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NATURALLY-OCCURRING HEMAGGLUTININ IN A TUNICATE *HALOCYNTHIA PYRIFORMIS*

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Naturally-occurring hemagglutinating proteins (lectins) are widely distributed in nature and are finding numerous applications in immunologic and cancer research. The most thoroughly studied lectins are those derived from plants such as concanavalin A (from the jack bean), red kidney bean agglutinin (PHA), wheat germ agglutinin, and soybean agglutinin. Phytohemagglutinins possess many interesting biological and chemical properties in addition to their ability to agglutinate red cells. Some lectins are specific for human ABO and MN blood groups and have been used in blood typing and studies of blood group specificity (Boyd, 1963; 1970). Certain lectins act as mitogens stimulating blast formation and mitosis of lymphocytes (Robbins, 1964; Naspitz and Richter, 1968). Since lectins bind specifically to cell-surface saccharides, they provide a new method for the study of the architecture of cell surfaces. Lectins are of particular interest because some are capable of preferential agglutination of cultured mammalian cells that have been transformed by oncogenic viruses or chemical oncogens, as well as cells from spontaneous tumors (Aub, Sanford and Cote, 1965; Burger, 1969; Inbar and Sachs, 1969; Sela, Lis, Sharon and Sachs, 1970).

The presence of these unusual biochemical properties in plant lectins has stimulated renewed interest in naturally-occurring hemagglutinins from other sources, particularly invertebrate animals. In the course of our study of humoral immune factors of tunicates, we found a quite active panagglutinin in *Halocynthia pyriformis,* which will be described in this paper. Tunicates occupy a unique phylogenetic niche between the vertebrates and invertebrates. In light of the great differences in immunological specificity and memory phenomena between vertebrate and invertebrates, tunicates assume a high degree of importance in the study of the phylogeny of immunity. Recently several studies of hemagglutinins in tunicates have appeared (Fuke and Sugai, 1972; Wright, 1974); the lectin described in this paper apparently differs in both biochemistry and activity from

hemagglutinins from other tunicate species. In addition to basic biophysical and chemical characteristics of the lectin, we present data on specificity, inhibition by saccharides, and sensitivity to chemically-modified erythrocytes.

Materials and Methods

Experimental animals and hemolymph collection

The ascidians, *Halocynthia pyriformis* and *Boltenia ovifera,* were collected in the Bay of Fundy by Marine Research Associates, New Brunswick, Canada; *Ciona intestinalis* was supplied by the Marine Biological Laboratory, Woods Hole, Massachusetts. The animals were held at approximately 15° C in 150 gallon marine aquaria (Aquarium Systems, Inc., Eastlake, Ohio) until used. After expressing the sea water held within the branchial sac, the ascidians were bled through incisions made in the tunic beneath the excurrent siphon. As much as 10 ml of hemolymph could be collected from one large ascidian. The hemolymph was collected on ice and used immediately after removal of hemocytes by centrifugation.

Hemagglutination assays

Pooled normal blood in Alsever's solution (1:1) from various mammalian and avian species were obtained from the Animal Blood Centre, Inc. (Syracuse, New York). The cells were washed in Alsever's solution and resuspended to give stock solutions of 10^9 cells/ml. The stock solutions were stored at 4° C. Prior to use, aliquots of the stock erythrocytes were diluted in 0.15 M NaCl to a concentration of 10^8/ml. Hemolymph (cell-free) was serially diluted in 0.15 M NaCl and an equal volume of dilute erythrocytes was added to each tube. Agglutination titers are expressed as the reciprocal of the lowest dilution of hemolymph which caused visible agglutination at a given time of incubation.

Hemolymph treatments

The effect of trypsin was determined by incubating tunicate hemolymph for 60 min at 37° C, in the presence of 5–500 μg/ml Type III trypsin. All chemicals, except where otherwise indicated, were obtained from Sigma Chemical Company, St. Louis, Mo.

