. Acta Societatis Med. Upsal. 57:230-241.

Wilson, E.B. and A.P. Mathews. 1895. Maturation, fertilization, and polarity in the echinoderm egg. New light on the "Quadrille of the Centers". J. Morph. 19:319.

Wilson, E.B. 1892. Lineage of Nereis. J. Morph. 6:361.

Wilson, E.B. 1925. The Cell in Development and Heredity. The MacMillian Co. New York.

Wolf, Don P. 1974. On the contents of the cortical granules from Xenopus laevis. Dev. Biol. 38:14-29.

Worthington Enzyme Manual. 1972 Worthington Biochemical Corp. Freehold, New Jersey.

Yamamoto, Toki-O. 1939a. Changes of the cortical layer of the egg of Oryzias latipes at the time of fertilization. Proc. of the Imp. Acad. 15:269-271.

Yamamoto, T. 1939a. Mechanism of membrane elevation in the eggs of Oryzias latipes at the time of fertilization. Proc. of the Imp. Acad. 15:272-274.

Yamamoto, T. 1954. Physiological studies on fertilization and activation of fish eggs. V. The role of calcium ions in activation of Oryzias eggs. Exptl. Cell Res. 6:56-68.

Yamamoto, T. 1956. The physiology of fertilization in the Medaka (Oryzias latipes). Exptl. Cell Res. 10:387-393.

Yamamoto, T. 1962. Mechanism of breakdown of cortical alveoli during fertilization on the Medaka Oryzias latipes. Embryologia. 7:228-251.