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The Immunological Mechanisms of the Horseshoe Crab, *Limulus polyphemus*

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ABSTRACT—The immunological system of Limulus polyphemus *is composed of a series of protein agglutinins which may be demonstrated to be distinct from one another by various immunochemical methods. The hemolymph agglutinates bacteria in vitro and inhibits bacterial growth. The levels of serum agglutinin vary after the injection of killed bacteria into* L. polyphemus. *The serum protein level falls after bacterial injection, but returns to normal within 12-24 h. The hemocyte hematocrit is directly proportional to the changes in hemolymph protein. Presumably the heteroagglutinin aids in removing bacteria from the hemolymph by preparing them for phagocytosis by hemocytes. Hemocyte lysates, which are endotoxin sensitive and remove endotoxin by gelatin, also agglutinate bacteria and inhibit bacterial growth in culture.*

A third and previously undescribed aspect of the immune system is a glycoprotein exudate produced by hypodermal glands and secreted through canals in the carapace. The exudate serves as a mechanical barrier to pathogens due to its viscosity and has agglutinating properties. The exudate is produced in response to injection of bacterial endotoxin or exposure to fouled seawater. Bacterial cultures are inhibited by the exudate. The exudate, like L. polyphemus *heteroagglutinin, immobilizes bacteria by binding the flagella or by rendering them inactive. RBC's and algae are also readily agglutinated by the exudate. During the secretory phase, large numbers of hemocytes move into the connective tissues under the carapace, around the hypodermal glands, and into the glandular ducts. The heteroagglutinin of the hemolymph, the hemocytes, and the exudate-producing glands provide an effective immunological defense against potential pathogens.*

INTRODUCTION

The anatomy and physiology of the horseshoe crab, *Limulus polyphemus*, has been examined in considerable detail. The attention this animal has received from the scientific community is justified on the basis that it is a very primitive arthropod. It is also the most primitive arthropod whose reactions to pathogens and injury have been studied. It has several immunological capabilities which have allowed it to survive from ancient times to the present. Its immune systems, which have been examined recently, are agglutinating proteins in the hemolymph and bacterial endotoxin reactive substances found in hemocytes. These two systems complement each other and function with a third, previously undescribed, system consisting of hypodermal glands which produce a viscous mucosubstance with agglutinating properties.

Natural humoral hemagglutinins are common among invertebrates and have been reported in most major phyla as discussed by Johnson (1968), Kahan and Reisfeld (1972), and Sparks (1972). The serum heteroagglutinin of *L. polyphemus* has been isolated and characterized by Cohen et al. (1965), Marchalonis (1964), and Marchalonis and Edelman (1968). In addition, Voightmann et al. (1971) and Sprenger and Uhlenbruch (1971) have shown that this heteroagglutinin is directed against N-acetylneuraminic acid receptors, certain carbohydrates, and basic proteins.

A great deal of research on the hemocytes of *L. polyphemus* has been performed (Bang, 1965; Levin and Bang, 1963, 1964, 1965, 1968; Young et al., 1972) which indicates the protein in hemocyte granules is coagulable upon exposure to bacterial endotoxin or to the lysis of hemocytes in wounds. The source of all coagulable protein in clotting reactions is apparently the hemocyte granules. Young et al. (1972) separated lysates prepared from hemocytes into two or three fractions. One fraction had an enzyme-like effect which increased the rate of gelation of a coagulable substrate fraction and could be activated by endotoxin. This reaction is very specific and has been used recently to detect gram-negative infections in humans (Rojas-Corona, 1969; Levin et al., 1970, 1972).

A previously undescribed facet of the immune mechanism of the horseshoe crab involves the carapace and hypodermal glands. The carapace, itself a mechanical barrier against invading pathogens, is penetrated by canals which serve as outlets for hypodermal glands. These canals have been reported by Karlson et al. (1968), but their function was unknown. This paper reports a description and characterization of the glands, the glycoprotein exudate, and their immunological role in *L. polyphemus*.

MATERIALS AND METHODS

Animal Collection and Maintenance

Animals were purchased from the Florida Marine Biological Specimen Company, Panama City, Fla., and maintained in Instant Ocean[1] aquarium systems and artificial sea salts at 15°C.

Bacterial Cultures

Cultures of *Pseudomonas atlantica* and *Vibrio marinopraesens* were purchased from the American Type

[1]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

Culture Collection, Rockville, Md., and were used for all agglutination and bactericidal studies, along with unidentified bacterial species cultured from *L. polyphemus* intestines. All bacteria were gram-negative and were grown on Difco marine agar throughout the study. Live bacteria were washed off slants with sterile seawater and diluted as required, or were killed by boiling for 30 min or exposure to 10 percent formalin in seawater overnight. The dead bacteria were centrifuged at 2,000 × g for 30 min and the pellet resuspended in sterile seawater and recentrifuged. This procedure was repeated three times. The killed bacteria were then stored in serum bottles at 4°C. The supernatant from boiling was retained and used as endotoxin.

Collection of Hemolymph

Hemolymph was collected by withdrawing blood into a precooled syringe fitted with a 20 gauge needle. The hemolymph sample was gently expelled from the syringe into a cold plastic centrifuge tube and centrifuged at 3,000 × g for 15 min at 4°C to remove hemocytes. Protein concentration in the hemolymph was measured with a hand-held refractometer calibrated with bovine serum albumin. The presence of the heteroagglutinin was determined by agglutination of RBC's (red blood cells) or bacteria.

Preparation of Hemocyte Lysates

Hemocyte lysates were prepared by centrifugation of the hemolymph as indicated above. The supernatant hemolymph was decanted and the pellet was washed by agitation in cold sterile seawater and centrifuged as discussed previously. The procedure was repeated three times and the resulting pellet was homogenized in a cold glass tissue grinder. Protein determinations were performed by the Folin-Lowry technique (Lowry et al., 1951) or spectrophotometrically at 280 nm using bovine serum albumin as a standard. Aliquots of lysate incubated with bacteria were observed for the removal of protein concomitant with agglutination of the bacteria.