Hemolymph was heated in a thermostatically-controlled water bath over a wide range of temperatures and for various time intervals, to determine the heat lability of the hemagglutinin. Hemagglutination was carried out at various pH values in appropriate buffers to determine its sensitivity to hydrogen ion concentration.

Rapid freezing and thawing of hemolymph was carried out by freezing in an ethanol-dry ice mixture followed by thawing under warm water.

Several methods were used to remove most of the divalent cations from hemolymph samples. The hemolymph was dialyzed at room temperature against Ca^{++} and Mg^{++}-free Hank's balanced salt solution (Grand Island Biological Company,

New York) containing 0.02% EDTA (ethylenediamine tetraacetic acid, disodium salt). Hemolymph and EDTA (1.56×10^{-3}–2.5×10^{-2} M final concentration) were incubated for 3 hr at room temperature followed by dialysis against 0.15 M NaCl, 60 hr at room temperature. The calcium and/or magnesium ion concentration in dialyzed or untreated hemolymph was adjusted by additions of $CaCl_2$ or $MgCl_2$.

Dialysis of hemolymph for 24 hr at room temperature against deionized water resulted in the production of a light flocculation. This flocculant material was removed by centrifugation at about 1090 g for 10 min and was readily soluble in 0.15 M NaCl. This fraction was shown to contain almost all of the hemagglutinating activity of native hemolymph. The hemagglutinin could also be obtained by treating the hemolymph with 30–50% ammonium sulfate (18 hr at 4° C). The resulting precipitate was centrifuged out as above, washed several times with deionized water, and dissolved in 0.15 M NaCl. Total hemagglutinating activity was recovered in the precipitate; the supernatant had no activity.

Adsorption studies were carried out to determine the specificity of the hemagglutinin. Hemolymph was adsorbed with 10^9 washed erythrocytes/ml hemolymph for 1 hr at 37° C. The red cells were removed by centrifugation, and the hemolymph was again adsorbed with a comparable quantity of fresh erythrocytes at the same temperature and time. The red cells were again centrifuged out and the hemolymph serially diluted for the agglutination assay. Control hemolymph was unadsorbed but was incubated at 37° C for 2 hr.

Since many plant and invertebrate lectins combine with specific sugar moieties, the inhibitory action of certain monosaccharides commonly found in the glycoproteins of red cell membranes on the tunicate hemagglutinin was assayed. The sugars tested included D-glucose, D-galactose, D-mannose, L-fucose, L-arabinose, D-xylose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, and N-acetylneuraminic (sialic) acid. Hemolymph was serially diluted in 10^{-5}–0.25×10^{-3} M solutions of the various sugars, and the mixture was incubated 1 hr at 23° C. Horse erythrocytes were then added, as previously described, and the activity of the hemagglutinin determined. When the indicator cells used were human red cells, the agglutination studies were carried out in the presence of 20 mM $CaCl_2$.

Chemical modification of erythrocytes

Enzyme-treated erythrocytes have been shown to be agglutinated more strongly by plant lectins than untreated cells. The effect of tunicate hemagglutinin on several kinds of chemically-altered cells is reported in this paper.

Red cells (10^8/ml) were incubated in an equal volume of 0.1 mg Sigma Type III (bovine pancreatic) trypsin/ml Hank's balanced salt solution for 10–30 min at 37° C. The cells were then washed 4 times in saline and resuspended so that the final red cell concentration was 10^8/ml. Horse erythrocytes (2 ml of 10^9/ml) were incubated in 20 ml 0.01% bromelain for 10–30 min at 37° C, followed by 4 washes and resuspended in saline. The agglutination of erythrocytes (1 volume packed red cells:1 volume enzyme solution) incubated at 37° C for 10–60 min in the presence of Sigma Type V protease ("pronase" from *Streptomyces griseus*) was also studied.

