Heterogeneity of Lobster Agglutinins. II. Specificity of Agglutinin–Erythrocyte Binding†

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ABSTRACT: Lobster agglutinins, which were shown in the preceding paper to be structurally heterogeneous, were analyzed to determine the chemical characteristics of their binding to erythrocytes. The analysis included agglutinin inhibition studies with simple saccharides, enzyme treatment of erythrocytes, studies of absorption of agglutinins, and microagglutinate preparations. One agglutinin (LAg-2) was shown to contain an *N*-acetylgalactosamine (GalNAc) site which bound to GalNAc residues on mouse, horse, and hamster erythrocytes. Another agglutinin (LAg-1) contained an *N*-acetylneuraminic acid (NANAc) site which bound to NANAc residues on human erythrocytes; LAg-1 also bound to mouse erythrocytes *via* the NANAc site or *via* a second binding site on the LAg-1 molecule. These observations represent the first substantial evidence for binding site heterogeneity in invertebrate agglutinins and illustrates a way in which agglutinins may be functionally similar to vertebrate antibodies. Since lobster hemocytes engulf erythrocytes *in vitro* only if the appropriate agglutinins are present, it may be that lobster agglutinins function similarly to opsonic antibodies in vertebrates by recognizing foreign organisms.

We studied the chemistry and biology of the agglutinins of a representative invertebrate, the lobster, *Homarus americanus*, in order to more fully understand how invertebrates defend themselves against foreign pathogens. In the preceding paper (Hall and Rowlands, 1974) we described experiments which demonstrated structural heterogeneity of invertebrate agglutinins. Two agglutinins differing in molecular weight, electrophoretic mobility, antigenic properties, and erythrocyte binding specificity were purified from lobster hemolymph. This finding of heterogeneity in invertebrate agglutinins provides evidence for the hypothesis that invertebrate agglutinins function as recognition factors for a variety of foreign substances leading to their phagocytosis and detoxification. The agglutinins would, in this respect, be functionally analogous to vertebrate antibodies.

The present communication describes experiments conducted to investigate the chemical structures involved in agglutinin–erythrocyte binding. From these experiments we could determine if differences between the two purified agglutinins included heterogeneity of erythrocyte binding specificity as well as the structural heterogeneity described previously (Hall and Rowlands, 1974). Binding specificity heterogeneity of agglutinins would be required to sustain the hypothesis that agglutinins function as recognition factors.

Materials and Methods

Agglutinin Preparation. Lobster agglutinins (LAg-1 and LAg-2) were purified from native hemolymph by ammonium sulfate precipitation, pevikon block electrophoresis, and gel chromatography as described previously (Hall and Rowlands, 1974).

Chemicals. The following chemicals were obtained from the sources indicated in the highest purity available: neuraminidase, Type V (0.1 U/mg) from *Clostridia perfringens*, trypsin, Type 111 (10,000 U/mg) from bovine pancreas, fluorescein isothiocyanate, bovine submaxillary gland mucin, porcine stomach mucin, D-galactose, α-L-fucose, *N*-acetyl-D-glucosamine, D-lyxose, D-melibiose, D-stachyose, *N*-acetyl-D-galactosamine, D-glucosamine, L-arabinose, L-rhamnose, D-mannose, D-fructose, D-mannosamine, *N*-acetyl-D-mannosamine, *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, D-glucuronic acid, and D-glucosamine 6-phosphate (Sigma Chemical Co.); D-glucose (Fisher Chemical Co.); sucrose (Baker Chemical Co.); lactose (Merck Chemical Co.); raffinose (Pfanstiehl Laboratories, Inc.); D-galacturonic acid (General Biochemicals). Partially purified A and B blood group substances were gifts from Dade Division, American Hospital Supply Corp.

Lymphocytes. Lymphocyte suspensions from AKR mice and from normal human volunteers were purified from peripheral blood using a Ficoll–Hypaque separation technique (Böyum, 1968).