Hemocyte Hematocrit Determination

Hemocyte numbers were determined by hematocrit. Siliconized capillary tubes were filled with precooled hemolymph, plugged with plasticine, and centrifuged 5 min in an International microcapillary tube centrifuge. In order to determine whether any change in hematocrit followed hemagglutinin change, hematocrits were determined at the same times as hemolymph protein concentrations.

Attempted Change of Hematocrit and Agglutinin Concentration

In order to induce and determine a change in the heteroagglutinin concentration in the hemolymph, animals were injected with 0.5 ml saline containing 5×10^6 heat- or formalin-killed bacteria per milliliter. Blood was drawn at 6 h intervals as previously described. The samples were divided to determine total protein, heteroagglutinin titer, and bactericidal activity. Secondary responses were produced by a bacterial challenge 36 h after the initial injection. To check for enhanced cellular numbers and activity, several horseshoe crabs were pre-immunized as described with 0.5 ml saline containing 2×10^5 bacteria per milliliter and hematocrit changes were followed at 6 h intervals.

Production of Exudate

Animals were injected with small amounts of endotoxin or were placed for several hours in fouled seawater prepared by allowing cod fish slices to spoil. The glycoprotein exudate was collected by pushing it to the edge of the prosoma with a rubber policeman and removing it with a Pasteur pipette.

Chemical and Physical Analysis of the Exudate

The protein moiety of the exudate was determined by absorbance at 280 nm or by the Folin-Lowry method (Lowry et al., 1951). The carbohydrate moiety was determined by the anthrone reaction (Bailey, 1958) or the phenol sulfuric acid reaction (Colowick and Kaplan, 1966). The Morgan-Elson reaction was performed on the exudate for the detection of glucosamine and N-acetylglucosamine (Morgan and Elson, 1934). Purification and fractionation was attempted with Sephadex chomatography using seawater as the eluant. Chemical and physical treatments of stability and function were carried out as outlined by Pauley et al. (1971) for: freezing and thawing, heat lability at 65° and 100°C; pH changes; pipetting; pre-adsorption with bacteria or RBC's; extraction with trichloroacetic acid, phenol, diethyl ether, and ethanol; and incubation with 2-mercaptoethanol. Samples were hydrolyzed in 10 N HCl for 24 h and run on a Beckman amino acid analyzer.

Exudate Agglutination Tests

Bacteria, algae, and human, dog, and rabbit RBC's were used for exudate agglutination. Agglutination tests were performed after each step in the physical and chemical tests outlined above. Protein and carbohydrate concentrations were examined after the addition of heat-killed bacteria to samples of the exudate. The mixture was incubated 4 h at 4°C, centrifuged at 2,000 × g for 30 min to remove bacteria, and then read for protein concentration at 280 nm and carbohydrate concentration by the phenol sulfuric acid reaction. Samples were dialyzed in either distilled water, seawater, or ethylenediaminetetraacetic acid (EDTA) decalcified seawater for 24 h and tested for agglutination with RBC's. Aliquots of tested samples were subsequently used for acrylamide gel electrophoresis. Phase microscopy was used to observe agglutination or phagocytosis of bacteria or RBC's. Hanging drop cultures of acellular hemolymph, cellular hemolymph, lysate, and exudate were prepared using depression slides and plastic or siliconized cover glasses. Bacteria or RBC's were added to a drop of the various samples and mixed by agitation of the slide preparation and examined immediately.

Electrophoresis

Acrylamide gel electrophoresis was performed by the method of either Davis (1964) or Hjerten et al. (1965a,b) using a 5 percent stacking gel and a 7 percent separating gel. Bromphenol blue was used as the tracking dye. Samples of 10-20 µl were layered on the surface of the stacking gel and run 1.5 h at 200 V and 2 A per tube. Samples of hemolymph, lysate, and exudate were run before and after treatment with RBC's or killed bacteria. The gels were stained for protein with amido Schwartz, and for glycoprotein with alcian blue, toluidine blue, and periodic acid Schiff (PAS) by the methods of

Maurer (1971). The molecular weight of the exudate was estimated by using sodium dodecyl sulphate (SDS) acrylamide gel electrophoresis by the method of Segrest and Jackson (1972).

Hypodermal Gland Anatomy and Histochemistry

Pieces of the dorsal surface of prosoma were fixed overnight in 10 percent neutral buffered formalin at 4°C, and then prepared for paraffin embedding. Other tissues were frozen in liquid nitrogen, freeze dried, formalin-vapor fixed, and paraffin embedded for histochemical staining. Some pieces also were embedded in methacrylate after the methods of Leduc and Bernhard (1967) and Rudell (1971) with dehydration in monomer at 4°C. Staining procedures were performed as outlined by Bancroft (1967) and Pearse (1968) for toluidine blue, mucicarmine, PAS, colloidal iron, alcian blue, azure A, low and high iron diamine alcian blue, periodic acidparadiamine, tetrazonium, Bial, Sakaguchi, 8-hydroxyquinoline, Millon reaction dimethylamino benzaldehyde performic acid-alcian blue, ninhydrin Schiff, and mercuric bromphenol blue. Specimens for scanning electron microscopy were frozen in liquid nitrogen, freeze dried, and coated with gold-palladium for observation of external surfaces. Samples were sonicated for cleaning, vacuum dried, and gold-palladium coated for the observation of internal surfaces.

Bactericidal Assays

The hemolymph was assayed for bactericidal qualities by the addition of 0.5 ml saline containing 5×10^3 bacteria per milliliter to various concentrations of serum, which were incubated 90 min at 20°C and then plated on marine agar. The same procedure was followed for samples of hemocyte lysate. Samples of exudate were centrifuged at 2,000 × g for 30 min to remove particulate matter and then placed in sterile culture dish halves and exposed to a germicidal ultraviolet lamp for 15 min. The samples were inoculated with an 18 h culture of *Vibrio marinopraesens* at a concentration of 5×10^3 bacteria per milliliter and incubated 1-4 h at 20°C before plating on marine agar. All plates were read 24 h after plating by comparing colony counts or areas with control plates.

