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Characterization of a Natural Agglutinin Present in the Hemolymph of the California Sea Hare, *Aplysia californica*[1, 2]

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A substance in the serum of the marine gastropod, *Aplysia californica*, capable of agglutinating marine bacteria and vertebrate red blood cells was subjected to physicochemical analysis in order to ascertain its possible nature. Our studies indicate that agglutinating activity is due to a heterogeneous group of high molecular weight molecules with two activity peaks exhibiting sedimentation coefficients centering ~18.5 S and ~31.0 S. This material has a protein component associated with the active site since it is sensitive to heat, *p*H extremes, and extraction with 2-mercaptoethanol, phenol, chloroform, and trichloracetic acid. Its physicochemical characteristics are different from other known invertebrate agglutinating substances and from classical vertebrate antibody.

INTRODUCTION

Recent reviews indicate that invertebrates possess a variety of humoral substances involved in immunity that differ considerably from classical vertebrate antibodies (Bang, 1967; Tripp, 1969; Sindermann, 1970). Marine invertebrates possess humoral substances capable of agglutinating many foreign substances, including protozoa (Bang, 1962, 1967); bacteria (Blitz, 1965; Cornick and Stewart, 1968a,b); vertebrate red blood cells (Johnson, 1964; Tripp, 1966; Marchalonis and Edelman, 1968); and invertebrate blood cells and spermatozoa (Tyler and Metz, 1945; Tyler, 1946). Only the hemagglutinins of the horseshoe crab, *Limulus polyphemus* (Marchalonis and Edelman, 1968) and the oyster, *Crassostrea virginica* (Li and Flemming, 1967; McDade and Tripp, 1967; Acton et al., 1969) have been characterized in any detail.

Recently McKay et al. (1969) found that serum from *Aplysia* sp. agglutinated vertebrate red blood cells. A study was undertaken to determine whether serum from the California sea hare, *Aplysia californica*, contained bacterial agglutinins and to characterize the physicochemical properties of any agglutinin found. This paper reports the detailed characterization of a natural agglutinin in the serum of *A. californica* capable of reacting with bacteria and vertebrate red blood cells. A small portion of this study has appeared in an earlier communication (Pauley, 1971).

MATERIALS AND METHODS

Animal collection and maintenance. Animals for experimental use were collected intertidally throughout the year at the Irvine Horse Ranch south of the Game Preserve in Corona del Mar, southern California. Animals were maintained in a

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200-gallon salt water aquarium cooled to 17 ± 2°C. This aquarium was supplied with airstones and subsand filters covered by crushed oyster shells to maintain a slightly basic *p*H, and overlaid with washed activated charcoal. Water was added to the aquarium to replace evaporation loss and 25 gallons of water was changed each month. Salinity was monitored with a hydrometer. Animals were fed fresh algae (*Egresia* sp.) twice a month; uneaten plants were immediately removed if they started to decompose. Sea hares thrive extremely well under these conditions for 2–3 months.

Bacterial cultures. All the test bacteria were grown on seawater agar slants (Johnson, 1968). Four of the organisms were obtained from Dr. Phyllis Johnson (Kerckhoff Marine Laboratory, Corona del Mar, California): *Gaffkya homari* (Gh); *Serratia marcescens* (SM); *Micrococcus aquivivus* (628); and a *Pseudomonas* sp. (Fr). These bacteria have been characterized in detail by Johnson (1969). A fifth bacterium used in this study is a chromagenic gram-negative rod (Ap5Y) isolated by the senior author from the foot mucous of *A. californica*. This organism forms smooth round, brilliant yellow colonies on seawater agar plates. Test cultures of all bacteria were grown for 48 hr except for Ap5Y which was grown for only 24–36 hr because it grew more quickly than others. Bacteria used in our tests were washed off the slants with sterile seawater.

Serum preparation. Whole hemolymph was withdrawn from the engorged pedal sinus of *A. californica* using a sterile syringe fitted with a 25-gauge needle after cleaning the pedal surface with 70% ethanol. This procedure caused little observable trauma if animals were held in moist paper towels. Withdrawn fluid was quickly transferred to sterile test tubes placed in an ice bath. Fluid from different sea hares was not pooled except for *p*H activity and molecular weight studies. Cells were removed from the hemolymph by centrifuging the tubes at 2000 rpm/4 min. All centrifugations in this study were carried out at 4°C. The supernatant fluid was drawn off and passed through a Swinnex-25 Millipore filter (0.45 μ pore size) into sterile test tubes. If the filtered fluid was not immediately used, the tubes were frozen-stored at −12 or −18°C. Filter-sterilized hemolymph prepared in the above manner will be referred to as serum. For molecular weight studies, the serum was concentrated by pressure dialysis to remove water and small ions using Amicon Ultrafiltration Cells fitted with a PM 10 Diaflow Ultrafilter having a molecular weight exclusion limit of 10,000 (Amicon Corp).