Results

Hemagglutinating activity in Halocynthia pyriformis *hemolymph*

Halocynthia hemolymph contains naturally-occurring hemagglutinin(s) for a wide range of avian and mammalian erythrocytes. The agglutinin is specific for certain blood cells; its titer for any given kind of erythrocyte shows little variation among individual ascidians of the same species. The red cells could be divided into two major groups with regard to sensitivity to the lectin. Weakly agglutinated erythrocytes (titers ranging from 2 to 32) include human, pigeon, rabbit, sheep, swine, goat, calf, and ox. Much higher titers (64–512) were consistently recorded for duck, goose, chicken, turkey, guinea pig, and horse red cells. Horse cells were the most readily agglutinated and were frequently used as indicator cells in further studies of the properties of the hemagglutinin. As discussed elsewhere, the presence of Ca^{++} in the median generally increased the titers for all red cell types. The hemagglutinin was not specific as to the A, B, O antigens of human erythrocytes.

Homogenates of branchial sac tissue also contained agglutinating activity for human and horse erythrocytes. It is quite possible that this activity resulted from the presence of hemolymph in this tissue; it was technically impossible to obtain hemolymph-free tissue samples.

The hemagglutinating activity of the hemolymph was not affected by incubation with low concentrations of trypsin for 60 min at 37° C. However, trypsin concentrations over 500 μg/ml hemolymph did substantially reduce hemagglutination.

Sensitivity of the lectin to heat, pH and freezing and thawing and storage

The lectin was shown to be heat-labile at temperatures exceeding 50° C. Incubation of hemolymph at temperatures ranging from 1°–48° C, for periods of 1–2 hr, did not inhibit its strong hemagglutinating activity for horse erythrocytes. This activity was abolished by any of the following time and temperature regimens: 120 min at 50°, 60 min at 53°, 30 min at 55°, or 15 min at 58° C.

The agglutination of horse erythrocytes by tunicate hemolymph was slight at extreme pH values (5 and 11). Activity was quite strong over a comparatively wide range of pH 6–10. The highest titers were recorded about pH 8–9.

Repeated (20 ×) freezing and thawing of the hemolymph had no appreciable effect on the titer of the lectin against horse red cells.

The hemagglutinin retained full activity for at least 2 days when held at 25° C. No activity was lost during the first 5 days at 4° C; thereafter the activity gradually declined but was still quite strong for as long as 2 months at this temperature. Stability could be considerably prolonged by storing the hemolymph at −5° C.

Role of divalent cations in hemagglutinin activity

Dialysis of hemolymph against EDTA-containing balanced salt solution for 24 hr resulted in marked diminution of the ability of the hemolymph to agglutinate horse or human B erythrocytes. Hemolymph incubated with 6.25×10^{-3} M (or higher) EDTA for 3 hr at room temperature lost its activity for human A, B, or

horse erythrocytes; the EDTA was removed by dialysis prior to determination of titers.

If the hemagglutination reaction was carried out in the absence of added Ca^{++}, no agglutination of human A red cells was detected; the presence of 20 mM $CaCl_2$ resulted in marked hemagglutination with titers of 128 or higher. Horse, swine, and calf erythrocytes agglutinated in the absence of added Ca^{++} but, with the exception of calf cells, were more extensively agglutinated in the presence of 20 mM $CaCl_2$. This stimulatory effect of Ca^{++} could be shown in concentrations of 1–100 mM added to untreated hemolymph. The effects of low concentrations of Ca^{++} on hemagglutinin activity are more pronounced if the hemolymph is dialyzed against water (24 hr, 25° C) and the flocculated protein redissolved in saline. The addition of 5 mM of $CaCl_2$ quadruples the titer observed in the absence of Ca^{++}; further increase in Ca^{++} concentration has no effect on the titer.

It would appear that Mg^{++} cannot substitute for Ca^{++} in these reactions. At low concentrations (0.1–1 mM) added Mg^{++} has little or no effect on hemagglutination titers; however, at higher concentrations (10–100 mM) Mg^{++} in the medium inhibits the reaction. The hemagglutinating activity of protein derived from hemolymph by dialysis against deionized water is markedly inhibited by 5 mM $MgCl_2$ and abolished by 30 mM Mg^{++} present in the medium.