Preparation of Antibody

Antibody was prepared against whole cell lysate, acellular hemolymph, and glycoprotein exudate by injecting rabbits subcutaneously with one of the materials mixed with Freunds complete adjuvant using the method of Clausen (1969). These immune sera were used to test for cross reactivity between the lysate, the hemolymph, and the exudate by using test tube precipitin and ring tests as outlined by Campbell et al. (1963) and Ouchterlony double diffusion tests.

HEMOLYMPH HETEROAGGLUTININ

The heteroagglutinin effectively agglutinates mammalian erythrocytes and certain bacteria as observed by phase microscopy. The material is heat labile and its action is calcium dependent. Heating at 65°C for 15 min destroys its activity as do pH extremes beyond pH 4 and pH 9. Compared to control plates, the heteroagglutinin inhibits bacteria growth as summarized in Table 1, and

Table 1.—Inhibition of bacterial growth by various concentrations of Hemolymph[1].

	Tube number									
	1	2	3	4	5	6	7	8	9	10
Colony number	0	0	0	2	4	2	5	10	30	103
	0	0	2	2	6	12	23	50	108	125
	0	3	4	16	12	14	18	18	26	89
	0	8	0	4	20	23	51	63	80	134
	0	0	0	0	11	18	35	67	93	100

[1]Tube numbers represent a dilution series of 1/10 with tube 10 as a bacterial control. A 0.05 ml/tube containing 5×10^3 bacteria was used throughout the experiment.

was most effective at high concentrations of hemolymph. Observations using phase microscopy suggest that bacterial flagella were bound or inactivated, rendering them nonmotile before death. Endotoxin ("0" antigen) prepared from *V. marinopraesens* also was agglutinated readily. These results indicate that the heteroagglutinin functions both as an agglutinin and as a bactericidin.

The level of heteroagglutinin was changed in the animal by the injection of killed bacteria. The protein concentration varied among animals, but always decreased about 6 h post-injection and rose above normal within 36 h. If the animals were reinjected at this time, the level fell and remained low for 24 h before returning to normal (Fig. 1). A decrease in the agglutination titer followed the decrease in hemolymph protein concentration and is presumably due to

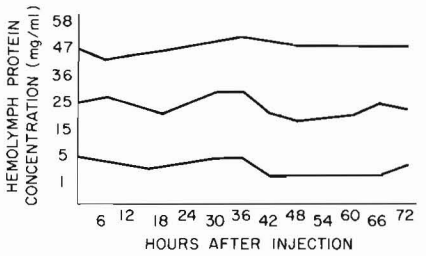


Figure 1.—Changes in hemolymph protein concentration after the injection of heat- or formalin-killed bacteria. The hemolymph protein concentration (mg/ml) differs between animals, as indicated in the three representative animals presented on graph.

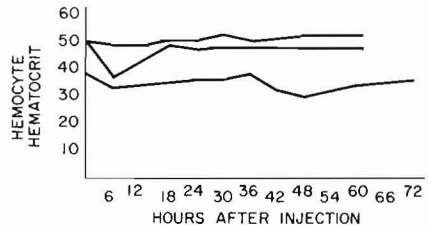


Figure 2.—Changes in circulation hemocyte hematocrits after the injection of heat- or formalin-killed bacteria. Individual variation of three animals is indicated on graph.

the immediate agglutination of injected bacteria which are cleared from the circulating hemolymph during this time. The origin of the heteroagglutinin is unknown.

HEMOCYTES AND COAGULATION

The injection of endotoxin into horseshoe crabs results in the coagulation of hemocytes, gelation of the hemolymph, and death apparently by asphyxiation due to intravascular clotting and paralysis. The animal becomes stiff and tetanic and the hemolymph is difficult to withdraw with an 18 gauge needle and syringe. This occurrence has also been noted by Bang (1956). The injection of killed bacteria causes a drop in circulating hemocytes for 6 h followed by a slow increase in cell numbers to about normal within 12 h (Fig. 2). The change in cell number parallels the change in hemolymph protein concentration. If an animal is bled repetitively 10 ml at a time and becomes stressed, the hematocrit increases markedly and the hemolymph becomes milky. The origin of the hemocytes is unknown, but from histological sections the storage areas appear to be in sinusoids located around muscles, the heart, the hepatopancreas, and gonadal

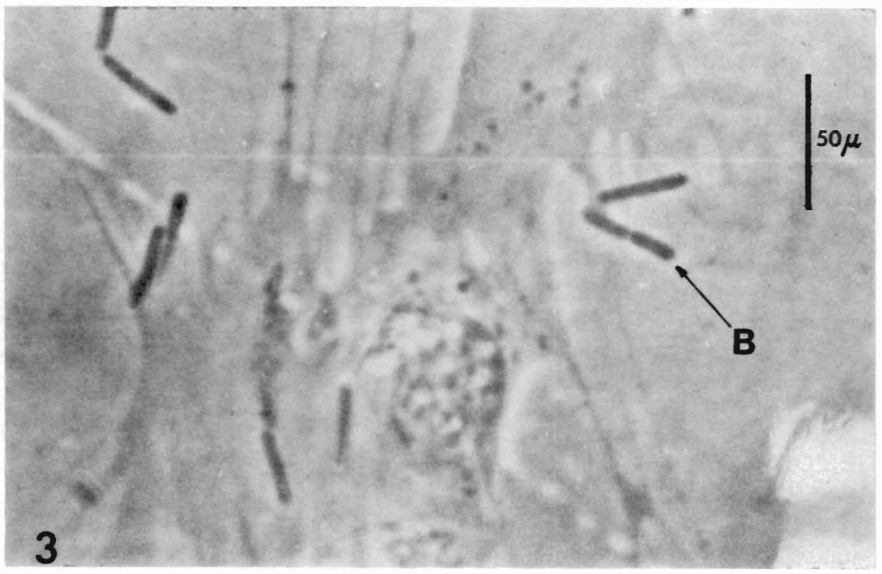


Figure 3.—The attachment of bacteria (B) to *Limulus polyphemus* hemocytes at the beginning of phagocytosis.