Erythrocytes (E) from pooled vertebrate blood were obtained as follows: rat, mouse, goat (Grand Island Biological Co.), horse, chicken, goose (Miles Laboratories), and from blood of individual animals as follows: toad (*Bufo marinus*) (Lemberger Co.), sheep (School of Veterinary Medicine of the University of Pennsylvania), rabbit, guinea pig, mouse, dog, hamster, opossum (obtained locally from laboratory animals); human group A, B, and O negative (Blood Bank, Hospital of the University of Pennsylvania). Group O negative human E were used unless otherwise stated.

Bacteria of the *Achromobacter* species were isolated from the hemolymph of some lobsters by culture on enriched agar (Adams, 1959) at 4 and 20°.

Hemocytes. Suspensions of washed lobster hemocytes were prepared in 0.01 M *N*-ethylmaleimide as described previously (Hall and Rowlands, 1974).

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Agglutination Assay. Agglutinin activity was assayed using the microtiter apparatus and Tris-buffered diluent (TBD)[1] as described previously (Hall and Rowlands, 1974). Replicate determinations were not performed routinely, although on occasions when duplicate determinations were done the results did not differ by more than one dilution. Activity (units or titer) was expressed as the reciprocal of the highest dilution giving positive agglutination.

Agglutination Inhibition Experiments. Various substances including simple sugars, polysaccharides, and glycoproteins were tested for their ability to inhibit agglutination as follows: solutions of inhibitor in TBD were prepared and the pH was adjusted to between 7.0 and 8.0 if required. Parallel dilutions of sample were made in TBD and TBD–inhibitor solution and the dilutions incubated for 1 hr at 20° prior to addition of 1.5% suspensions of E.

Alteration of Erythrocytes. A neuraminidase solution was prepared by dissolving 5 mg of neuraminidase in 1.0 ml of 0.05 M acetate-buffered normal saline, pH 5.5, made 0.1% in $CaCl_2$. A 3.0-ml quantity of a 5% saline suspension of E was added to this preparation and incubation was carried out at 37° for 15 min. Trypsinized E was prepared by incubating 3 ml of a 5% suspension of E with 1 ml of a trypsin solution (10 mg/ml in 0.1 M phosphate buffer (pH 7.3)) for 10 min at 37°. The suspensions were quickly cooled to 20° and centrifuged at 3000 rpm for 5 min and the cells washed three times in TBD and suspended in TBD.

Fluorescein-conjugated human E were prepared by washing human E three times in TBS and suspending in a saturated solution of fluorescein isothiocyanate in TBS for 16 hr at 22° in the dark with gentle agitation. The conjugated cells were then washed six times in TBS and resuspended as a 2% solution in TBD.

Erythrocyte stroma were prepared as described by Zmijewski (1968).

Absorption of Hemolymph. Samples of hemolymph and purified agglutinin were absorbed with E, E stroma, and insolubilized mucin. All of these absorptions were performed in the presence of 0.02 M $CaCl_2$ and incubated at 4° for 16–20 hr. Following incubation the suspension was centrifuged and the absorbed hemolymph (supernatant) decanted and assayed.

Microagglutinate Experiments. Binding of the agglutinins (mixtures and purified preparations) to mixtures of two cell types was studied microscopically. Pure cell suspensions of human, mouse, or chicken E, human or mouse lymphocytes, or lobster hemocytes were prepared. The two cell suspensions to be mixed were adjusted to the same concentration (±5%). One drop of the mixed suspension and of each of the two pure suspensions was placed on glass slides. One drop of a dilution of agglutinin adjusted to give small microagglutinates (less then ten cells) was mixed with the cell suspension on the slide, a cover slip was added, and the slide was examined under the microscope. In cases where an inhibitor was used, the inhibitor was incorporated into the drop containing the diluted agglutinin. The composition of the microagglutinates was observed on a Leitz Ortholux microscope using phase contrast and fluorescent illumination and photographs of typical fields were taken using Kodak high speed Ektachrome film (3200K) and the Leitz Orthomat camera. Incident light fluorescent illumination was provided by a mercury arc lamp (Hb0200) using a K490 barrier filter with matching dichroic beam splitter and a K510 suppression filter. The composition of a series of microagglutinates composed of three, four, or five to ten cells was recorded and the percentage of cells in unmixed and mixed microagglutinates calculated

