To Carl
With best wishes —
Elias

IMMUNOLOGIC OBSERVATIONS OF THE AGGLUTININS OF THE HEMOLYMPH OF *LIMULUS POLYPHEMUS* AND *BIRGUS LATRO*

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IMMUNOLOGIC OBSERVATIONS OF THE AGGLUTININS OF THE HEMOLYMPH OF *LIMULUS POLYPHEMUS* AND *BIRGUS LATRO**

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It has not been established[1] that invertebrate animals produce specific antibodies, as do vertebrates, in response to stimulation by foreign antigens.[2–6] However, there have been extensive reviews of invertebrate natural antibody-like agglutinins to cellular components of other species, by Bisset,[7] Huff,[2] Cushing and Campbell.[8] Comprehensive studies of natural invertebrate agglutinins conducted by Tyler and Metz[9] indicated that chemically purified hemocyanin did not have agglutinating activity for sheep erythrocytes and spermatozoa of other marine species.

Limulus polyphemus, (horseshoe crab) a primitive marine anthropod, often classified as a member of an order of the Arachnida, was selected for such studies because of the large volume of hemolymph that can be harvested from individual animals. As much as 250 milliliters of hemolymph has been collected from a single specimen. *Limulus* has been labeled a "living fossil" because remains of this animal have been observed from the Triassic period of about 200 million years ago. Species of this genus are known from the Atlantic coast of North America, the East coast of Africa, the East coast of Asia, the Malayan Archipelago, and the Indian Ocean.

Throughout the Pacific Islands, especially in association with coconut palms, occurs a formidably large species of hermit crab, *Birgus latro* (coconut crab). This crustacean, through evolution, has adapted to living on land without the utilization of gastrapod shells as a home, except in its juvenile stages. Although its life history is poorly known, it has been recorded in early history as a dominant land animal on many islands in the Pacific Ocean. Its large size, from 1000 to 2670 grams, suggested its potential value as another invertebrate blood donor of suitable volumes of hemolymph for intensive immunochemical and serologic study.

Eriksson-Quensel and Svedberg,[10] first described an ultracentrifical pattern of four components with sedimentation rates of 57, 34, 16, and 6, respectively, in *Limulus* serum. Cohen, Rowe and Wissler[11] found four components with sedimentation rates of 80, 70, 51, and 14, respectively.

Noguchi[12] was the first to call attention to the agglutinins present in the serum or hemolymph of *Limulus polyphemus* (the horseshoe crab). These agglutinins differentiated red blood cells of amphibians and reptiles, without hemolyzing them. Marchalonis,[13] as well as Cohen *et al.*,[11] independently confirmed Noguchi's work and demonstrated agglutinins to a variety of vertebrate species. Marchalonis[13] and Cohen *et al.*[11] reported that hemacyanin

*This paper, illustrated with slides, was presented at a meeting of the Section on December 11, 1967. Reprints available from Buffalo, N. Y. address.

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of *Limulus* had no agglutinating activity. Tripp[5] reported that the hemagglutinin in oyster blood was similar to that of the agglutinin of the *Limulus*, as reported by Cohen *et al.*,[11] because of the following features:

a) agglutination of the erythrocytes of several vertebrate species
b) stability at 56°C but not at 65°C
c) reactivity with a common antigen or complex of antigens on the erythrocytes of several vertebrate species
d) electrophoretic ability similar to that of vertebrate alpha or beta globulins. However, whereas, *Limulus* hemolymph contains a respiratory pigment, the blood of the oyster has no such functional protein, according to Galtsoff.[14]

Collaborative work with Dr. Alvin Watne, Joseph A. Migaiolo[15] and Vergil E. Lyon[15,16] has indicated that *Limulus* agglutinins could be utilized to harvest leukocytes and/or tumor cells from blood of patients with cancer.

A progress report is presented of recent immunologic observations of agglutinins of *Limulus* and *Birgus*.

MATERIALS AND METHODS

Limulus serum was obtained from the hemolymph of living specimens, obtained from Cape May Beach along the Atlantic Ocean, through the energetic efforts of Drs. Alan and Mabel Boyden[17] of the Serological Museum, Rutgers University. Such hemolymph was allowed to clot and the serum, clarified by centrifugation, was then shipped unfrozen, by air, to Buffalo, New York. On the other hand, serum from the hemolymph of *Birgus latro* was obtained at Eniwetok Atoll during the course of two biomedical missions, supported by the Atomic Energy Commission and the University of Hawaii. Gel formation, without associated clot retraction of the *Birgus* hemolymph, necessitated fragmentation of gel with applicator sticks and centrifugation to collect and clarify serum.

Hemagglutination tests were performed with serially diluted invertebrate sera-M/150 phosphate in 0.85% saline buffered to pH 7.2, identified as DHB (Difco hemagglutination buffered saline). Human or other vertebrate erythrocytes were prepared in 2% cell suspension for testing. Incubation was for 60 minutes at room temperature (25°C in Buffalo, New York, and 29°C in Eniwetok Atoll, Marshall Islands), or 4 to 6°C in the refrigerator, followed by centrifugation at 1000 rpm for one minute in an International Clinical Model Centrifuge. Reading of agglutination was done macroscopically with score (summation of degree of agglutination values of each tube), as well as titer recorded. The "score" was utilized to demonstrate differences in avidity of agglutination.