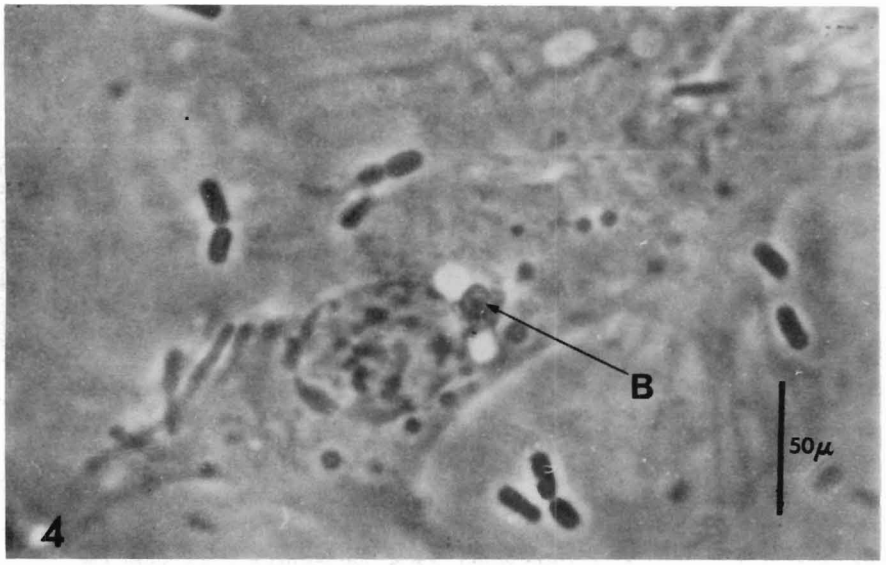


Figure 4.—The phagocytized bacteria (B) are contained within phagocytic vacuoles.

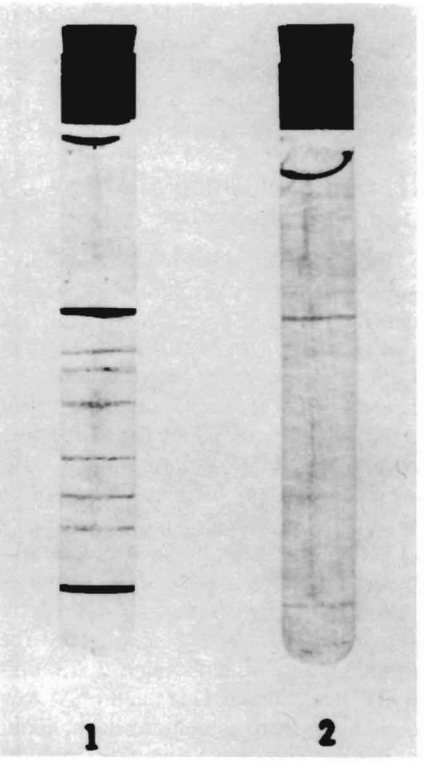


Figure 5.—Acrylamide gel electrophoresis of hemocyte lysate before (tube 1) and after (tube 2) the addition of bacterial endotoxin. Gels were stained with amido Schwartz.

tissues. Therefore, the replenishment of hemocytes is apparently due to their release from storage areas and is not due to an immediate increased production of cells. An injection of live bacteria may be successfully dealt with if the concentration of bacteria is below 2×10^5 per milliliter as reported by Smith (1964), since an LD_{50} represents approximately 10^6 bacteria per milliliter. No long-term enhancement of cell number or activity was found in immunization experiments with killed bacteria.

The hemocytes aggregate in vitro and form a fibrous clot with gel around it, which traps and immobilizes bacteria.

Table 2.—Inhibition of bacterial growth by various concentrations of lysate[1].

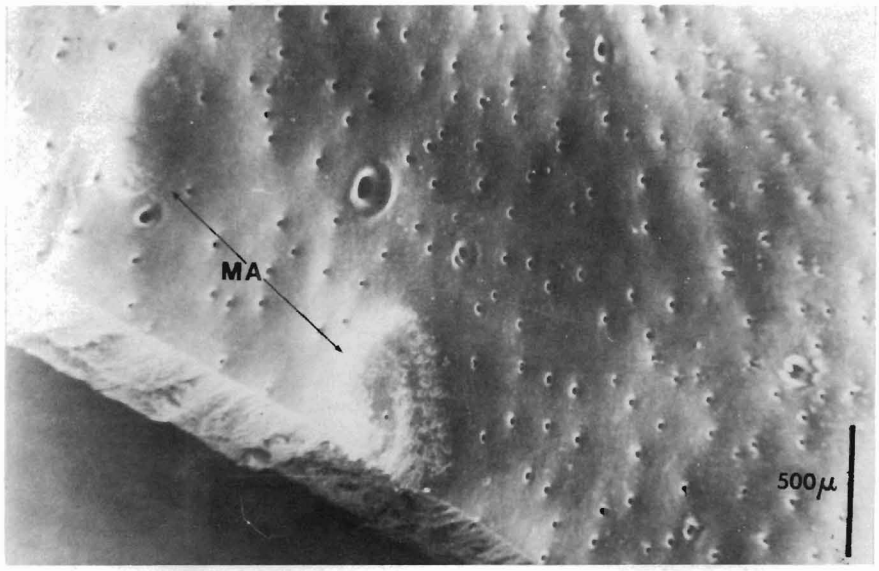
	Tube number									
	1	2	3	4	5	6	7	8	9	10
Colony number	0	0	0	0	0	0	3	55	78	200
	0	0	0	0	43	67	89	97	131	156
	0	0	0	0	0	18	39	53	80	140

[1]Refer to Table 1 for procedure.

This was observed also by Shirodkar et al. (1960) and Bang (1956). In hanging drop cultures of hemocytes, the cells degranulate and some agglutination occurs. Upon addition of bacteria, the agglutination of hemocytes is enhanced with bacteria attached at the periphery of the degranulated cells, which subsequently phagocytize the bacteria (Figs. 3, 4). Shirodkar et al. (1960) found that cells remained granular in culture, but degranulated with the addition of bacteria and became antibacterial. However, no phagocytosis was observed (Levin and Bang, 1968; Sparks, 1972).