In Vivo Phagocytosis. Lobsters received by air freight were maintained in the laboratory at 4° in a moist environment for the following experiment. A human E suspension (10^9 washed cells in 2 ml of TBS) was injected into the ventral abdominal sinus of each of a series of lobsters. The lobsters were sacrificed at 30 min, 60 min, and 14 hr and sections for histologic examination were removed from the heart, lymphatic gland, and hepatopancreas (liver). The tissue was fixed in Zenker's fixative, and the final sections were stained with hematoxylin and eosin.

Results

The agglutinins bound to a wide variety of cells including erythrocytes, lymphocytes, and bacteria. The agglutinins were active against erythrocytes from all 14 species tested in titers ranging from 2 (rabbit E) to 512 (horse, rat, and goose E). Group A, B, and O human E gave titers of 256, 256, and 128, respectively. Lymphocytes of both mouse and human origin were agglutinated when mixed with 1:10 dilutions of hemolymph or with purified agglutinins. Bacteria of the *Achromobacter* species were agglutinated by hemolymph and could absorb agglutinins to human E (but not mouse E) from hemolymph.

The agglutination of human E could be inhibited by several simple sugars. *N*-Acetylneuraminic acid was the most potent inhibitor, followed by *N*-glycolylneuraminic acid (NANGly), *N*-acetylmannosamine (ManNAc), glucosamine (GlcN), and *N*-acetylglucosamine (GlcNAc) (Table I). *N*-Acetylgalactosamine (GalNAc) (0.2 M) had no effect on agglutination of human E by hemolymph. The agglutination of E from several other species could be inhibited using other sugars; for example, toad E agglutination was reduced from a titer of 32:2 by 0.5 M GlcN, and inhibition of goose (512 → 64) and goat (32 → 8) E occurred in the presence of 0.05 M *N*-acetylneuraminic acid (NANAc). Human E agglutination by native hemolymph was also inhibited by 1% bovine and porcine mucins and by human B blood group substance.

TABLE I: Inhibition of Agglutination of Human Erythrocytes by Simple Saccharides.[a,b]

	Molarity					
Inhibitor	0	0.01	0.025	0.05	0.1	0.2
NANAc	256	128	32	4	2	
NANGly	128	64	32	16		
ManNAc	128	128	64	16	4	4
GlcN	256	128	128	128	64	64
GlcNAc	256	128	128	256	128	64

[a] Native hemolymph diluted in TBD containing the following inhibitors: NANAc, NANGly, ManNAc, GlcN, and GlcNAc. [b] Additional inhibition experiments using concentrations of inhibitors which gave at least four times inhibition gave substantially the same results as those shown in the table.

[1] Abbreviations used are: GalNAc, *N*-acetylgalactosamine; NANAc, *N*-acetylneuraminic acid; TBD, Tris-buffered diluent; TBS, Tris-buffered normal saline; E, erythrocytes; NANGly, *N*-glycolylneuraminic acid; ManNAc, *N*-acetylmannosamine; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine.