Agglutination Assay. Test bacteria or red blood cells (RBCs) from chickens and pigs (obtained preserved in Alsevers Solution from Flow Laboratories) were washed three times in either sterile seawater or sterile 0.15 *M* saline. Bacteria were centrifuged at 3600 rpm/3 min and adjusted to a final concentration of 3.0×10^9 cells/ml by visual comparison with a barium sulfate nephelometer. RBCs were centrifuged at 2000 rpm/5 min and adjusted to a 2.0% final concentration. Serial 2-fold dilutions of 1.0 ml sea hare serum were made in Kahn tubes to which was added 0.05 ml of bacteria or RBCs. Dilutions were made with either sterile sea water (bacteria) or saline (RBCs). Agglutination titers were read after 18–24 hr incubation at 26°C or after 36–48 hr incubation at 13°C. We considered agglutination activity to be strong (+++) when all bacteria seemed to be agglutinated into numerous large clumps. In weak reactions (+), not all of the bacteria were agglutinated, although many small clumps were observed. When clumping did occur in control tubes, there were significantly fewer clumps than were found in weak reaction (+) tubes. Control and experimental tubes were always assayed side by side and only those experimental tubes

exhibiting a marked difference were considered positive for agglutinating activity. The titer end point was defined as the highest serum dilution which permitted visible agglutination (+) as determined with a dissecting microscope at ×30 and agglutinating activity was expressed as the reciprocal of the titer dilution. Three controls were used in each of the experimental agglutinin tests: (1) control tubes containing untreated sterile seawater; (2) control tubes containing sterile seawater which received the experimental treatment; and (3) control tubes containing untreated or dialyzed serum. Tests were performed in triplicate on the serum from each of five donors. These tests were repeated twice, usually against two different organisms. Agglutinin specificity was tested by incubating 1.0 ml serum overnight at 26°C with 7×10^9 bacteria and then assaying for agglutinating activity against the same organism, other bacteria strains, or RBCs. To determine whether "O" antigen is involved in the attachment site of the agglutinin, 0.2 ml of purified Difco *E. coli* "O" antigen was employed as a blocking antigen and incubated in a test tube with 0.5 ml of sea hare serum for 24 hr at 26°C.

Physical tests of stability. The effect of lowered temperatures was studied by (1) storing serum at −12°C and testing agglutinin activity after 1, 2, 3, 4, 5, and 6 months, and (2) measuring agglutinin activity of serum after repeated freezing and thawing as described by Krassner and Flory (1970). Heat stability was studied by exposing serum to one of the following temperatures for 20–30 min: 40°, 50°, 60°, 70°, 80°, and 100°C, after which the serum was rapidly cooled by plunging the tube into an ice bath. Any formed precipitates were removed by centrifuging the fluid at 5000 rpm/20 min; the withdrawn supernatant was tested for agglutinating activity. Serum was dialyzed in 8 × 100 Visking tubing (Union Carbide Corporation) for 24–48 hr at 4°C against one of the following solutions: (1) 0.15 M NaCl (*p*H 6.0), (2) sterile seawater, or (3) 0.15 M NaCl, 0.01 M Tris-HCl buffer (*p*H 8.6). This last solution will be abbreviated as THB throughout the paper. Sera from a number of donors showed a *p*H range of 8.4–8.7, with a mean value of 8.6. These sera were then pooled and placed into a number of test tubes and the *p*H was adjusted to various levels by adding either 1 M HCl, 10 M HCl, 1 M NaOH, or 10 M NaOH. After 24 hr incubation at 26°C, precipitates were removed by centrifugation at 5000 rpm/20 min and the supernatant dialyzed 24 hr against three changes of THB (*p*H 8.6) at 4°C.

Chemical tests of stability. Test and control sera were extracted with phenol as described by Kolb and Granger (1968, 1970) with the exception that dialysis was carried out against 0.15 M NaCl (*p*H 6.0). Diethyl-ether extractions were used as one type of control in these tests. Chloroform extractions were carried out as follows: chloroform, glassware, and serum were precooled at 4°C and 3 parts of chloroform were gently mixed with 1 part of serum in a separatory funnel for about 3 min. The chloroform phase containing precipitate was separated from the aqueous (serum) phase and the latter centrifuged at 5000 rpm/3–4 min to remove excess precipitate. The serum phase was then dialyzed for 24 hr at 4°C against THB (*p*H 8.6). Toluene and xylene extractions were carried out in the same manner as chloroform extraction except that 5 parts of toluene or xylene were mixed with 1 part of serum. Trichloroacetic acid (TCA) extraction was performed by incubating 0.5 ml serum in a test tube containing 0.5 ml 20% TCA for 2 hr at 4°C. Formed precipitate was removed by centrifuging at 3000 rpm/20 min, and the supernatant was dialyzed for 24 hr at 4°C against THB (*p*H 8.6). Inactivation with 2-mercaptoethanol was done by precooling the chemical, sea hare serum, and glassware to 4°C. One milliliter of serum was gently mixed with 0.2 ml of 1 M 2-mercaptoethanol and

incubated for 24 hr at 4°C. The mixture was then centrifuged at 3000 rpm/20 min to remove any precipitate and dialyzed 48 hr at 4°C against THB (*p*H 8.6). Sodium citrate chelation was carried out by dialyzing serum for 24 hr against 0.4 M sodium citrate (Acton et al., 1969), and then dialyzing against THB (*p*H 8.6) for 24 hr at 4°C. To determine the effect of urea on agglutinin, an equal volume of serum and 8 M urea were gently mixed and incubated overnight at 26°C. The mixture was then dialyzed for 24 hr at 4°C against THB (*p*H 8.6).

Enzyme sensitivity tests. Five enzymes (Worthington Laboratories) were tested for their effect on agglutinin activity of sea hare serum. These were used at concentrations of 1.0 or 10.0 μg/ml serum. Enzymes were incubated with sera overnight at the *p*H and temperatures indicated: trypsin—*p*H 8.0, 37°C; pronase—*p*H 7.0, 37°C; neuraminidase—*p*H 5.1, 37°C; ribonuclease—*p*H 7.3, 37°C; and deoxyribonuclease—*p*H 7.0, 26°C. Precipitates were removed by centrifuging at 3000 rpm/5 min. As a control, proteolytic enzymes were checked for their activity against hemoglobin at the *p*H and temperatures listed. Hemoglobin was then eluted on Brinkman MN-polygram Cel 300 to 20 × 20 cm precoated thin-layer chromatography (TLC) sheets, using an eluting solvent consisting of pyridine, isoamyl alcohol, and water in the ratio of 45:35:35.