The lysate agglutinated bacteria and inhibited bacteria growth (Table 2). Acrylamide gel electrophoresis demonstrated that normal lysate has two major protein bands with some minor staining bands between them. These lighter staining bands may be subunits of the other two darker, more pronounced bands, since column chromatography has shown the presence of only two groups of proteins. This has been corroborated by Young et al. (1972). Incubation with endotoxin or bacteria removes protein from the lysate and decreases the band densities as shown in Figure 5. This diminution of protein is due to its agglutination and precipitation by the endotoxin or bacteria. This reaction, like that of the heteroagglutinin, requires calcium ion and is effective at pH 6-8 with maximal activity at pH 7.5 (Levin and Bang, 1968; Young et al., 1972).

Figure 6.—Scanning electron micrograph of the inner surface of the dorsal prosomium of *Limulus polyphemus*. The indentations are muscle attachments (MA). Numerous canals are apparent.



HYPODERMAL GLAND MUCOPROTEIN

The histology of the carapace demonstrates the secretion pathways of the hypodermal glands through the carapace, with two types of canals penetrating the carapace (Figs. 6, 7). Canals housing gland ducts are $16 \times 22\,\mu$ and the larger canals are $55 \times 77\,\mu$. There are an average of 10-12 gland ducts for every large canal. These large canals are probably associated with sencilla on the dorsal surface of the carapace. Figure 8 shows the hypodermal glands and

Figure 7.—Scanning electron micrograph of the inner surface of the dorsal prosomium. The large canals (LC) are associated with sencillia and the small canals (SC) are associated with gland ducts.

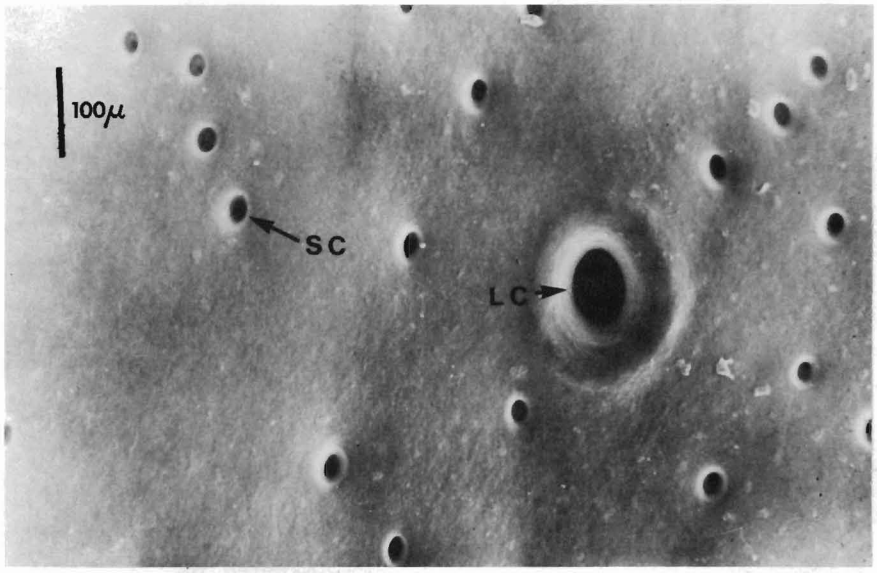
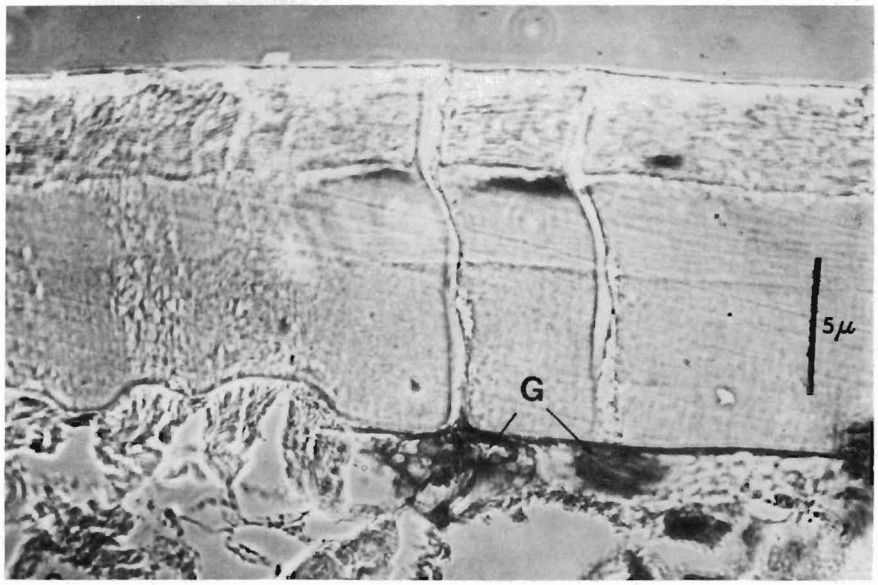


Table 3.—Extraction procedures and inactivation tests to determine the stability and nature of exudate.

Experimental procedure	Complete inactivation of agglutinin	Agglutinin partially inactivated	Agglutinating activity unaffected
Physical treatments:			
Prolonged freezing (4-6 months)			+
Repeated freezing and thawing			+
Heat (65°C)	+		
Heat (100°C)	+		
Acidic pH extremes	+		
Alkaline pH extremes	+		
Dialysis:			
Distilled water	-		
Artificial seawater		+	
EDTA treated seawater	+		
Forceful pipetting			+
Preadsorption with bacteria or RBC	+		
Chemical treatments:			
Trichloracetic acid	+		
Phenol extraction	+		
Diethyl-ether extraction			+
2-mercaptoethanol incubation	+		
Ethanol	+		

Figure 8.—Section through the dorsal prosomium. Glands (G) with their associated ducts are seen to penetrate the carapace through canals.