Effect of temperature of incubation

The effect of incubation temperature on the hemagglutinin titer for human A, horse, sheep, rabbit, guinea pig, ox, and goat erythrocytes was determined. Generally, the titers obtained at 1° C were about the same as those measured at 25° C for all erythrocytes tested; however, the reactions were less intense at the lower temperature. Stronger reactions and higher titers were usually recorded at 37° C, particularly in the case of sheep, guinea pig, and goat erythrocytes.

Dialysis and salting out of hemagglutinin

The hemagglutinin is resistant to dialysis against 0.15 M NaCl for 24 hr at room temperature, provided Ca^{++} is present during hemagglutination, particularly in the case of human erythrocytes. If the hemolymph is dialyzed against deionized water, a light flocculation takes place; by 48 hr no additional material will come out of solution. The material was shown to represent almost the total serum protein (on the basis of Lowry protein determinations) and could be easily centrifuged out of suspension and redissolved in 0.15 M NaCl. The hemagglutinating activity resides only in this protein fraction; the soluble material remaining after dialysis against deionized water is devoid of activity.

It was also found that the hemagglutinin could be rendered insoluble by treatment of the hemolymph with 30% or 50% ammonium sulfate for 18 hr at 4° C. This precipitate can be washed in deionized water and dissolved in 0.15 M NaCl with restoration of agglutinating activity.

Adsorption studies

The specificity of the natural hemagglutinin(s) in *Halocynthia pyriformis* hemolymph was tested by adsorption studies using a number of mammalian eryth-

TABLE I

Adsorption studies of Halocynthia pyriformis *lectin*

Test erythrocytes	Hemagglutination titer after adsorption with erythrocytes of:				
	Unadsorbed	Horse	Human A*	Goat	Calf
Horse	256	32	256	32	32
Swine	32	0	4	†	0
Human A	32	2	32	0	2
Calf	32	0	2	†	0
Goat	16	0	4	0	†
Ox	4	0	2	0	†
Sheep	8	0	4	0	†
Rabbit	4	0	4	0	†

* In the presence of 20 mM $CaCl_2$.
† Not done.

rocytes (Table I). Horse red cells are strongly agglutinated by *Halocynthia* hemolymph; this activity is unaffected by adsorption with human A cells, but can be substantially reduced by adsorption with horse, goat, or calf erythrocytes. Adsorption of hemolymph with horse, goat, or calf red cells also markedly reduces or eliminates hemagglutinating activity against swine, human A, calf, goat, ox, sheep, and rabbit cells in all instances tested. Hemolymph adsorbed with human A erythrocytes retains its ability to agglutinate these cells and all other mammalian red cells tested. The data indicate that, particularly with the nonhuman mammalian erythrocytes, adsorption with any given red cells will not only decrease or abolish activity of the agglutinin for the cells in question, but will also alter the hemagglutination of many other kinds of erythrocytes.

Agglutination of chemically-modified erythrocytes

Bromelain-treated horse erythrocytes were agglutinated to the same extent as untreated cells. However, both trypsinized and protease-treated mammalian red cells were agglutinated more strongly than untreated cells (Table II). Human A erythrocytes, which are relatively refractory to the action of the lectin in the absence of Ca^{++}, were strongly agglutinated in the absence of Ca^{++} after only 10 min incubation in a 0.1 mg/ml trypsin solution. Similar marked increases in titer of the agglutinin for human B and O, ox, sheep, horse, and calf erythrocytes could be produced by 10 or 30 min incubations in trypsin. Treatment of horse, calf, or swine red cells with protease (0.1 mg/ml) caused progressive increases in hemagglutinin titers with increasing time of incubation from 10 to 60 min.

Another ascidian, *Boltenia ovifera,* was shown to possess a lectin which reacts weakly with a variety of mammalian erythrocytes. The titer of this lectin was no greater for trypsinized sheep cells than for untreated sheep red cells. However, trypsinization did increase agglutinin titers for horse and human erythrocytes.