Molecular weight studies. Molecular sieve column chromatography using various Sephadex gels was employed initially in an attempt to approximate the molecular weight of sea hare agglutinin. A slurry of Sephadex G-50 or G-100 was poured into a Siliclad (Clay-Adams) coated glass column, 1.3 cm in diameter, to a final height of 20 cm. Sephadex G-200 slurry was poured into a coated glass column, 1.6 cm in diameter to a final height of 26 cm. In all cases the columns were equilibrated for 24 hr with THB (*p*H 8.6). A sample (0.5 ml or 1.0 ml) of concentrated (10×) *A. californica* serum was loaded on the column and eluted with THB. The flow rate was adjusted to 1 ml/12.5 min. Two-milliliter fractions were collected and tested first for the amount of protein present by absorption at 280 nm in a Gilford 2000 spectrophotometer and then assayed for agglutinating activity. Subsequently, sucrose density gradients (10–40%) using ultracentrifugation were employed to ascertain the molecular weight of the agglutinin. Beckman cellulose nitrate tubes (1″ × 3″) were used to make 26-ml continuous sucrose gradients by diluting 40% sucrose with THB (*p*H 8.6) on a Buchler polystaltic pump. The gradients were stored in a 4°C cold room overnight before use. Concentrated (5×) *A. californica* serum and protein markers were carefully layered on the top of the gradient. Protein markers used were bovine serum albumin (mol. wt. 67,000 or 4.4 S) and human γ-globulin (mol. wt. 150,000 or 7 S). Samples were centrifuged for 6 hr in a Beckman L-2 ultracentrifuge at 22,000 rpm, using a SW 25.1 rotor. By use of the polystaltic pump, 45% sucrose containing phenol red as a marker was layered beneath the sucrose gradient, and 2-ml fractions were collected from the top of the tube as the lighter gradient was moved upward. Each fraction was tested for protein by absorption at 280 nm in a Gilford 2000 spectrophotometer, dialyzed 24 hr against THB (*p*H 8.6) at 4°C to remove the sucrose, and then assayed for agglutinating activity.

Results

Agglutination Titers

Normal serum agglutination titers of *Aplysia californica* varied between individuals although we never saw more than a 2- to 4-fold difference in activity (Table 1). All four marine bacteria tested were agglutinated, but *Serratia marcescens*, a terrestrial pathogen of insects, was not agglutinated. The absence of agglutination by sea hare serum in this case may be due to the fact that

the capsule of *S. marcescens* masks bacterial cell wall antigens (Nowotny, 1969). Normal sea hare serum agglutinated both chicken and pig RBCs with very strong activity against chicken cells. A prozone sometimes noted in the most concentrated serum tubes, was especially frequent when assay was made using the gram-negative rod Ap5Y. The results of the cross agglutination tests show that any of the marine bacteria are capable of completely absorbing out of normal sea hare serum the agglutinin against itself, the other marine bacteria, and the two RBCs, indicating the agglutinin is nonspecific (Table 2). However, *S. marcescens* is incapable of absorbing the agglutinin out of the serum, which is not surprising since it is not agglutinated by normal serum. Purified *E. coli* "O" antigen acts as a blocking agent which partially inactivates the agglutinin (Table 2). The hemagglutinins in the snail *Viviparus malleatus* (Cheng and Sanders, 1962) and the oyster *Crassostrea virginica* (Tripp, 1966) have been found to have agglutinating titers directly proportional to the total serum protein concentration. Therefore, we analyzed the total protein concentration in the serum of several *A. californica* by the Folin-Ciocalteau method described by Lowry et al. (1951). Normal serum protein concentration in the sea hares was found to vary among individual animals and may be inversely proportional to the agglutination titer (Table 3).

TABLE 1

NORMAL RANGE OF AGGLUTINATION TITERS AGAINST BACTERIA AND RBCs FOUND OCCURRING NATURALLY IN THE SERUM OF *Aplysia californica*[a]

Test cells	Normal titer range
Pseudomonas sp. (Fr)	32–64
Gaffkya homari (Gh)	16–32
Chromagenic gram-negative rod (Ap5Y)	16–32
Micrococcus aquivivus (628)	4–8
Serratia marcescens (SM)	0
Chicken RBC	128–512
Pig RBC	32–64
Sterile seawater controls	0

[a] It is noted that on occasion bacteria and chicken RBCs clumped in the saline control tubes. In these cases, control and experimental tubes were assayed side by side and only those experimental tubes exhibiting a marked difference were considered positive for agglutinating activity.

TABLE 2

EFFECT OF CROSS ABSORPTION ON AGGLUTINATING ACTIVITY OF NORMAL *Aplysia californica* SERUM[a]

Absorbing material	Cells used to assay for agglutinin after absorption					
	M. aquivivus (628)	*Pseudomonas* sp. (Fr)	Chromagenic gram-negative rod (Ap5Y)	*Gaffkya homari* (Gh)	Chicken RBC	Pig RBC
M. aquivivus (628)	–	–	–	–	–	–
Pseudomonas sp. (Fr)	–	–	–	–	–	–
Chromogenic gram negative rod (Ap5Y)	–	–	–	–	–	–
S. marcescens (SM)	+	+	+	+	+	+
E. coli "O" antigen	±	NP	±	NP	NP	NP

[a] Results indicate that the agglutinin is nonspecific. + = agglutinin not absorbed out of serum; ± = agglutinin partially absorbed out of serum; – = agglutinating activity completely absorbed out of serum; NP = test not performed.