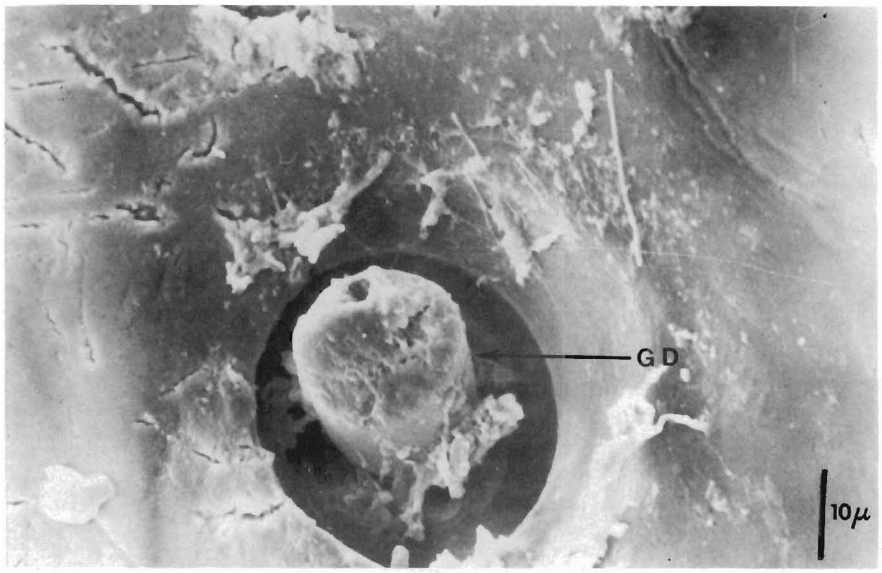


Figure 9.—Scanning electron micrograph of the outer surface of the dorsal surface of the prosomium. A gland duct (GD) is protruding from the outer opening of a canal.

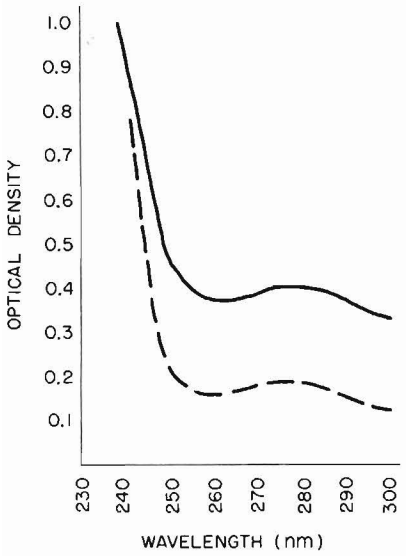


Figure 10.—Spectral absorption curve of exudate before and after the addition of killed bacteria. The solid line is untreated exudate and the broken line is bacteria treated exudate from the same sample demonstrating the adsorption of the protein agglutinin onto the bacteria.

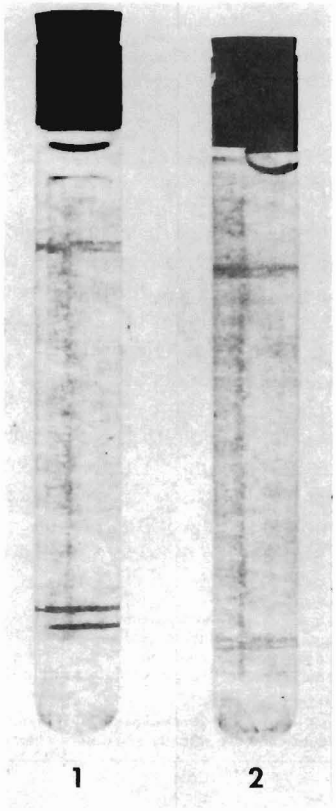


Figure 11.—Acrylamide gel electrophoresis of exudate. Duplicate gels were stained for protein (tube 1) with amido Schwartz or carbohydrate (tube 2) with alcian blue.

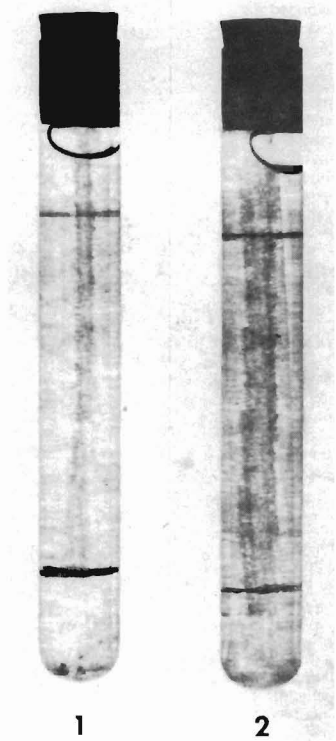


Figure 12.—Acrylamide gel electrophoresis of exudate treated with killed bacteria. Tube 1 was stained with amido Schwartz and tube 2 with alcian blue, indicating bacteria adsorb and remove the agglutinin.

canals penetrating the carapace. The external ending of a duct may be seen in a canal opening (Fig. 9).

After stimulation, the production of viscous and elastic exudate persists for 12-24 h. Its presence may be evidenced by swirling a sample in a flask or stirring with a glass rod which produces spirals of the material in seawater. The exudate has both a protein moiety and a carbohydrate moiety and may contain 0.5-1.0 mg/ml of protein and 300-1,000 μg of carbohydrate per milliliter.

The function of the exudate is apparently twofold. It functions first as a mechanical barrier to pathogens due to its viscosity and secondly as an agglutinin. In vitro it readily agglutinates human, dog, and rabbit RBC's, algae, and both live and heat-killed bacteria. Its agglutinating ability was demonstrated by adding heat-killed bacteria or "O" antigen to samples of exudate and then reading the 280 nm absorbance before and after the incubation step. The bacteria adsorb protein from the hemolymph as shown in Figure 10, where 0.37 mg/ml of protein was adsorbed from an initial concentration of 0.82 mg/ml of protein. Hemolymph carbohydrate also shows a decrease of about 35 percent after treatment with bacteria or "O" antigen.