Inhibition of hemagglutinin by sugars

Ascidian hemolymph was reacted with a number of monosaccharides which are found in glycoproteins in order to give information concerning the nature of

receptor sites of the lectin. The sugars used in this study included D-glucose, D-galactose, D-mannose, L-fucose, L-arabinose, D-xylose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, and N-acetyl-neuraminic (sialic) acid. None of these sugars exerted inhibitory activity against the lectin at concentrations of 10^{-5} to 10^{-4} M. However, incubation of hemolymph with 0.25×10^{-3} M sialic acid markedly inhibited the agglutination of both horse and human A erythrocytes; the other sugars were not inhibitory at this concentration.

Hemagglutinins in several other ascidians

Boltenia ovifera, like *Halocynthia pyriformis,* is a large ascidian which is common on subtidal ledges in the Bay of Fundy. However, the hemolymph of *Boltenia* lacks a powerful hemagglutinin for either avian or mammalian red cells. Calf, duck, goat, and guinea pig erythrocytes were not agglutinated by *Boltenia* hemolymph; titers of 2–4 were obtained using sheep, goose, chicken, turkey, pigeon, ox, horse, and human red cells.

Ciona intestinalis is probably one of the most frequently studied ascidians. We bled a number of these animals and found a low level of hemagglutinating activity in their hemolymph. After 2 hr of incubation, titers of 2–4 were recorded for ox, goat, and human B and O cells; the titer for human A and horse cells was somewhat higher (about 16). Sheep and guinea pig erythrocytes were not agglutinated.

Discussion

The presence of hemagglutinins in the hemolymph of invertebrates has been known since at least 1903 (Noguchi, 1903). Tyler (1946) reported the presence of heteroagglutinins in the hemolymph of a number of invertebrates. A resurgence of interest in these factors has taken place recently, largely as a result of their implication as a part of a system for the recognition of foreign antigens (Boyden,

Table II

Agglutination of enzyme-treated erythrocytes

Treatment*	Erythrocyte type	Hemagglutinin titer			
		Time of incubation with enzyme			
		0′	10′	30′	60′
Pronase	Swine	16	64	64	128
	Horse	128	128	256	512
	Calf	8	16	16	32
Trypsin	Human A	4	64	128	†
	Human B	32	†	64	†
	Human O	16	†	64	†
	Horse	256	†	1024	†
	Sheep	4	†	32	†
	Ox	4	64	512	†

* 1 vol. 0.1 mg enzyme/ml saline: 1 vol. packed erythrocytes, 37° C.

† Not done.

1966; Tripp, 1966; Cushing, 1967) and because of the unusual properties of plant lectins already mentioned. Recently natural hemagglutinins have been reported in numerous invertebrates including sponges (Gold, Phelps, Khalap and Balding, 1974), pelecypod mollusks (Jenkin and Rowley, 1970; Acton, Bennett, Evans and Schrohenloher, 1969; Cornick and Stewart, 1973), gastropod mollusks (Pemberton, 1970a; 1970b; Bizot, 1971; Hammarström and Kabat, 1971; Matsubara and Boyd, 1974; Pauley, Granger and Krassner, 1971); annelids (Cooper, Lemmi and Moore, 1974); arthropods (Miller, Ballback, Pauley and Krassner, 1972; Marchalonis and Edelman, 1968; Finstad, Litman, Finstad and Good, 1972; Pauley, 1973; Scott, 1971; Anderson, Day and Good, 1972), and echinoderms (Finstad, Litman, Finstad and Good, 1972).

Halocynthia hemagglutinin was assayed in hemolymph cleared of blood cells by centrifugation. The source of this lectin is not known at the present time. The possibility of its being released by hemocytes *in vivo* cannot be ruled out. However, it is unlikely that this factor was released by hemocyte lysis during hemolymph preparation. Extracts of osmotically-lysed blood cells had no hemagglutinating activity even after concentration by ultrafiltration.