Physical Stability

Sea hare agglutinin is active over a wide range of *p*H values between 5 and 10 and was reduced but never completely inactivated at extreme acid or alkaline values (Fig. 1). Reduction of titer was most pronounced below *p*H 5. The naturally occurring agglutinin in *A. californica* serum is stable to dialysis, storage at low temperatures, and repeated freezing and thawing (Table 4). Sea hare agglutinin is stable after incubation at 60°C/30 min but is completely inactivated after incubation at 70°C/20 min (Table 4).

TABLE 3

PROTEIN CONTENT AND AGGLUTINATION TITER OF NORMAL SERUM FROM INDIVIDUAL *Aplysia californica*[a]

Animal No.	Milligrams protein/ml serum	Agglutinin titer[b]
1	1.950	32
2	1.325	32
3	2.910	16
4	1.685	16
5	2.275	16
6	1.510	32
7	1.085	32
8	1.280	32

[a] Duplicate analyses were performed on the serum of each animal and averages are listed below.

[b] Test bacteria was chromagenic gram-negative rod (Ap5Y).

Chemical Stability

A number of experiments were run to determine the stability and nature of the bacterial agglutinating substance present in the serum of *A. californica*. Results of these tests are listed in Table 4. Chloroform, TCA, and phenol were the only treatments that completely inactivated the agglutinin. Incubation with 2-mercaptoethanol partially inactivated the agglutinin.

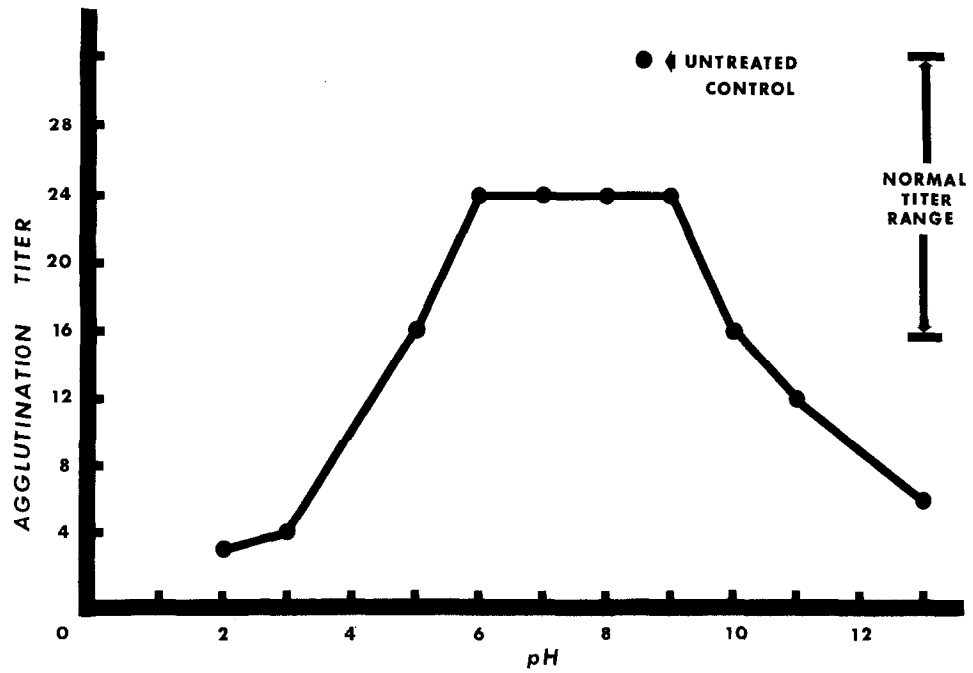

FIG. 1. *p*H stability of *Aplysia californica* serum agglutinin. Activity was determined against the chromagenic gram-negative rod Ap5Y. Sera from several donors was pooled, and the pH was adjusted by adding HCl or NaOH. Treatment of sera with HCl or NaOH reduces agglutinating activity although the reduced activity between *p*H 5–10 falls within the normal range. Tests were run in duplicate, assayed against the gram-negative rod Ap5Y, and the averaged duplicate titer values are reported.

Molecular Weight Studies

The stability of the agglutinin to dialysis indicated a macromolecule was involved, and subsequent tests using molecular sieve column chromatography and sucrose gradient separations were employed in an attempt to approximate the size of the agglutinin molecule. Sephadex separation indicates that the molecular weight is probably greater than 150,000 assuming it is a globular protein. The elution pattern on Sephadex G-50, G-100, and G-200 is the same as blue dextran which has a molecular weight of 2×10^6. An example of the separation pattern in a G-200 column is shown in Fig. 2 where the agglutinin appears in the single major protein fraction which is eluted with the blue dextran. After ultracentrifugation of *A. californica* serum in sucrose gradients, agglutination activity was found in all fractions containing sea hare protein (Fig. 3). Additionally, a secondary peak was observed in fractions 13 and 14 which contained some protein detectable by optical density measurements (Fig. 3). The major agglutination activity appears to be a heterogeneous group of molecules with a sedimentation coefficient centering around 18.5 S, while the minor peak has a sedimentation coefficient of approximately 31 S. These observations indicate that the agglutinin present in sea hare serum is a very large molecular weight material. The sedimentation coefficients of the two agglutinin peaks may be subject to some error, since we made the estimate based on the ultracentrifugation pattern of only two protein markers. However, the sucrose density ultracentrifugation studies do corroborate earlier findings that the agglutinin has a molecular weight greater than 150,000 as indicated by its elution pattern on a Sephadex G-200 column.