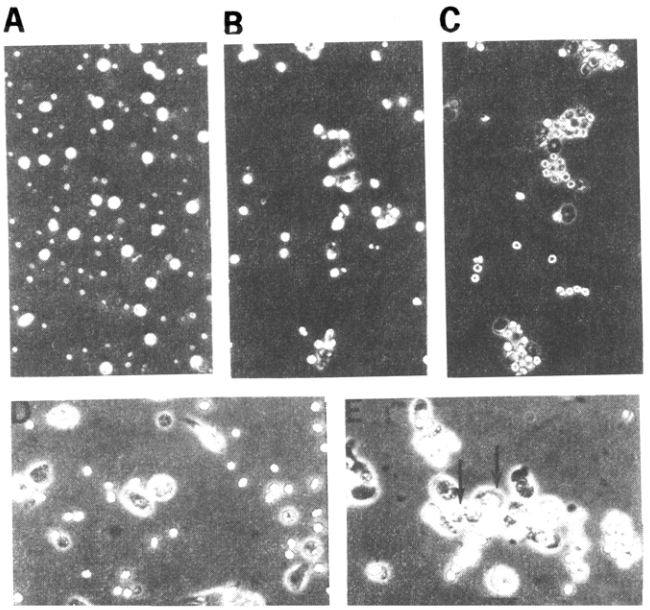

FIGURE 1: Mixed microagglutinates of erythrocytes, lymphocytes, and hemocytes. Mixtures of equal numbers of two types of cells were prepared. In some cases an equal volume of an agglutinin source was mixed with the cells. Preparations were examined under the microscope using phase contrast and ultraviolet illumination as indicated: (A) mixture of fluoresceinated human E and native mouse E; (B) mixture A plus purified LAg-1; (C) mixture of human E and human lymphocytes plus hemolymph diluted 1:10; (D) mixture of human E and lobster hemocytes; (E) mixture D plus purified LAg-1. The arrows indicate possible phagocytosis of E by the hemocytes.

While mouse E showed no distinct pattern of inhibition when tested with native hemolymph, a pattern of inhibition could be demonstrated using purified agglutinins. The agglutinin LAg-2 could be inhibited by GalNAc and to a lesser extent by either ManNAc or GlcNAc (Table II). The degree of inhibition with GalNAc was dose dependent with concentrations as low as 0.01 M giving a fourfold reduction in titer (64 → 16). The agglutinin LAg-1 could not be inhibited by any of the simple saccharides (Table II) or by a combination of GalNAc, NANAc, and GlcN (each 0.1 M).

The nature of the chemical binding of agglutinins to E was further investigated by enzyme treatment of human, sheep, and mouse E. While trypsin had no effect in any case, neuraminidase treatment of human and sheep E significantly reduced agglutinin titers to human E (256 → 4) and to sheep E (64 → 1), but had no effect on mouse E.

TABLE II: Inhibition of Agglutination of Mouse Erythrocytes by Simple Saccharides.[a]

Inhibitor	Titer: Native Hemolymph	Titer: LAg-1	Titer: LAg-2
None	256	32	64
0.05 M NANAc	128	32	32
0.1 M ManNAc	256	32	16
0.5 M GlcN	256	32	128
0.5 M GlcNAc	256	16	16
0.1 M GalNAc	128	32	1

[a] Agglutinin preparations diluted in TBD containing the inhibitors listed.

TABLE III: Absorption of Agglutinins by Human and Mouse Erythrocytes.[a]

Agglutinin Preparation	Titer to: Human E	Mouse E	Hamster E	Horse E
Native hemolymph	128	256	128	1024
Hemolymph absorbed with human E	0	128	16	64
Hemolymph absorbed with human E[b]	0		1	2
Hemolymph absorbed with mouse E	16	1		

[a] A 0.5-ml quantity of washed, packed E was incubated with 0.3 ml of native hemolymph at 4° for 18 hr. The E were sedimented and the supernatants tested for activity. [b] In the agglutination assay, the Tris-buffered diluent was made 0.025 M in *N*-acetylgalactosamine.

The specificity of agglutination was investigated by absorption of hemolymph with E. Absorption with human, sheep, and chicken E greatly reduced agglutinin activity to those E as well as E from toad, goat, goose, and rat. Absorption with human E, however, did not significantly affect titers to mouse E and only slightly reduced titers to horse and hamster E (Table III).