Halocynthia pyriformis hemolymph contains naturally-occurring hemagglutinin(s) against a wide range of avian and mammalian red cells. The activity was directed against the erythrocytes of all (14) species tested and was characterized by relatively high titers (64–512) for the red cells of 6 species. The hemagglutinin of the tunicate *Ciona intestinalis* is quite weak in comparison, with reactivity against many red cell types but with titers never exceeding eight (Wright, 1973; 1974). The natural lectins reported in several other tunicates (*Styela plicata* and *Halocynthia hilgendorfi*) are stronger and show more specificity (Fuke and Sugai, 1972). These agglutinins were directed against the erythrocytes of rats, rabbits, and mice but were inactive against fish, frog, snake, sheep, and guinea pig red cells.

Human blood group specificity, which is marked in albumin gland hemagglutinins from certain gastropod mollusks (Prokop, Uhlenbruck, Rothe and Cohen, 1974), is lacking in *H. pyriformis*. This lack of specificity has been reported in other urochordates (Wright, 1974) and is typical of most hemolymph lectins (Brown, Almodovar, Bhatia and Boyd, 1968; Tripp, 1966; Hall and Rowlands, 1974b).

A lack of sensitivity of invertebrate lectins to proteolytic enzymes has been reported for a number of species (Uhlenbruck, Reifenberg and Prokop, 1971; Pauley, 1974; Fuke and Sugai, 1972; Ryoyama, 1974). We report that *H. pyriformis* lectin is inactivated by certain trypsin concentrations as is the case for lobster agglutinin (Hall and Rowlands, 1974a). The protein nature of this tunicate lectin was indicated in several ways. Treatment of the hemolymph with trichloroacetic acid precipitated essentially all of the proteins present, protein-free hemolymph was lacking in hemagglutinating activity. Most of the hemolymph protein could be removed by ultrafiltration; the total activity of the lectin resided in this material and hemagglutination titers were proportional to protein concentrations over the dilution range tested.

H. pyriformis hemagglutinin begins to show heat lability at about 50° C and can be totally inactivated by incubating 15 min at 58° C. Wright (1974) reports

that *Ciona* lectin is heat labile at temperatures exceeding 70° C. However, *Styela plicata* coelomic fluid retains full activity even after 30 min at 100° C (Fuke and Sugai, 1972). The lectins of most invertebrates so far studied, such as arachnids, crustaceans, mollusks and insects, are heat labile in the range 50°–70° C (Finstad, Litman, Finstad and Good, 1972; Hall and Rowlands, 1974a; Tripp, 1966; Johnson, 1964; Cornick and Stewart, 1973; Scott, 1971; Pauley, 1974). The hemagglutinin of the sea urchin *Hemicentrotus pulcherrimus* is not affected by temperatures of 100° C; however, the lectins of other sea urchnis are heat labile from 70°–85° C (Ryoyama, 1974).

H. pyriformis lectin is active over a wide pH range (6–10) and is most active around pH 8–9. Fuke and Sugai (1972) report essentially the same properties for hemagglutinin from another tunicate species. The hemagglutinins of the lobster, sea hare, crayfish, sea urchin and oyster are also active over broad ranges of pH values (Hall and Rowlands, 1974a; Pauley, 1974; Ryoyama, 1974; Cornick and Stewart, 1973).

We report that tunicate hemagglutinin does not lose activity after repeated freezing and thawing. This is also a property of the lectins of the sea hare, crayfish and blue crab (Pauley, 1974); these agglutinins could be stored with full activity for at least 6 months at −12 or −25° C. *H. pyriformis* hemagglutinin is fully active for at least several months at −5° C and loses little activity when stored at 4° C. *Ciona* lectin responded to storage in a similar fashion (Wright, 1973).