TABLE 4

EXTRACTION PROCEDURES AND INACTIVATION TESTS TO DETERMINE THE STABILITY AND NATURE OF THE NATURAL AGGLUTININ IN *Aplysia californica* SERUM

Experimental procedure	Complete inactivation of agglutinin	Agglutinin partially inactivated	Agglutinating activity unaffected
Physical treatments			
Prolonged freezing (6 months)			+
Repeated freezing and thawing			+
Heat (60°C)			+
Heat (70°C)	+		
Alkaline pH extremes		+	
Acidic pH extremes		+	
Dialysis			+
Chemical treatments			
Trichloroacetic acid extraction	+		
Phenol extraction	+		
Diethyl-ether extraction			+
Chloroform extraction	+		
Toluene extraction			+
Xylene extraction			+
2-Mercaptoethanol incubation		+	
Sodium citrate chelation			+
Urea incubation			+
Enzyme treatments			
Trypsin digestion			+
Pronase digestion			+
Neuraminidase digestion			+
Ribonuclease digestion			+
Deoxyribonuclease digestion			+

DISCUSSION

The insusceptibility of *Aplysia californica* agglutinin to RNase, DNase, and neuraminidase, in conjunction with its sensitivity to phenol and TCA extraction, suggest that its biological activity is not dependent upon nucleic acids or polysaccharides. Its sensitivity to TCA, phenol, and chloroform extraction indicate that the activity may be due to a protein or lipoprotein. Although lipoproteins are dissolved in phenol (Nowotny, 1969), a lipid component in the agglutinin

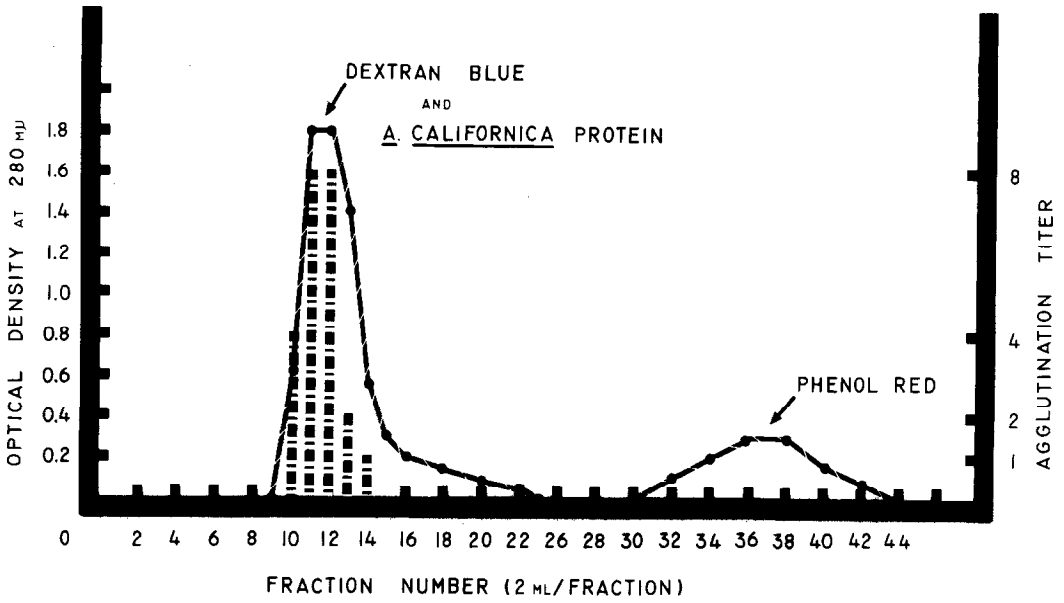

FIG. 2. Sephadex column chromatography of *A. californica* serum. Concentrated serum was separated by Sephadex G-200 column chromatography as outlined in the text. Collected fractions were assayed for agglutinating activity against *M. aquivivus* (628). Line graph shows the optical density of each fraction as measured at 280 nm. Agglutinating activity is indicated by bar graphs. Agglutinating titer of concentrated control serum not subjected to column chromatography was 32. Blue dextran was eluted in tubes 11 and 12, while phenol red was eluted in tubes 34–38.

was ruled out on the basis of several other tests. Toluene and xylene will inactivate lipids (Krassner and Flory, 1970), and most lipoproteins are easily denatured by diethyl ether at temperatures as low as 0°C (Scanu, 1965), freezing, or repeated freezing and thawing (Hatch and Lees, 1968). However, *A. californica* agglutinin is not inactivated by any of these lipid and lipoprotein tests. Current evidence, therefore, indicates that the agglutinin is protein or contains a major protein component. This assumption is based on the agglutinin's susceptibility to heat, *p*H extremes, and to extractions with 2-mercaptoethanol, chloroform, phenol, and TCA. Protein is precipitated by chloroform (unpublished data), phenol (Palmer and Gerlough, 1940; Nowotny, 1969), and TCA (MacInnis and Voge, 1970). The inactivation of *A. californica* agglutinin by heat and extremes of *p*H is characteristic of proteins (Fox and Foster, 1957; Florkin and Stotz, 1963). However, it should be pointed out that some proteins, such as mouse lymphotoxin (LT), are extremely tolerant to extremes of both heat and *p*H (Kolb and Granger, 1970). Cleavage of cystine disulfide bonds in proteins is accomplished with 2-mercaptoethanol (Fougereau and Edelman, 1965). The lack of sensitivity of the agglutinin to proteolytic enzymes indicates that either the enzymes were not active or they do not affect the active site. The first of these possibilities can be eliminated because the enzymes were active as determined by their effect on hemoglobin eluted on TLC sheets. Kolb and Granger (1968, 1970) observed that although both human and mouse LT were protein as determined by their bouyant densities, they were resistant to trypsin digestion. This may be due to the fact that trypsin acts upon specific sites which are not exposed or are not present in LT molecules. Pronase, on the other hand, has no specific site of action and if the agglutinin is proteinaceous it should therefore be