The material's viscosity may be lost irreversibly with repeated forceful pipetting, heating to 60°C for 15-30 min, and changes of pH beyond 7-7.6. Its activity is destroyed by heating, pH

changes, TCA (trichloroacetic acid), and alcohol treatments, but it retains its viscosity and function after freezing and thawing several times and may be frozen for several months. The material has not been separated effectively by Sephadex chromatography, since it binds to the Sephadex beads. A similar problem has been encountered by Ishiyama and Uhlenbruch (1972) with snail agglutinin, which had to be eluted from Sephadex with 0.5 M glucose. However, this technique was not successful when applied to horseshoe crab exudate. The exudate is dialyzable with distilled water, artificial seawater, and EDTA decalcified seawater. When dialyzed against artificial seawater, the materials retained and passed through the dialysis membrane are both active in agglutination. However, distilled water and EDTA treated artificial seawater effectively inactivate the exudate as tested with RBC's, indicating it is dependent upon calcium ions. A summary of chemical and physical treatments on the exudate is presented in Table 3.

Acrylamide gel electrophoresis of the exudate resulted in the separation of one or two protein bands and two carbohydrate bands (Fig. 11). The farthest migrating protein and carbohydrate bands approximate each other very closely. Incubation of exudate with RBC's or killed bacteria decreases or removes the protein bands and decreases the staining of the carbohydrate bands due to the adsorbance of these materials to the bacterial cell walls or RBC's (Fig. 12). Preliminary work using SDS acrylamide gel electrophoresis for molecular weight estimation suggests a molecular weight for the complex below, 6,000 daltons (one dalton equals the mass of one hydrogen atom). However, due to decreasing binding of SDS with glycoproteins an anomalous decrease in mobility may occur, so that molecular weights less than 10,000 may be only approximated by this method.

Histochemically the hypodermal glands are PAS positive and give metachromatic reactions for acid mucopolysaccharides as discussed by Pearse (1968) and Bancroft (1967). Pearse (1968) assumes this as evidence of sulfated mucosubstances which may contain hyaluronic acid or sialomucins. Tetrazonium reaction for sulfate groups are positive as are performic acid-alcian blue reactions for thiols and disulfides. Bial tests for sialic acid are also positive. Low and high iron diamine alcian blue staining and paradiamine staining also indicate the presence of sulfated mucosubstances with a content of uronic acid or sialomucin. Modified carbazole reactions for uronic acid and the Elson-Morgan reaction for Hexos-Protein stains are also positive. The reactions are summarized in Table 4 and indicate the presence of arginine, tyrosine, tryptophan, amino groups, and perhaps cysteine or thiol groups. Amino acid analysis shows the presence of proline, threonine, and aspartic acid. This indicates the exudate is a glycoprotein containing a small polypeptide and sulfated mucopolysaccharides made up of sialic acid, uronic acid, hexosamine, and perhaps hyaluronic acid.

After incubation in exudate, bacterial growth is inhibited and is most effective

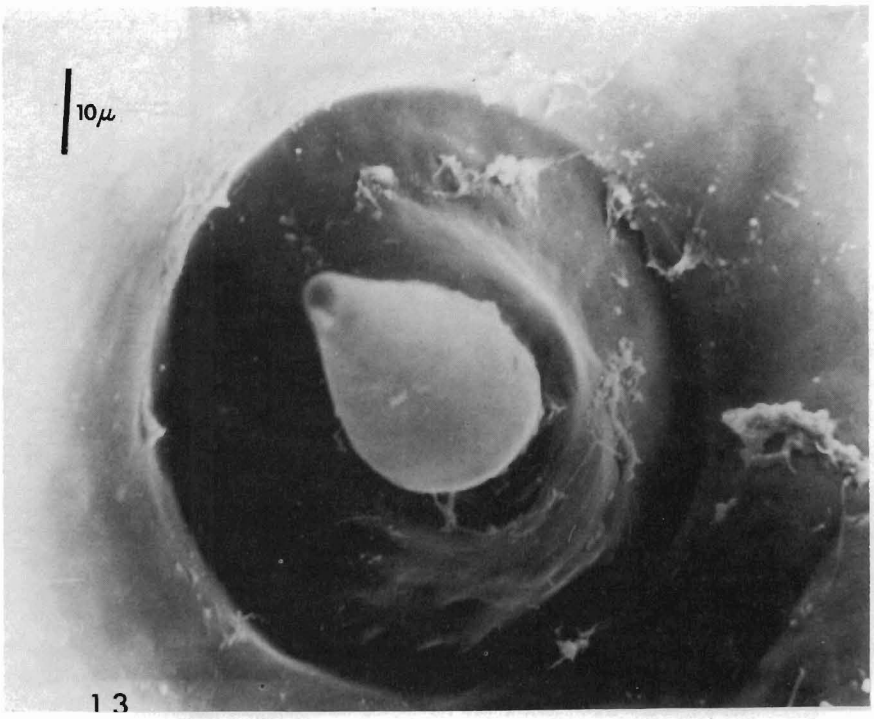


Figure 13.—Scanning electron micrograph of a sencillum on the dorsal surface of the prosomium.

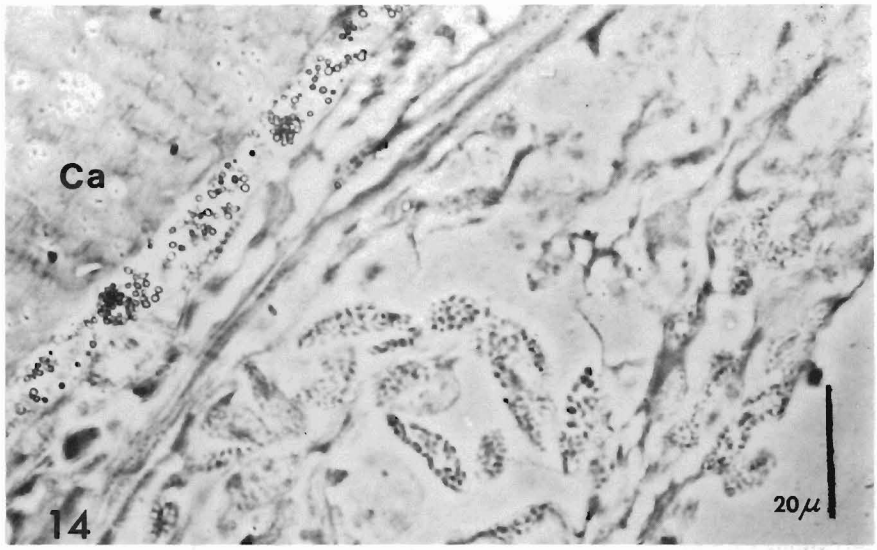


Figure 14.—A section through the dorsal prosomial carapace (Ca) demonstrating the presence of hemocytes in connective tissues.