Ca^{++} is required for the expression of biological activity of many naturally-occurring invertebrate hemagglutinins, such as those from *Limulus* (Finstad, Litman, Finstad and Good, 1972; Marchalonis and Edelman, 1968); lobster (Hall and Rowlands, 1974a); oyster (Acton, Bennett, Evans and Schrohenloher, 1969), and tunicate (Wright, 1973). A similar Ca^{++} dependency exists for *H. pyriformis* lectin based on its inactivation with EDTA and the augmented titers observed in the presence of added Ca^{++}. McDade and Tripp (1967) found that oyster hemagglutinin was active against sheep and rabbit erythrocytes in the absence of Ca^{++}, but did not agglutinate human red cells without Ca^{++} in the medium. McDade and Tripp (1967) also observed that Ca^{++} was required for heat stability of the lectin; this was confirmed by Cornick and Stewart (1973). Ca^{++} enhanced the activity of sea urchin lectin, whereas Mg^{++} was ineffective (Ryoyama, 1974); similar results were reported in the case of *Limulus* (Marchalonis and Edelman, 1968). The inability of Mg^{++} to substitute for Ca^{++} in lectin activation is demonstrated in the tunicate *H. pyriformis*. However, Wright (1973) reports that both Ca^{++} and Mg^{++} enhance the hemagglutinin titer in *Ciona* and both ions will restore the activity of EDTA-treated hemolymph. It has been reported that Ca^{++} is not required for activity of *Styela plicata,* sea hare or blue crab hemagglutinin (Fuke and Sugai, 1972; Pauley, 1974).

We report that *H. pyriformis* lectin loses no activity after dialysis against 0.15 M NaCl for 24 hr. If the agglutinin is dialyzed against deionized water for a similar period, a light flocculation occurs. This material can be centrifuged out of suspension, is quite soluble in 0.15 M NaCl, and can be shown to be a protein by the Lowry and biuret methods. The total hemagglutinating activity of the hemolymph can be recovered in this fraction. The natural hemagglutinins of

other tunicates are also stable after dialysis *vs.* saline (Fuke and Sugai, 1972; Wright, 1973). Most invertebrate lectins are nondialyzable and their activity will precipitate out with the serum proteins when dialyzed against distilled water (McDade and Tripp, 1967; Scott, 1971)

Adsorption studies of *H. pyriformis* hemagglutinin indicate a considerable degree of cross-reactivity. Similar results were obtained by Wright (1973) using *Ciona* and Fuke and Sugai (1972) using several other ascidian species. This general lack of specificity of hemagglutinins has been reported for most invertebrate species. In arthropods and mollusks adsorption with erythrocytes from a given species usually reduces lectin titers against that cell, as well as affecting the activity against other types of red cells (Tripp, 1966; Scott, 1971; Hall and Rowlands, 1974b).

The agglutination of enzyme-treated erythrocytes by tunicate lectin has not been previously reported. Incubation of red cells with either trypsin or pronase greatly increased their susceptibility to the hemagglutinin of *H. pyriformis.* Untreated human erythrocytes are weakly agglutinated in the absence of Ca^{++}; however, the same cells when trypsinized agglutinate strongly regardless of Ca^{++} concentration. Hall and Rowlands (1974b) reported that trypsinized cells were not agglutinated more strongly by lobster agglutinins; however, increased titers for trypsin-treated red cells were found in the hemolymph of the sea urchin (Ryoyama, 1974) and the sponge (Gold, Phelps, Khalap and Balding, 1974).

The activity of many lectins can be significantly altered by reacting them with various simple sugars prior to exposure to test erythrocytes. N-acetylneuraminic (sialic) acid was the only sugar (of nine tested) which markedly inhibited the tunicate hemagglutinin studied here. Sialic acid-binding lectins have been described in the horseshoe crab (Bird, Uhlenbruck and Pardoe, 1971) and the lobster (Hall and Rowlands, 1974b). *Limulus* lectin (limulin) has been recently reinvestigated by Roche and Monsigny (1974). Limulin was purified and shown to be a glycoprotein of 13.9 S, 335,000 MW consisting of 19,000 MW subunits held together by noncovalent bonds. Agglutination of horse red cells was inhibited by N-acetylglucosamine, free sialic acid, and particularly by glycoprotein-bound sialic acid (human orosomucoid). On this basis it was suggested that the limulin receptor is a complex carbohydrate containing sialic acid and N-acetylglucosamine and is not a monosaccharide.