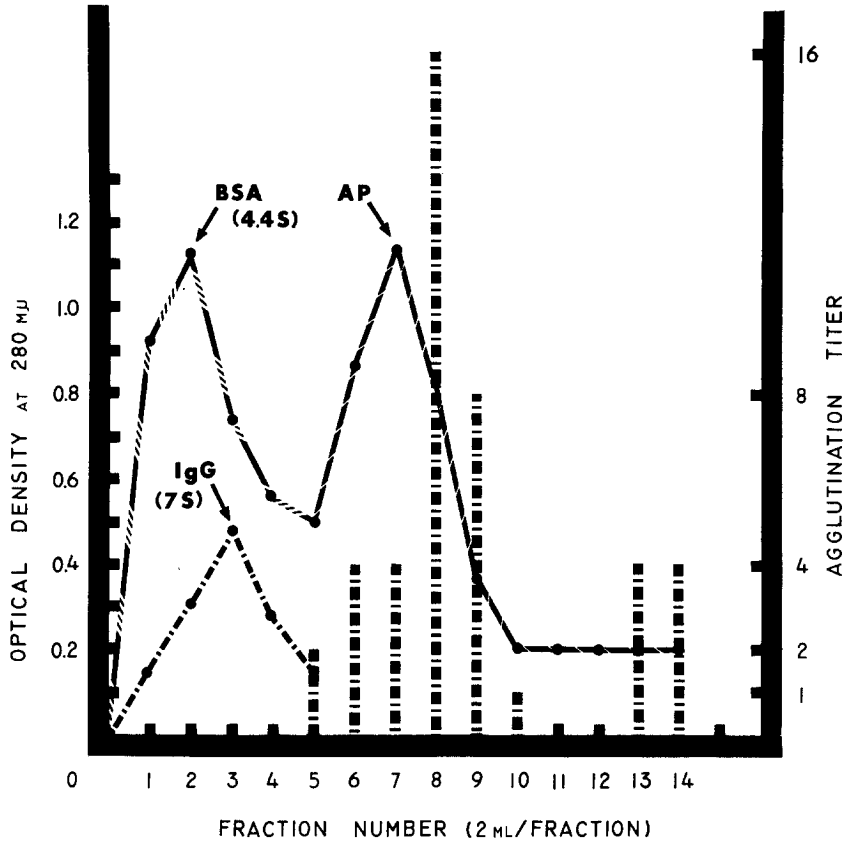

FIG. 3. Ultracentrifugation profile of concentrated *A. californica* serum (AP) was subjected to a 10–40% sucrose gradient as outlined in the text. Collected fractions were assayed for agglutinating activity against *Pseudomonas* sp. (FR). Line graph shows the optical density of each fraction as measured at 280 nm. Agglutinating activity is indicated by bar graphs. Concentrated control serum not subjected to ultracentrifugation had an agglutination titer of 128. Bovine serum albumin (BSA) was found to have a maximum optical density in tube 2, while the maximum optical density of human γ-globulin (IgG) was in tube 3.

inactivated by this enzyme. The apparent insensitivity of the agglutinin to pronase is unknown, but there are three possible explanations for this. It may be that pronase affects the *A. californica* agglutinin nonspecifically only in a specific region of the molecule, as does pepsin, which degrades only the Fc fragment of vertebrate antibody into small peptides (Fougereau and Edelman, 1965). Since the agglutinin molecule is extremely large, it may be present as a complex that is not easily degraded by pronase. It is possible that pronase can cleave the agglutinin into subunits, but is not capable of acting on the subunits due to the formation of a protective complex (e.g., with a polysaccharide that protects the active site but is not itself involved in the biological reaction of agglutination). Sea hare agglutinin was not affected by urea, a compound which causes dissociation of hydrogen bonds in proteins and will often separate a protein into component subunits, as occurs in horseshoe crab hemagglutinin (Marchalonis and Edelman, 1968). Sodium citrate had no effect on the agglutinin, indicating that it is not dependent upon bivalent cations for stabilization. The presence of disulfide bonds in vertebrate antibody may be detected by the use of 2-mercaptoethanol (Fougereau and Edelman,

1965), and the partial inactivation of sea hare agglutinin by this chemical indicates that disulfide bonds are present. Incomplete inactivation with this chemical may be because alkylation with iodoacetamide was not performed, thereby permitting the disulfide bonds to recombine under the alkaline conditions of the experiment to yield some agglutinin-active sites. The possibility also exists that the molecule is coiled in such a way as to prevent complete unwinding upon breaking of the disulfide bonds, thereby partially preserving the integrity of the active site.