The heterogeneity of binding sites within a given agglutinin molecule was investigated by observing microagglutinates of mixtures of two types of cells. In experiments with mouse and human E, the human E were first conjugated with fluorescein to permit them to be distinguished from mouse E. Mouse and human E were then combined in equal proportions, the appropriate source of agglutinin was added, and the composition of the resulting microagglutinates was examined under the microscope. Microagglutinates containing both mouse and human E (Figure 1B) were the most common finding when either hemolymph or purified LAg-1 was used as the source of agglutinin, although the precise composition of the microagglutinates in these two experiments was slightly different (Table IV). On the other hand, when LAg-2 was used as the agglutinin, or if 0.05 M NANAc was present with LAg-1, most microagglutinates were composed of mouse E only and the few mixed microagglutinates present contained primarily mouse E (Table IV). When human E were mixed with human lymphocytes in the presence of dilute hemolymph, microagglutinates of mixed composition formed (Figure 1C); addition of NANAc inhibited the agglutination of both E and lymphocytes.

When washed lobster hemocytes and human E were mixed in the absence of hemolymph almost no aggregation of cells was seen (Figure 1D). Microagglutinates composed of both hemocytes and E formed when diluted LAg-1 was added to the mixtures of lobster hemocytes and human E. Binding of E to hemocytes as well as to other E was observed (Figure 1E). In a few cases it appeared that E may have been engulfed by the hemocytes (Figure 1E, arrows), but only occasional instances of this phenomenon were encountered. In a supplementary experiment in which human E were injected into a lobster and the tissues examined 30 min after injection, un-

TABLE IV: Composition of Mouse–Human Erythrocyte Microagglutinates.[a,b]

Agglutinin Source	Types of Microagglutinates (%)			Composition of Mixed Microagglutinates		
	Mouse E only	Mixed	Human E only	Mouse E (%)	Human E (%)	1 SD
Native hemolymph	5	85	10	52	48	2
LAg-1	32	65	3	78	22	3
LAg-1 plus 0.05 M NANAc	86	9	5	94	6	4
LAg-2	69	26	5	95	5	1

[a] Mixtures of equal numbers of mouse and fluoresceinated human E were prepared, an equal volume of an agglutinin source was mixed with the cells and the microagglutinates were studied under the microscope. [b] Data shown are from one of two experiments which showed similar results.

mistakable phagocytosis of E by many hemocytes located in the cardiac sinuses was evident (Figure 2).

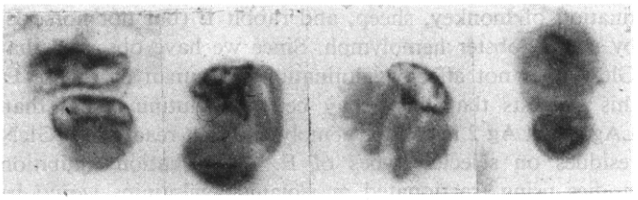

FIGURE 2: *In vivo* phagocytosis of erythrocytes by lobster hemocytes. Lobsters were injected 30 min prior to sacrifice with 10^9 human E. Sections were taken from the cardiac sinuses. Hematoxylin and eosin. 300×.

Discussion

The lobster agglutinins bind to a variety of different types of cells, and the chemical determinants to which they bind may be inferred from inhibition studies. For example, the binding of LAg-2 to mouse E probably involves GalNAc residues, or closely related chemical structures, on the erythrocyte membrane. The evidence for this is that GalNAc (0.01 M) significantly inhibits agglutination, and that as the concentration of GalNAc is increased, the degree of inhibition increases proportionately. The agglutination of horse and hamster E by LAg-2 is inhibited by GalNAc in a similar fashion. Therefore, the binding of LAg-2 to E in general is dependent on GalNAc, so the binding site on LAg-2 for E may be referred to as a "GalNAc" site. The actual binding determinant on the erythrocyte membrane probably includes more than just GalNAc, since LAg-2 does not bind to group A human E which have terminal GalNAc residues.