Enzyme treatment of erythrocytes was carried out with 10,000 micrograms per milliliter of pancreatic protease (Nutritional Biochem. Co.), as described by Neter *et al.*[18]

Immunodiffusion was carried out in Difco agar 1% in saline, buffered to pH 7.2 by M/150 phosphate in Petri dishes. Reaction wells contained 0.2 milliliter in volume and were spaced one centimeter apart. Rabbit anti-*Limulus* serum and rabbit anti-*Birgus* serum were produced by two courses of subcutaneous immunizations of white New Zealand adult rabbits.

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Electrophoresis in cellulose acetate medium was performed with a Gelman Model 51170-1 electrophoresis chamber, 30 minutes at 1.5 millionamperes per strip, in a pH 8.6.

Immunoelectrophoresis was performed on microscope slides by the method of Scheidigger.[19]

RESULTS AND DISCUSSION

Agglutinins of Limulus polyphemus

The average agglutinin titers of nine lots of *Limulus* sera with 2% human erythrocyte suspensions were found to be 1/32, 1/16, and 1/16 at 4°C, 25°C, and 37.5°C, respectively. Summations of degrees of agglutination were 15–19, 13–15, and 12–15, respectively. The same erythrocyte donor was used for all titrations. Minimal low temperature enhancement of agglutination could be demonstrated with cells of other donors. Temperature optima for 60 minutes incubation were concluded to be 4°C and 20°C. All experiments were conducted with M/150 phosphate buffer.

A differentially greater specificity has been noted of *Limulus* agglutinins for human group A and B erythrocyte antigens than for group O (H) antigen, as shown in TABLE 1. Two lots of *Limulus* serum sent to the Ortho Research Foundation showed anti-A plus anti-B and anti-A activity when absorbed with group O Rh_O(D) cells and group AB Rh_O(D) cells at room temperature, but were nonspecific at 4.0°C, according to Miss Marjory Stroup.

Inhibition of hemagglutination of human erythrocytes by commercial purified group A or group B substance was investigated. *Limulus* serum incubated at room temperature with equal volumes of substance (10 to 25 mg/ml) prior to testing would give more than a two-tube depression of titers. However, no significant difference of degree of inhibition was observed between the A or B blood group specific substance. Neither has precipitin arc formation been observed between *Limulus* serum and blood group substances in agar gel diffusion plates.

Limulus serum gave a higher titer and scoring of the agglutination of human erythrocytes in contrast to those of rhesus and stump-tail monkeys, based on tests with the erythrocytes of three different individual rhesus monkeys and three different individual stump-tail monkeys, as shown in TABLE 2. Agglutination, absorption, and elution experiments with erythrocytes of human and selected nonhuman primate species would be interesting to critically evaluate taxonomic application of this property of the *Limulus* agglutinins.

Experiments with erythrocytes of human and sheep have suggested that a common antigen exists on the cells of both species. TABLE 3 presents results of a representative experiment. Absorption of *Limulus* serum by sheep cells does not completely remove agglutinins to sheep cells after the first absorption. However, subsequent absorptions with sheep erythrocytes can completely remove agglutinin activity for human erythrocytes. This experiment has been repeated at different temperatures and with reciprocal species cell absorptions, but with the same result.

Sheep erythrocytes are agglutinated, but with lower titers and scores than human erythrocytes. Alligator erythrocytes are not agglutinated by all lots of *Limulus* serum. Yet, it had been observed that alligator erythrocytes could

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TABLE 1

EVALUATION OF ABO SPECIFICITY OF *LIMULUS* AGGLUTININS

	Reciprocals of titer	Agglutination Score
Absorbed *Limulus* with O cells		
Tested with O cells	16	14
A cells	32	16
B cells	32	14.5
AB cells	16	12
Absorbed *Limulus* with B cells		
Tested with O cells	16	12
A cells	16	12
B cells	16	12
AB cells	16	12
Unabsorbed *Limulus*		
Tested with O cells	64	18
A cells	64	18
B cells	64	17.5
AB cells	32	17
Absorbed *Limulus* with A cells		
Tested with O cells	16	9
A cells	16	10
B cells	16	8.5
AB cells	8	8
Absorbed *Limulus* with AB cells		
Tested with O cells	16	12
A cells	16	12
B cells	16	12
AB cells	16	12

absorb out as much agglutinin activity as can human erythrocytes. It was hypothesized that the geometry of agglutinin receptor cites on alligator cells did not permit agglutination or else allowed only a small degree of agglutination. Protease treatment of alligator cells, prior to incubation with test serum, rendered them agglutinable to the same titer and score as human cells. Protease-treated alligator cells did not absorb any more agglutinin from serum than did untreated cells. It was concluded that receptor sites on