The partial inactivation of the agglutinin by *E. coli* "O" antigen indicates that the attachment site of the agglutinin is, at least in part, a polysaccharide similar in structure to "O" antigen. This in part helps to explain the inability of sea hare serum to agglutinate *S. marcescens*, since the "O" antigens are protected by a capsule in this bacterium (Nowotny, 1969).

Of the few invertebrate agglutinins that have been characterized in detail, oyster hemagglutinin appears to be most similar to sea hare agglutinin. *C. virginica* hemagglutinin is unaffected by dialysis, although aging renders it dialyzable; it exhibits cross reactivity; it is heat labile; and it is a protein with an extremely large sedimentation coefficient of 33.4 S (Tripp, 1966; Li and Flemming, 1967; Acton et al., 1969). Oyster hemagglutinin differs from sea hare agglutinin in that it is stable over a narrower *p*H range (*p*H 6–9) and breaks down into subunits beyond *p*H 7–8 (Li and Flemming, 1967; Acton et al., 1969). However, the reduced titer of sea hare agglutinin at *p*H extremes may indeed indicate dissociation of the molecule into subunits. Another difference between the two agglutinins is that oyster hemagglutination activity is proportional to serum protein levels (Tripp, 1966), whereas an inverse correlation is found between sea hare agglutinin and serum protein concentration. However, more data are needed before any definitive statement can be made. Li and Flemming (1967) found oyster hemagglutinin activity associated with two distinct protein peaks after separation by Sephadex G-75 columns, indicating a molecular weight less than 75,000. This is in fair agreement with McDade and Tripp (1967), who estimated a molecular weight above 65,000. These authors were probably working with the subunits rather than the intact molecule because Acton et al. (1969) has since shown that oyster hemagglutinin is composed of noncovalently linked subunits, with a molecular weight of 20,000, and that the entire molecule with all its subunits intact has a very high sedimentation coefficient of 33.4 S. This is fairly close to the minor 31 S activity peak of sea hare agglutinin. Two studies have shown that oyster hemagglutinin is stabilized by calcium ions (McDade and Tripp, 1967; Acton et al., 1969), but this is not true for *A. californica* agglutinin since it was not inactivated by sodium citrate.

A hemagglutinin present in the mussel *Velesunio ambiguus* has recently been purified and characterized (Jenkin and Rowley, 1970). The material was precipitated by 50% saturated ammonium sulfate, and subsequently purified by sucrose density gradient centrifugation. Its sedimentation coefficient of 28 S is similar to the minor 31 S activity peak of sea hare serum. The mussel hemagglutinin was capable of being cross absorbed with different vertebrate RBCs, as was sea hare agglutinin. Purified mussel hemagglutinin was found to be protein and subsequent amino acid analysis revealed no sulfur-containing amino acids, differing from sea hare agglutinin which has disulfide bonds.

Horseshoe crab (*Limulus polyphemus*) hemagglutinin has been studied extensively by Marchalonis and Edelman (1968), who estimated a molecular weight of about 400,000 for this molecule, which like oyster hemagglutinin could be separated into subunits of 22,500 molecular weight by exposure to *p*H 3.0 or 9.6 followed by treatment with

8.0 M urea. They also showed that horseshoe crab hemagglutinin was stabilized by calcium ions and did not have covalent bonds, such as disulfide bonds, between the subunits. Cornick and Stewart (1968a) found that the bacterial agglutinin in another arthropod (*Homarus americanus*) was unaffected by dialysis and could be absorbed out of the serum by preincubation with various bacteria, which is what we found with sea hare serum. All the marine invertebrate agglutinins are heat labile (Cornick and Stewart, 1968a; Jenkin and Rowley, 1970; Marchalonis and Edelman, 1968; Tripp, 1966).

When compared with vertebrate immunoglobulin (see Fougereau and Edelman, 1965), sea hare agglutinin shows some obvious differences in molecular weight, stability to urea, and *p*H stability. In addition, the titer of *A. californica* agglutinin cannot be increased by prior bacterial challenge (Pauley, 1971) as would occur with vertebrate immunoglobulin. Current knowledge indicates that all invertebrate agglutinating substances are very different from the highly specific, inducible agglutinating antibodies that vertebrates possess.

Agglutinins in certain marine invertebrates appear to play an important role in the defense mechanisms of these animals against potential pathogens. Bang (1962, 1967) found that spider crabs lacking the agglutinin against the marine ciliate *Anophyrs sarcophaga* were killed by this protozoan. Cornick and Stewart (1968b) suggested that the lack of a serum agglutinin in the lobster against *Gaffkya homari* may be the reason why this bacterium causes a pernicious disease in *Homarus americanus*, while bacteria susceptible to agglutination are not pathogenic in these crustaceans. This protection may be maintained by the ability of these molecules to act as opsonins as in the case of oyster hemagglutinin (Tripp and Kent, 1967). A selective protection as in the case of the lobsters appears to be the function of the sea hare agglutinin and this will be discussed in detail in a subsequent paper concerning in vivo bacterial clearance in these gastropods.

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References

Acton, R. T., Bennett, J. C., Evans, E. E., and Schrohenloher, R. E. 1969. Physical and chemical characterization of an oyster hemagglutinin. *J. Biol. Chem.*, **244**, 4128–4135.

Bang, F. B. 1962. Serological aspects of immunity in invertebrates. *Nature (London)*, **196**, 88–89.

Bang, F. B. 1967. Serological responses among invertebrates other than insects. *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **26**, 1680–1684.

Blitz, R. R. 1965. The clearance of foreign material from the coelom of *Phascalosoma agassizii*. Ph.D. Thesis, University of California, Berkeley. 177 pp.