Johnson (1964) found that N-acetyl-D-galactosamine (GalNAc) and N-acetyl-D-glucosamine (GlcNAc) inhibited the agglutinin of the butter clam. McDade and Tripp (1967) reported that no single saccharide would inhibit the agglutination by oyster lectin of all red cell types tested; however, D-galactosamine (GalN), D-glucosamine (GlcN), GlcNAc, and GalNAc inhibited the agglutination of human red cells, while D-ribose inhibited rabbit erythrocytes. Ryoyama (1974) found that 9 sugars (of 36 tested) could inhibit the lectin of the sea urchin *Pseudocentrotus depressus.* The hemagglutinin of the sponge can be inhibited by galactose (Gold, Phelps, Khalap and Balding, 1974). Hall and Rowlands (1974b) found that lobster agglutinins are not only sensitive to sialic acid (NANAc) but are inhibited by N-glycolylneuraminic acid, N-acetylmannosamine, GlcN and GlcNAc.

The physiological significance of this lectin and many other invertebrate hemagglutinins is as yet undefined. It may function as a humoral recognition factor, a

property of certain invertebrate lectins originally described by Tripp (1966). However, Fuke and Sugai (1972) found that ascidian (*Styela plicata*) hemolymph did not stimulate *in vitro* phagocytosis of fixed rabbit erythrocytes. Hall and Rowlands (1974a) point out the structural and physical chemical similarities between some invertebrate hemagglutinins and certain nonimmunoglobulin vertebrate agglutinins and suggest that similar molecules may have been preserved during many stages of animal evolution. The contribution of these molecules to the natural defense mechanisms of higher animals has not been determined.

Other possible roles of invertebrate lectins in nature include Ca^{++} transport, particularly in shell-bearing mollusks. They could also function in sugar transport or storage, or could serve for the attachment of glycoprotein enzymes in organized multienzyme systems (Sharon and Lis, 1972). At the present time there is little evidence to support or refute these hypotheses.

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Summary

An active, naturally-occurring panhemagglutinin has been described in the hemolymph of the protochordate *Halocynthia pyriformis*. This protein lectin is inactivated by temperatures exceeding 50° C, is active in the range pH 6–10, and is resistant to repeated freezing and thawing. Frozen hemolymph retains full hemagglutinating activity for several months. Activity is not reduced by dialysis against saline; however, serum proteins with total hemagglutinating activity can be precipitated by dialysis against deionized water or by treatment with appropriate ammonium sulfate concentrations. This lectin will not agglutinate human erythrocytes in the absence of Ca^{++}; Ca^{++} potentiates the agglutination of red cells from all other species tested. However, Mg^{++} does not stimulate hemagglutination and will inhibit it at certain concentrations.

Halocynthia pyriformis hemolymph will cause the agglutination of red cells from many avian and mammalian species. Human, rabbit, sheep, swine, goat, calf, ox, and pigeon erythrocytes agglutinate weakly with titers of 2–32; whereas, titers of 64–512 were consistently recorded for the red cells of guinea pig, horse, duck, goose, chicken, and turkey. The lectin was not specific for the ABO blood group antigens of man. The hemagglutinin shows considerable cross-reactivity; adsorption with nonhuman mammalian erythrocytes not only decreases activity toward the adsorbing cells but also alters the agglutination of cells from other species. Treatment of red cells with pronase or trypsin causes markedly increased hemagglutination titers; such treatment allows human erythrocytes to agglutinate in the absence of Ca^{++} in the medium. Incubation of hemolymph with N-acetylneuraminic acid strongly inhibits subsequent hemagglutinating activity, suggesting that sialic acid residues are present in the binding site of the lectin.

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