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TABLE 2

HEMAGGLUTINATION OF MONKEY AND HUMAN ERYTHROCYTES WITH *LIMULUS* (#62-1) SERUM

	Reciprocal of dilution								
	UD	2	4	8	16	32	64	128	Saline
Human									
O Rh_0(D)	4	3.5	2.5	1.5	1.5	1-	0	0	0
O Rh_0(D)	4	3.5	2.5	1.5	2	1-	0	0	0
O Rh_0(D)	4	4	2.5	2	2	0	0	0	0
Monkey									
R 1	4	4	2.5	0	0	0	0	0	0
R 2	4	4	2.5	0	0	0	0	0	0
R 3	4	4	3	0	0	0	0	0	0
S 1	4	4	2.5	0	0	0	0	0	0
S 2	4	4	2.5	0	0	0	0	0	0
S 3	4	4	3	0	0	0	0	0	0

R—Rhesus monkey (Macaca Mulatta)
S—Stump-tail monkey (Macaca Speciosa)

TABLE 3

ABSORPTION OF *LIMULUS* AGGLUTININS TESTED WITH 2% HUMAN CELL SUSPENSION

	Reciprocal of titer							
	UD	2	4	8	16	32	64	Saline
Unabsorbed *Limulus* serum	3	2.5	2.5	1.5	1	1	0	0
Absorbed with human cells 1X	1.5	.5	1.5	0	0	0	0	
Absorbed with human cells 2X	0	0	0	0	0	0	0	
Absorbed with human cells 3X	0	0	0	0	0	0	0	
Absorbed with sheep cells 1X	2.5	1.5	1.5	1	0	0	0	
Absorbed with sheep cells 2X	1	1	1	0	0	0	0	
Absorbed with sheep cells 3X	0	0	0	0	0	0	0	

All incubation done at 4°C.

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TABLE 4

EFFECT OF PROTEASE TREATMENT ON HEMAGGLUTINATION BY *LIMULUS* SERUM

Erythrocytes					
Alligator		Sheep		Human	
Untreated titer-score	Treated titer-score	Untreated titer-score	Treated titer-score	Untreated titer-score	Treated titer-score
0-0	64-22	8-9	64-19	16-13	64-20
U-0.5	128-23	8-11	32-16	16-16	128-22
Control					
0-0	0-0	0-0	0-0	0-0	0-0

Titer—Reciprocal of titer
Score—Summation of agglutination readings
U—Undiluted
Control—Saline substituted for serum

alligator erythrocytes are accessible to the agglutinin molecules, whether cells are protease treated or not. Enzyme treatment alters the surface of the erythrocyte so that cell-agglutinin-cell lattice formation is possible with subsequent clumping of cells. A similar explanation of the mechanism of action of proteolytic enzyme was proposed as necessary to permit agglutination of bacterial lipopolysaccharide-coated alligator red cells by homologous antibodies for the lipopolysaccharide that was used.[20] TABLES 4 and 5 present the results of experiments with protease-treated alligator erythrocytes.

Confirmation was made independently in our laboratory, and by Marchalonis,[13] of the earlier observation by Noguchi,[12] that *Limulus* serum does not hemolyze untreated erythrocytes. Addition of human or guinea pig complement did not facilitate demonstration of any hemolysin activity at 4°C, 25°C or 37.5°C. However, recent experiments in our laboratory have suggested that protease-treated human erythrocytes would be lysed when guinea pig complement was added to *Limulus* serum, but not by addition of guinea pig complement alone. This lysis occurred with the addition of complement to heat-inactivated (56°C for 30 minutes) *Limulus* serum. Native serum could lyse protease-treated cells, but not as well. This serologic observation was not made with every batch of *Limulus* serum and requires confirmation.

Absorption of *Limulus* serum with stromata of human erythrocytes removed agglutinins to erythrocytes, but also removed all agglutinins to leukocyte (lymphocytes) suggesting the presence of a common antigen. TABLE 6 presents a representative experiment.

The marked avidity of *Limulus* agglutinins to human erythrocytes was applied to the harvest of tumor cells and leukocytes from whole blood of cancer patients.[16] *Limulus* serum additive was compared with additives of other currently utilized sedimentation techniques, such as phytohemagglutinin, 6% dextran, and fibrinogen. *Limulus* serum was found equal to, but not superior to, saline additive control for the isolation of neutrophils and monocytes. Sedimentation of erythrocytes by *Limulus* serum yielded significantly greater amounts of monocytes from the whole blood of a particular

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TABLE 5

EFFECT OF PROTEASE TREATMENT ON HEMAGGLUTINATION BY *LIMULUS* SERUM

	Unabsorbed titer-score	Absorbed with untreated ARBC titer-score	Absorbed with treated ARBC titer-score
Tested with Human Erythrocytes			
	32-15	0-0	U-1
	32-18	U-1	2-3
	32-18	U-1	2-3
Control	0-0	0-0	0-0
Tested with Alligator Erythrocytes (ARBC)			
	512-31	0-0	0-0
	128-26	0-0*	0-0
Control	0-0	0-0	0-0

Titer—Reciprocal of titer
Score—Summation of agglutination readings
U—Undiluted
Control—Saline substituted for serum