Cheng, T. C., and Sanders, B. G. 1962. Internal defense mechanisms in mollusks and electrophoretic analysis of a naturally occurring serum hemagglutinin in *Viviparus malleatus* Reeve. *Proc. Pa. Acad. Sci.*, **36**, 72–83.

Cornick, J. W., and Stewart, J. E. 1968a. Interation of the pathogen *Gaffkya homari* with natural defense mechanisms of *Homarus americanus*. *J. Fish Res. Bd. Can.*, **25**, 695–709.

Cornick, J. W., and Stewart, J. E. 1968b. Pathogenicity of *Gaffkya homari* for the crab *Cancer irroratus*. *J. Fish. Res. Bd. Can.*, **25**, 795–799.

Florkin, M., and Stotz, E. H. 1963. "Comprehensive Biochemistry, Proteins," Part 1, 280 pp. Elsevier, Amsterdam.

Fougereau, M., and Edelman, G. M. 1965. Corroboration of recent models of the γ G

immunoglobulin molecule. *J. Exp. Med.*, **121**, 373–393.

FOX, S. W., AND FOSTER, J. F. 1957. "Introduction to Protein Chemistry," 459 pp. Wiley, New York.

HATCH, F. T., AND LEES, R. S. 1968. Practical methods for plasma lipoprotein analysis. *Advan. Lipid Res.*, **6**, 1–68.

JENKIN, C. R., AND ROWLEY, D. 1970. Immunity in invertebrates. The purification of a haemagglutinin to rat and rabbit erythrocytes from the haemolymph of the murray mussel (*Velesunio ambiguus*). *Aust. J. Exp. Biol. Med. Sci.*, **48**, 129–137.

JOHNSON, H. M. 1964. Human blood group A_1 specific agglutinin of the butter clam *Saxidomus giganteous*. *Science*, **146**, 548–549.

JOHNSON, P. T. 1968. A new medium for maintenance of marine bacteria. *J. Invertebr. Pathol.*, **11**, 144.

JOHNSON, P. T. 1969. The coelomic elements of sea urchins (*Strongylocentrotus*) III. In vitro reaction to bacteria. *J. Invertebr. Pathol.*, **13**, 42–62.

KOLB, W. P., AND GRANGER, G. A. 1968. Lymphocyte in vitro cytotoxicity: characterization of human lymphotoxin. *Proc. Nat. Acad. Sci. U. S.*, **61**, 1250–1255.

KOLB, W. P., AND GRANGER G. A. 1970. Lymphocyte in vitro cytotoxicity: characterization of mouse lymphotoxin. *Cell. Immunol.*, **1**, 122–132.

KRASSNER, S. M., AND FLORY, B. 1970. Antibacterial factors in the sipunculid worms *Goldfingia gouldii* and *Dendrostomum pyroides*. *J. Invertebr. Pathol.*, **16**, 331–338.

LI, M. F., AND FLEMMING, C. 1967. Hemagglutinins from oyster hemolymph. *Can. J. Zool.*, **45**, 1225–1234.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MCDADE, J. E., AND TRIPP, M. R. 1967. Mechanism of agglutination of red blood cells by oyster hemolymph. *J. Invertebr. Pathol.*, **9**, 523–530.

MACINNIS, A. J., AND VOGE, M. 1970. "Experiments and Techniques in Parasitology," 232 pp. Freeman, San Francisco, California.

MCKAY, D., JENKIN, C. R., AND ROWLEY, D. 1969. Immunity in the invertebrates. I. Studies on the naturally occurring haemagglutinins in the fluid from invertebrates. *Aust. J. Exp. Biol. Med. Sci.*, **47**, 125–134.

MARCHALONIS, J. J., AND EDELMAN, G. M. 1968. Isolation and characterization of a hemagglutinin from *Limulus polyphemus*. *J. Mol. Biol.*, **32**, 453–465.

NOWOTNY, A. 1969. "Basic Exercises in Immunochemistry," 197 pp. Springer Publ. New York.

PALMER, J. W., AND GERLOUGH, T. D. 1940. A simple method for preparing antigenic substances from the typhoid bacillus. *Science*, **92**, 155–156.

PAULEY, G. B. 1971. Bacterial clearance in the marine gastropod mollusk, *Aplysia californica* (Cooper). *Proc. Nat. Shellfish. Ass.*, **61**, 11–12.

SCANU, A. M. 1965. Factors affecting lipoprotein metabolism. *Advan. Lipid Res.*, **3**, 63–138.

SINDERMANN, C. J. 1970. "Principal Diseases of Marine Fish and Shellfish," 369 pp. Academic Press, New York.

TRIPP, M. R. 1966. Hemagglutinin in the blood of the oyster *Crassostrea virginica*. *J. Invertebr. Pathol.*, **8**, 478–484.

TRIPP, M. R. 1969. General mechanisms and principles of invertebrate immunity. *In* "Immunity to Parasitic Animals" (Jackson, Herman, and Singer, eds.), Vol. 1, pp. 111–128. Appleton, New York.

TRIPP, M. R., AND KENT, V. E. 1967. Studies on oyster cellular immunity. *In Vitro*, **3**, 129–135.

TYLER, A. 1946. Natural heteroagglutinins in the body fluids and seminal fluids of various invertebrates. *Biol. Bull.*, **90**, 213–219.

TYLER, A., AND METZ, C. B. 1945. Natural heteroagglutinins in the serum of the spiny lobster, *Panulirus interruptus*. I. Taxonomic range of activity, electrophoretic and immunizing properties. *J. Exp. Zool.*, **100**, 387–406.