A second example in which inhibition studies have provided clues to binding chemistry is the agglutination of human E by LAg-1. In this case, NANAc in low concentration (0.05 M) significantly inhibited agglutination of human E. The degree of inhibition was proportional to the concentration of NANAc, and substances similar to NANAc in structure such as NANGly and ManNAc also gave inhibition, although to a lesser degree. The role of NANAc in binding is further supported by the fact that human E which have had NANAc residues removed by treatment with neuraminidase do not agglutinate when exposed to LAg-1. This binding site on LAg-1 may be referred to as a "NANAc" site.

In contrast to the above examples, the nature of the binding of LAg-1 to mouse E is not well defined. Neither GalNAc, NANAc, nor a combination of both, even in relatively high concentration (0.1 M), inhibits agglutination of mouse E by LAg-1. For this reason the binding sites for mouse E on LAg-1 and LAg-2 must be different. Since in this case the substrate for binding mouse E is the same for both LAg-1 and LAg-2, and the inhibitor is the same (GalNAc), the difference in results (inhibition of binding with LAg-2 but not with LAg-1) must be due to a structural difference in the binding sites on LAg-1 and LAg-2 for mouse E. Because none of the simple sugars tested inhibited agglutination of mouse E by LAg-1, this binding site on LAg-1 will be referred to as the "ME" site.

The relationship between the two binding sites[2] associated with LAg-1 (NANAc and ME) is not completely clear. The possibility that the two sites might be located on separate molecules was raised, because most preparations of LAg-1 contained several molecular species giving a complex of closely related bands on polyacrylamide gel electrophoresis. This possibility was dismissed after the microagglutinate experiments showed frequent binding of human to mouse E when LAg-1 was added to a mixture of the two cells. The possibility was also raised that the two sites might actually be the same site. In order to explain the fact that NANAc inhibits LAg-1 agglutination of human but not mouse E, this one-site hypothesis would require a differential reactivity of receptors. In a competitive situation the mouse E receptors would be most reactive, NANAc of intermediate reactivity, and human E least reactive. This competitive situation was created by adding LAg-1 to a mixture of equal numbers of human and mouse E. The microagglutinates which formed contained mostly mouse E (78%) while the cells remaining unagglutinated were mostly human E. When NANAc was present under the same circumstances, the microagglutinates contained almost exclusively mouse E (95%). Since mouse E predominated in the microagglutinates, they showed greatest reactivity; since NANAc blocked reactivity to human E but not to mouse E, it showed an intermediate reactivity; and since human E were always in the minority in agglutinates, they showed the least reactivity. While this evidence for differential reactivity of receptors is consistent with the one-site hypothesis, it does not rule out the possibility of the presence of two separate sites on LAg-1.

The structural information regarding the binding sites on the lobster agglutinins may be summarized as follows: LAg-2 contains a GalNAc site which binds to GalNAc residues on mouse, horse, and hamster E; LAg-1 contains a NANAc site which binds to NANAc residues on human E; LAg-1 also binds to mouse E either *via* the NANAc site or *via* a second binding site on the LAg-1 molecule. In any case, it is clear that there is heterogeneity of binding sites in lobster agglutinins.

In an independent study of the nature of the erythrocyte receptor site for lobster agglutinin, Cornick and Stewart (1973) showed that glucosamine significantly inhibited agglu-

[2] The number of binding sites referred to in this discussion is the number of *types* of binding sites rather than the absolute number of sites.

tination of monkey, sheep, and rabbit E (but not horse E) by native lobster hemolymph. Since we have observed that GlcN does not affect agglutination of human or mouse E, this suggests that there may be an agglutinin other than LAg-1 or LAg-2 in lobster hemolymph that reacts with GlcN residues on selected types of E. Agglutination inhibition studies using fractionated or isolated agglutinins would be necessary to confirm this possibility.