TABLE 6

INHIBITION OF HEMAGGLUTINATION BY STROMATA

	Reciprocal of dilution						
	4	2	4	8	16	32	Saline
Tested with 0.5% RBC Suspension							
Limulus absorbed with:							
Saline	3	2	2	2	2	±	0
Stromata	2.5	2.5	2	1.5	1	0	
RBC	2	1.5	1.5	0	0	0	
WBC	3	3	2.5	2	±	0	
Tested with WBC							
Limulus absorbed with:							
Saline	2	1	0	0	0	0	0
Stromata	0	0	0	0	0	0	
RBC	0	±	0	0	0	0	
WBC	0	0	0	0	0	0	

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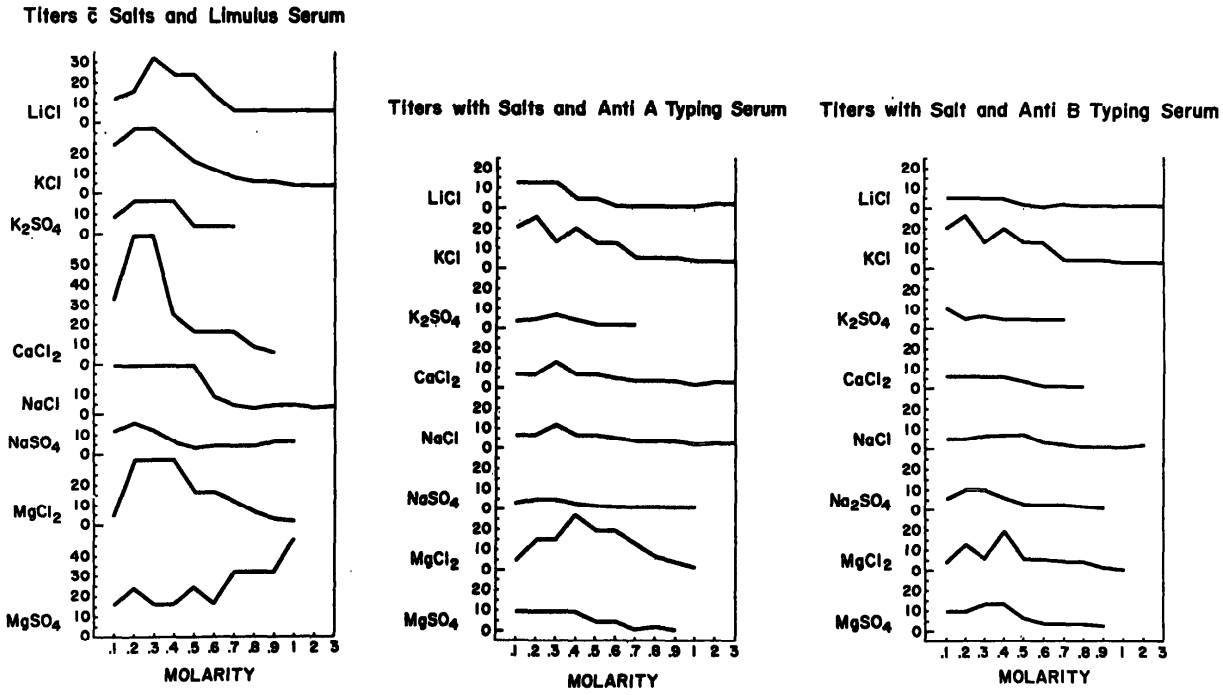

FIGURE 1. Effects of cations on hemagglutination titers. Values of titers are $\times 10^{-1}$ as shown on the vertical (ordinate) axis.

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human patient.[16] In addition, tumor cells harvested by use of *Limulus* serum, as an additive, gave better cytologic preparations for cytologic study. Nevertheless, the use of whole *Limulus* serum additive for sedimentation still leaves much to be desired.[20]

Cations of lithium, potassium, calcium, and sodium depressed *Limulus* and human (Anti-A and Anti-B) serum titers, but magnesium ions enhanced the avidity of the agglutinins of human and *Limulus* origin. These experiments, summarized in FIGURE 1, were a follow-up of the observations of Yokoyama and Finlayson[21] that indicated that the clumping of erythrocytes by isoagglutinins was influenced by the qualitative and quantitative nature of cations. It is evident that the pattern of the agglutinin titers of *Limulus* is similar to the patterns obtained with the human isoagglutinins anti-A and anti-B, respectively. Increased molarity of salt solutions may have caused changes in surface charges due to carboxylate groups, with complexes formed with the multivalent cations.[22] One might suggest that an increase in molarity of salt solutions may have altered the agglutinogens on the erythrocytes, rather than altering or salting out the agglutinins. The last possibility is excluded since, as was observed by Yokoyama and Finlayson[21] with human isoagglutinins, Na_2SO_4, a protein precipitant, decreased the agglutinin titer of *Limulus* agglutinins at 0.3 M, yet 0.95 M can be used to precipitate gamma globulins from serum.