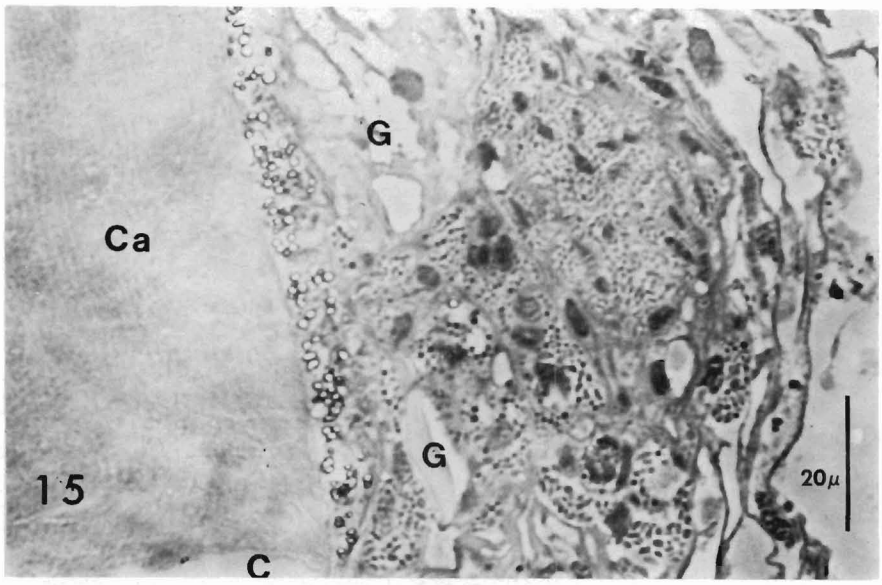


Figure 15.—A section through the dorsal prosomial carapace (Ca) demonstrating the presence of hemocytes in connective tissues and in association with glands (G) and canals (C).

Table 4.—Summary of histochemical staining reactions on hypodermal glands.

Method	Application	Result	Reference[1]
Toluidine Blue	Acid mucopolysaccharides	-	B
Mucicarmine	Acid mucopolysaccharides	+	B
PAS	Carbohydrates	+	B, P
Colloidal iron	Acid mucopolysaccharides	+	B
Alcian blue pH 1.0 and pH 2.5	Sulfated mucopolysaccharides Hyaluronic acid and sialomucins	+	B, P
Azure A pH 0.5 to pH 5.0	Sulfated mucopolysaccharides and sialomucins	+	B, P.
Low iron diamine Alcian blue	Sulfated mucopolysaccharides and sialomucins	+	B, P
High iron diamine Alcian blue	Uronic acid containing muco-polysaccharides and sialomucins	+	B, P
Periodic acid-paradiamine	Periodate reactive and neutral mucopolysaccharides	+	B, P
Tetrazonium	Sulfate esters and sulfonic acid	+	B, P
Bial	Sialic acid	+	B, P
Sakaguchi	Arginine	+	B, P
8-hydroxyquinoline	Arginine	+	P
Million reaction	Tyrosine	+	B, P
D.M.A.B.	Tryptophan	+	B
Performic acid-Alcian blue	Disulfides	+	B, P
Ninhydrin-Schiff	Amino groups	+	B, P
Mercuric-brom-phenol blue	Proteins	+	P

[1]Bancroft, 1967 (B) and Pearse, 1968 (P).

at short incubation times (Table 5). Observations with phase contrast microscopy show that the bacteria were agglutinated in clumps of 10-12 and were nonmotile after exposure to the exudate. These results suggest that the viscosity and the mechanical barrier action of the exudate are due to its carbohydrate moiety as discussed by Hunt (1970) and that its agglutinating property is due to the protein moiety directed against protein-polysaccharide complexes found on bacterial cell walls ("O" antigens) and membrane sites on RBC's. Since the results of the antigen-antibody test of cross-reactivity between heteroagglutinin, lysate, and glycoprotein exudate were negative, it is assumed that there is no serological relationship between these groups of agglutinating molecules although they are similar in action and antigenic sensitivity.

Table 5.—Inhibition of bacterial growth by hypodermal gland exudate[1].

Incubation time in hours	Experimental	Control
1	14.5	55.4
2	21.12	55.4
3	30.45	55.4
4	27.72	55.4

[1]Areas occupied by bacterial colonies were measured by planimetry and averaged for five samples of exudate.

The secretion of a protective glycoprotein by a marine arthropod has not been reported in the literature. This may be an adaptive mechanism of *Limulus polyphemus* and other primitive arthropods which has been lost through evolution or has been overlooked by investigators. Scanning electron microscopy and light microscopy reveal the presence of sencilla on the dorsal side of the carapace (Fig. 13). These structures are 19.2-48 μ in length and 22.8-29.5 μ in width. The presence or function of these structures has not been reported in the literature. However, chemoreceptors on the legs and gnathobases have been described by Hayes (1971). Perhaps these sensory structures are responsible for the detection of contaminants or endotoxin and trigger the secretion of exudate. Slightly before and during the active exudate secretion phase, a large number of hemocytes move into the connective tissues under the carapace, surround the hypodermal glands, and move into the canals of the glands and sencilla (Figs. 14, 15). This provides an effective connecting link among the several protective systems. In fouled or bacteria-contaminated water, exudate is initially produced, but if this and the carapace itself is penetrated, the heteroagglutinin of the hemolymph and the endotoxin sensitive phagocytic hemocytes are positioned to wall off or entrap invading pathogens.

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