This description of binding site heterogeneity in an invertebrate's agglutinins is important for several reasons. It establishes that binding sites of different specificity are present on each of two structurally different agglutinins. Previous studies of agglutinin binding chemistry in the snail (Hammarström and Kabat, 1969) and the horseshoe crab (Cohen, 1968; Bird *et al.*, 1971) have failed to demonstrate binding site heterogeneity at all. In one previous study heterogeneity was suggested (McDade and Tripp, 1967) by the fact that agglutination of rabbit E by oyster hemolymph was best inhibited by ribose, while agglutination of human E was best inhibited by galactosamine, glucosamine, or their *N*-acetyl derivatives. However, these "agglutinins" were not separated from each other or purified either by absorption experiments or by other means, so that whether or not the two binding specificities were on the same or on different molecules could not be determined. Heterogeneity has also been inferred from serologic experiments in the mussel (McKay *et al.*, 1969) and in the spiny lobster (Tyler and Metz, 1945), but these experiments did not include chemical separation of the agglutinins or study of the binding site chemistry.

The association of a particular binding site with a particular agglutinin is important support for the hypothesis that agglutinins are a family of recognition factors, each with a different specificity for foreign substances (Cushing, 1967). In this way the agglutinins may also be analogous to the vertebrate antibody system which also consists of a family of proteins, each with a characteristic binding specificity.

Carbohydrate binding is a property not only of invertebrate agglutinins, but also of plant agglutinins (Sharon and Lis, 1972) and certain non-immunoglobulin agglutinins (Springer and Desai, 1971; Harisdangkul *et al.*, 1972; Pollara *et al.*, 1970). This evidence of functional similarity (carbohydrate binding) between invertebrate and vertebrate non-immunoglobulin agglutinins together with their structural similarities already described (Hall and Rowlands, 1974) suggests that these proteins may be related phylogenetically. The occurrence of a NANAc binding agglutinin (LAg-1) is of interest because it is only the second such agglutinin known, the other being found in the horseshoe crab (Bird *et al.*, 1971) which, of all species tested, is the one most closely related to the lobster. The sialic acid binding properties of this agglutinin may make it useful as a probe of membrane structure and cell receptors (Pardoe and Uhlenbruck, 1970).

The lobster agglutinins are probably involved in the phagocytosis of E by lobster hemocytes. There is no question that E are rapidly engulfed by the hemocytes *in vivo*. Attempts to study phagocytosis *in vitro* were complicated by the fact that the *N*-ethylmaleimide (required to prevent clumping and rupture of hemocytes) may have diminished phagocytic activity of the hemocytes. Nevertheless, occasional phagocytosis of E was observed when washed hemocytes and E were incubated with a source of agglutinin *in vitro*. There was no phagocytosis of E in experiments where washed hemocytes were incubated without agglutinin present. These results are in accord with those of more extensive experiments in the crayfish (McKay and Jenkin, 1970), sea hare (Pauley *et al.*, 1971), snail (Prowse and Tait, 1969), oyster (Tripp, 1966), and octopus (Stuart, 1968) which demonstrate that agglutinins are facilitative in *in vitro* phagocytic systems. This function of invertebrate agglutinins as opsonins is additional evidence for their proposed role in defense in invertebrates and is another way in which agglutinins may be functionally analogous to vertebrate antibodies.

The evidence presented in this and the preceding paper establishing the heterogeneity of agglutinins in an invertebrate gives important support to the concept that the agglutinins are part of an invertebrate "immune" system (McKay *et al.*, 1969). Before we may firmly conclude that such a system of natural immunity exists in the invertebrates, however, studies of the role of agglutinins in protecting an invertebrate against pathogens *in vivo* and of the control mechanisms for agglutinin synthesis will be required. Such studies may also lead to a greater understanding of and appreciation for the natural immune system in mammals.

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