Hemagglutination inhibition experiments were carried out with the serum of *Limulus*. It was evident that n-acetyl-D-glucosamine (NADG) could inhibit agglutination of human erythrocytes regardless of the ABO group, as shown in TABLE 7. A group of other selected saccharides or their derivatives did not inhibit agglutination. Inhibition by saccharides was the same for whole serum, ultracentrifugal supernatants, or eluates from agglutinated erythrocytes. Experiments with equimolar concentrations of the same saccharides also demonstrate inhibition by NADG. Inhibition by saccharides is of particular interest in view of a recent report of the inhibition of the weak A agglutinin of clam *(Saxidemus gigantus)* by NADG.[23]

TABLE 7

INHIBITION OF HEMAGGLUTINATION BY WHOLE *LIMULUS* SERUM WITH SELECTED SUGAR OR SUGAR DERIVATIVES

Inhibitor Sugar*	Reciprocal of dilution of *Limulus* serum								
	2	4	8	16	32	64	128	256	Saline
Melibiose	3	2	1	1	1	0	0	0	0
N-acetyl-D-glucosamine	4	±	0	0	0	0	0	0	0
Lactose	3	2	2	1	±	0	0	0	0
D-galactose	4	2	2	1	±	0	0	0	0
Saline control	3	2	2	1	±	0	0	0	0

*Concentration in 16 mg/0.2 ml.

NOTE: Group O, Rh_0(D) erythrocytes were used in 2% suspension. Same results with group A and B cells. No inhibition by: D-glucosamine, 2-desoxy-D-glucose, D-glucose, L-fucose.

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In view of the inhibitory effect of NADG, suggestive of sialic acid receptors, neuraminidase treatment of human erythrocytes was studied with the collaboration of Dr. F. Stig Nordling, of Finland, who was a Visiting Fellow working with Dr. Leonard Weiss of the Department of Experimental Biology at Roswell Park Memorial Institute. Treated cells gave minimal to completely negative agglutination, with two different lots of *Limulus* serum. Also, the supernatants of neuraminidase-treated cells could inhibit agglutination. The experiments suggested the possible importance of NADG of the erythrocyte coat, for agglutination by *Limulus* agglutinins.

Human rheumatoid factor (a 19S macroglobulin) is known to precipitate smaller molecular weight 7S (IgG) human and rabbit immunoglobulins.[24] However, *Limulus* serum heated to 65°C for 15 minutes to destroy its agglutinin activity was found to inhibit hemagglutination of rabbit gamma globulin-sensitized alligator erythrocytes by rheumatoid factor.[25] If the human erythrocyte agglutinins were absorbed from *Limulus* serum or from ultracentrifugal supernatants, such preparations would not inhibit hemagglutination by rheumatoid factor. That experiment suggested that the inhibitor of rheumatoid factor and the agglutinin of human erythrocytes were identical. However, the quality and quantity of carbohydrates associated with *Limulus* agglutinins has not been determined. Nevertheless, the possibility of applying this reaction to detection of rheumatoid factor is under investigation.

Cellulose acetate electrophoresis of *Limulus*, *Birgus*, and human sera reveals marked differences in the electrophoretic mobility of the protein constituents of those sera, as shown in FIGURE 2. *Birgus* serum contains a component of faster mobility than human albumin, whereas *Limulus* serum contains components of slower mobility than human albumins.

Ouchterlony agar-gel analysis of antigens of *Limulus* serum has been made with precipitating antisera produced in rabbits, alligators, and crocodiles. The rabbits "recognized" three to five antigenic components, whereas crocodiles and alligators "recognized" one antigenic component, hemocyanin. No immunochemical cross reaction could be shown in agar gel diffusion tests between *Birgus* and *Limulus* components.

Limulus agglutinins absorbed with human erythrocytes at 4, 20, or 37.5°C could be eluted by 56°C saline washes of erythrocyte agglutinates. Eluate from one milliliter of packed cells contained 10–30 mg/100 ml of total protein. The eluate protein analyzed by immunoelectrophoresis gave one arc, with mobility characteristics like an alpha human globulin. In Ouchterlony agar-gel diffusion analysis, the eluate yielded one major and minor band. Eluate protein is being isolated for further study.

Agglutinins of Birgus Serum

Total protein determinations by the biuret technique of sera of ten individual *Birgus* specimens ranged from 4.6 to 12.6 gm/100 ml. These results are in sharp contrast to the total protein values obtained for the serum of *Limulus* which usually average 4 to 8 gm/100 ml.

Sera collected in the field were tested at that time at room temperature (29°C) and at refrigerator temperature (6°C) for agglutination of human erythrocytes of groups A, G, and O. In TABLE 8 can be found the results obtained when the sera of seven individual crabs were titered. Other experi-

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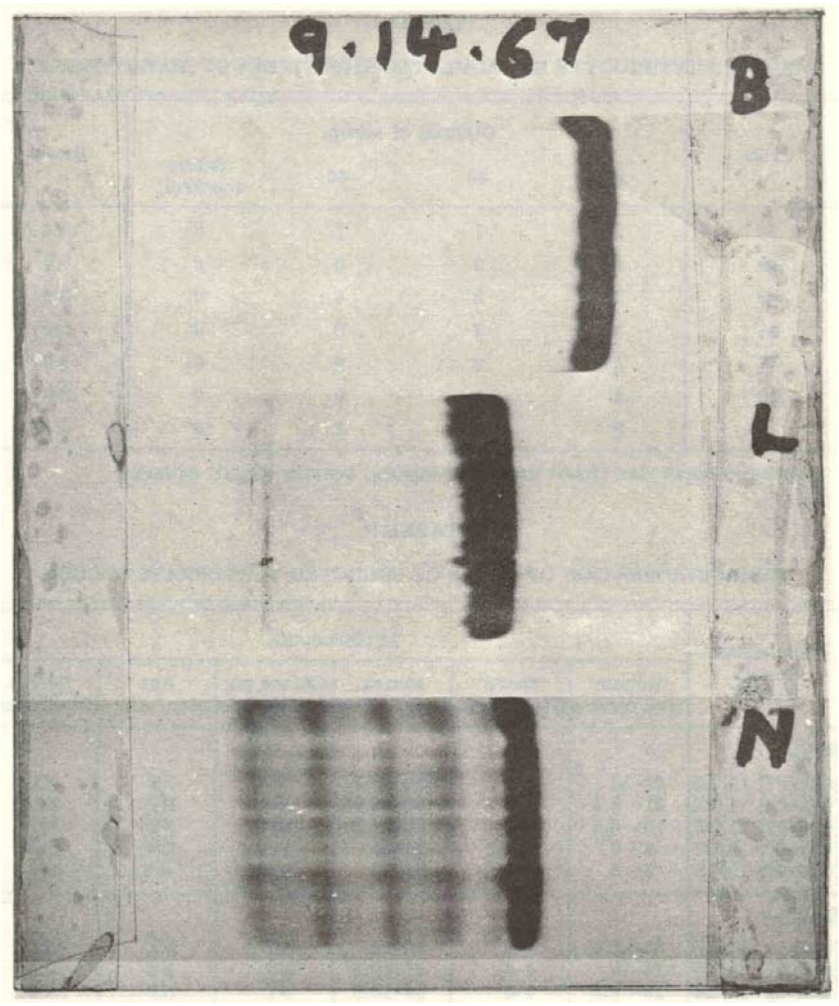

FIGURE 2. Cellulose acetate electrophoresis of *Birgus*, *Limulus*, and normal human serum.

ments indicated that no higher titer and score occurred with agglutination of human A or B cells than with O cells.

Titers and scores were compared using specimens of serum obtained at more than one bleeding, from five individual crabs over 628 grams in weight. Crabs 7, 8, 9, 10, and 15 gave hemagglutination scores of 6, 1, 5, 1, and 4, respectively, at bleedings on two different dates. It was possible to distinguish these individuals by their hemagglutination scores. However, no agglutinin activity was detectable in sera of seven individuals weighing 400 grams or

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TABLE 8

HUMAN ERYTHROCYTE HEMAGGLUTINATION TITERS OF *BIRGUS* SERUM

Crab	Dilution of serum				Score
	10	20	40	Saline (control)	
11	2	1	±	0	3.5
13	4	2	0	0	6.0
15	3	2	0	0	5.0
16	3	2	0	0	5.0
17	2	2	0	0	4.0
18	2	±	0	0	2.5
19	3	2	1	0	6.0

Human erythrocytes (donor graves) group A_1, bearing $Rh_o(D)$ antigen.

TABLE 9

-HEMAGGLUTINATION OF CELLS OF SELECTED VERTEBRATE SPECIES

Invertebrate Serum Tested	Erythrocytes					
	Human titer/score	Sheep titer/score	Mouse titer/score	Guinea pig titer/score	Rat titer/score	Rabbit titer/score
Birgus						
(#19-67) 11/22	32-13	0-0	256- 9.5	NT	NT	NT
(#20-67) 11/28	32- 9.5	0-0	64-10.5	NT	NT	NT
(#18-67) 11/28	16- 6.5	0-0	32- 7.5	NT	NT	NT
(#16-66) 9/27	4- 6	U-0.5	16-16	4-9	4-7	2-3
(#16-66) 10/18	2- 5	0-0	32-15	8-8	4-6	NT
Limulus						
(#67) 11/22	32-12.5	4-6	64-15	NT	NT	NT
(#63-3) 11/28	16-10	8-8	64-10.5	NT	NT	NT
(#63-8) 11/28	16- 8.5	4-6	64-10.5	NT	NT	NT

Human—Group O, bearing $Rh_o(D)$ antigen
Sheep—Pooled
Mouse—Swiss ICR/HA, male
Titer—Reciprocal of titer
Score—Summation of agglutination readings
U—Undiluted
NT—Not tested

less. Of 18 individuals collected in 1966, serum agglutinin activity appeared related to weight of the animals.

The sera of the larger (older?) animals gave the highest titers and/or scores, except the serum of one 900-gram animal, with no activity at all. Only one of the crabs tested was a female.

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Under favorable laboratory conditions, the temperature optima were determined for the serum of ten animals. Refrigerator (4 to 6°C) and the room temperature (25 to 29°C) gave the highest titers and scores in contrast to 37.5°C, as has been the experience with the serum of *Limulus*.

In tests of erythrocytes of different vertebrate origins, higher agglutination scores were obtained for ICR/HA Swiss mouse cells than for the human, English smooth-haired guinea pig, Charles River rat, or New Zealand white rabbit erythrocytes. Sheep erythrocytes gave the weakest agglutination of all. Tests were repeated with human, sheep, and mouse cells, as shown in TABLE 9.

In another series of 15 animals collected in 1967, a direct relationship was observed between agglutinin scores and carapace width. No agglutinin activity was detected in seven of the eight smallest animals of the series.

Variations in serum protein or agglutinin levels during the moulting process were not studied. Although the agglutinin of *Limulus* is not hemocyanin, it is important to note that Zuckerkandl[26] reported almost complete loss of hemocyanin in *Maia squinado* (spider crab) during the moulting cycle. The protein biochemistry of the moulting cycle is worthy of further study paralleled by immunochemical study of protein constituents, as well as extensive electrophoretic analyses, such as those by Manwell and Baker.[27] Immunochemical and serologic studies of *Birgus* agglutinins are incomplete and still in progress.

SPECIAL STUDIES OF *LIMULUS* SERUM AND SPECULATIONS

Removal of agglutinins from *Limulus* can be accomplished by multiple absorptions of the serum with equal volumes of fresh packed human erythrocytes. Changes can be observed in the ultracentrifugal pattern by the depression and virtual disappearance of the "peaks" of lighter (slower) components. However, there is also a depression of the peak of the 57S component (fast) (hemocyanin). Immunodiffusion analysis in agar gel indicates a similar phenomenon, weakening of the minor (agglutinin?) precipitin bands, accompanied by a decreased density of the major arc (hemocyanin), following repeated absorption.

One explanation may be that the hemocyanin molecules are trapped in the intercellular spaces of the packed erythrocyte mass during the centrifugation phase of absorption. Another explanation might be that the hemocyanin molecules are polymers that are in equilibrium with the agglutinin molecules, which are monomers. This idea was suggested by the physical studies of hemocyanins by Van Holde and Cohen,[28] who found that, with rises in pH above 7.5, the "molecule" divided into five identical pie-shaped pieces. Reassociation of two monomers to form a dimer, and of five dimers to form a decamer, occurred as the pH was first lowered below 10 and then below 7.5. Absorption of agglutinin molecules by erythrocytes could remove the magnesion ion which stabilizes the 57S component (hemocyanin). More agglutinin molecules would appear, at the expense of the polymer.

Although heating at 65°C or above destroyed agglutinin activity, freezing at -193°C and rapid thawing at 37.5°C for ten times was necessary before (two-tube dilution) reduction of agglutinin titer was observed. From that experiment, repeated several times, it was noted that the agglutinins were

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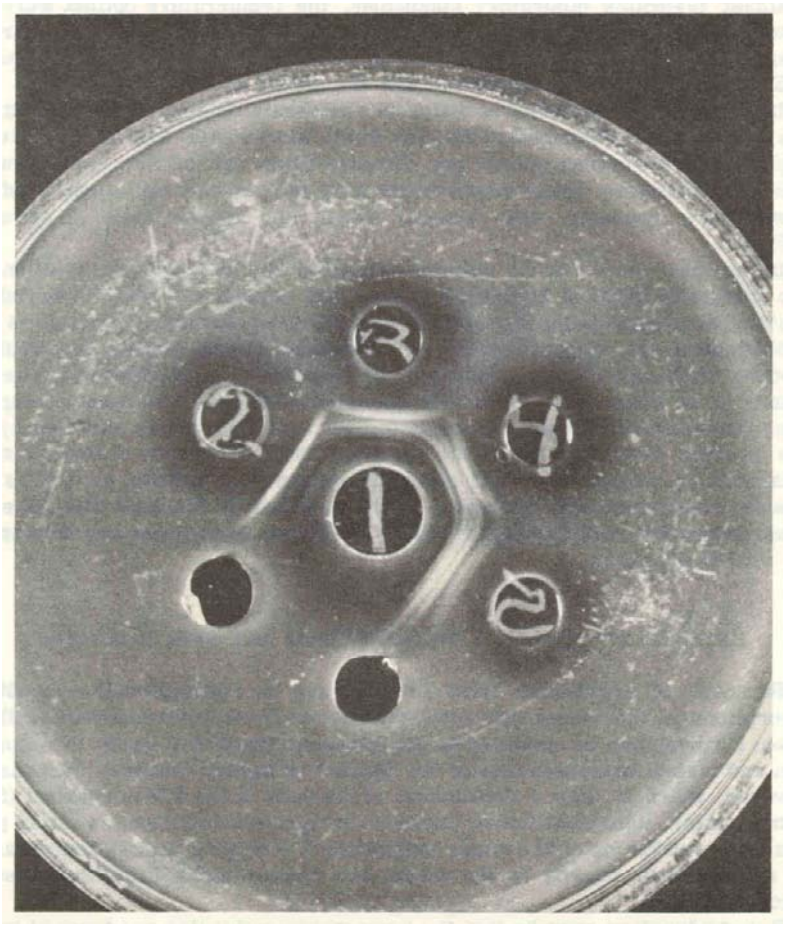

FIGURE 3. Effect of repeated freeze-thaw treatment. Well #1—rabbit anti-*Limulus* serum (C-175); well #2—unfrozen *Limulus* 62#2; well #3—*Limulus* freeze-thaw 1X; well #4—*Limulus* freeze-thaw 5X; and well #5—*Limulus* freeze-thaw 10X.

relatively stable to freeze-thaw treatment. Immunodiffusion analysis of *Limulus* serum freeze-thawed one or five or ten times, respectively, demonstrated an increase in the number of precipitin bands following successive freeze-thaws. This was accompanied by a decrease in the density of the major arc (hemocyanin), as described above, after absorption of agglutinins. A representative freeze-thaw experiment is shown in FIGURE 3.

Ultracentrifugal analysis of mercaptoethanol-treated *Limulus* serum showed an increase in the concentration (area) of the slow components (less than 57S), with accompanying decrease in hemagglutination activity. This breaking into subunits is also common with vertebrate agglutinins of the

gamma M(19S) class in contrast to those of the gamma G(7S) class, as described for vertebrate agglutinins by Deutsch and Morton.[29]

Triple arc immunodiffusion patterns obtained by Tornabene and Bartel[30] at pH 7.5, between rabbit anti-Keyhole Limpet hemocyanin (KLH) serum and its antigen, replicate the patterns observed in our laboratory with rabbit anti-whole *Limulus* serum and its antigen, at pH 7.5. However, Malley, Saha and Halliday[31] also purified *Limulus* hemocyanin by centrifugation and observed a single arc on immunodiffusion. Weigle[32] has emphasized that the degree of dissociation of hemocyanin, which is a function of pH, affects the qualitative and quantitative results of immunochemical analyses.

Marcholonis and Edelman[33] isolated and studied the natural hemagglutinin of *Limulus* in order to compare its structure with that of vertebrate immunoglobulins. They find it to be a protein with a molecular weight of approximately 400,000, which sediments with $s^{o}_{20,W} = 13.5S$ and migrates electrophoretically as a beta-globulin. The molecule is composed of subunits, each with a molecular weight of 23,000, linked through monovalent interactions. Agglutinating activity was potentiated by calcium ions, as observed in our own experiments presented in FIGURE 1. Starch gel electrophoresis, amino acid analysis, two-dimensional high voltage electrophoresis of tryptic peptides and immunologic analyses were performed on the purified hemagglutinin. The hemagglutinin structure was found to be different than that of mammalian immunoglobulins. Marcholonis and Edelman[33] do not believe that there is evidence that *Limulus* hemagglutinin and immunoglobulins are related evolutionary developments.

The expressed body fluids of the land snail, *Otala lactea*, were reported recently by Boyd and Brown[34] to contain strong high titer agglutinins for human A_1 erythrocytes that could differentiate A_1 from A_2 cells. In our laboratory, *Limulus* agglutinins could distinguish A_1 and A_2 human erythrocytes and, in particular, A_1 from A_x cells. The degree to which *Limulus* agglutinin can identify homozygous AA from heterozygous AO erythrocytes was not established.

In summary, serum obtained from the hemolymph of *Limulus polyphemus* or from *Birgus latro* contains avid agglutinins for erythrocytes of most of the vertebrate species tested. Less data are available on the properties of agglutinins of *Birgus* than for those of *Limulus*. Although *Limulus* hemocyanin has been investigated extensively or used as an immunizing antigen by immunologists for a half of a century, little attention has been given to the potential or biomedical application of the agglutinins present in the serum. Differences and similarities of such agglutinins to vertebrate antibodies or phytohemagglutinins have just begun to be studied. Levin and Bang,[35] in an elegant study, indicated that the blood of *Limulus* contains only one type of cell, the amoebocyte, which resembles and functions like the mammalian platelet in presence of endotoxin. One might speculate that the phylogeny of the immune response in the invertebrate is reflected by the presence of only one or more blood proteins with manifold properties, i.e., hemocyanin as a source of agglutinins. However, there is no laboratory evidence that *Limulus* agglutinins are fragments of the hemocyanin molecule. The agglutinins of *Limulus* or *Birgus* may have provided some selective advantage to the animals during the course of their evolution, although it is possible that these proteins that we label "agglutinins" may be part of a saccharide transport or storage

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mechanism associated with the shell formation of the animals. It is anticipated that beyond provocative speculations as to how *Limulus* or *Birgus* agglutinins fit in the phylogeny of the immune response, the biomedical application of natural cellular agglutinins of invertebrates will be investigated in the same manner as were the phytohemagglutinins.[36] Research into *Limulus*, *Birgus* and other invertebrate sera, as immunohematologic reagents for blood grouping, tissue culture and cytogenetics are already in progress in our laboratory.

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