1971

Characterization Of Erythrocyte-Reactive Factors Of Panulirus Argus--A Contribution To Immunophylogeny.

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CHARACTERIZATION OF ERYTHROCYTE-REACTIVE FACTORS OF *PANULIRUS ARGUS*--A CONTRIBUTION TO IMMUNOPHYLOGENY

by

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A DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in the
Department of Microbiology of the
Graduate School of the
University of Alabama in Birmingham

Birmingham, Alabama

1970

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA IN BIRMINGHAM
DISSERTATION APPROVAL FORM

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Major Subject Microbiology

Title of Dissertation Characterization of Erythrocyte-Reactive Factors of Panulirus Argus -- A Contribution to Immunophylogeny

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Acknowledgements

I wish to express my appreciation to Dr. E. E. Evans, chairman of my dissertation committee, for his never-ending interest, encouragement, instruction, and example.

I also wish to thank the members of my dissertation committee, Dr. Gerald Carlson, Dr. Thomas Feary, and Dr. Raymond Hiramoto for their close interest in my course work and research activities.

Special thanks is due Dr. J. Claude Bennett. In many ways he has been helpful to me, and I wish to record here my deep personal respect for him.

Support was provided by Training Grant 5-TOI-AI-00293 from the United States Public Health Service. Various aspects of this investigation were supported in part by United States Public Health Service Grants AI-02693 and AI-00293.

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CHAPTER I

INTRODUCTION

There have been numerous investigations of immunophylogeny during the past decade. Some groups have stressed the molecular evolution of the immunoglobulins, while others have concentrated on complement and complement-mediated responses. The rationale behind such a phylogenetic approach has been that the development of immunologic complexity might parallel the anatomic features which formed the original basis of the evolutionary sequence. Thus, the study of existing animal species at different phylogenetic levels might serve to elucidate the evolution of immunity. This approach should offer insights on some of the basic unresolved questions regarding the nature and mechanisms of immune responses.

One current view of immunologic phylogenesis is based on results which revealed molecular structures in some primitive vertebrates to be as fully complex as those described in mammals. This is well illustrated by the primary structural data accumulated from the immunoglobulin polypeptide chains of many phylogenetically distant species. Comparison of these data reveal a structural complexity common to all. Even the primitive shark has been shown to possess both heavy and light polypeptide chains; the latter existed as both kappa and lambda types and displayed a high degree

of heterogeneity (41).

It is recognized that the evolutionary process is slow and extremely conservative. Therefore, it is not common to find abrupt changes leading to entirely new structures or molecules which are without precedent in simpler forms. This striking conservatism is best illustrated by various functional molecules such as the respiratory proteins of mammals. These proteins have been shown on the basis of primary structural data to have evolved from a common, phylogenetically distant, ancestor. In this light it seems appropriate to ask questions concerning the ancestral precursors of vertebrate immune responses such as antibodies and complement systems, and to look for answers to these questions in representative members of the invertebrate phyla (23).

The present investigation was undertaken to determine whether certain of the humoral factors observed in a representative crustacean, the West Indian spiny lobster, Panulirus argus, represent primitive or precursor immune phenomena related to those of vertebrates. Specifically, experiments were conducted to: (1) characterize the naturally-occurring hemolytic system of the lobster, (2) explore the natural hemagglutinin of this species to see whether it is structurally related to vertebrate immunoglobulins, and (3) study the evolutionary significance of these humoral factors.

Review of Literature

The mechanisms by which members of the invertebrate phyla combat diseases of viral and bacterial origin are not as well characterized as the immune mechanisms of the vertebrate species, but evidence indicates that the invertebrates possess "immunity" and can protect themselves against potentially harmful foreign substances. In most instances this is accomplished at the cellular level by the processes of phagocytosis (5, 40, 71), encapsulation, segregation and nodule formation (72).

The general literature on invertebrate immunity has been discussed in detail by a number of reviewers (26, 43, 95, 34) and need not be repeated here. However, certain papers pertinent to invertebrate humoral immunity will be described because they are related to the major contributions of this thesis.

Phylum PORIFERA

Studies by Jakowska and Nigrelli (44) showed that fluids extracted from freshly collected subtropical sponges had a high degree of activity against microbes isolated from the spongocoel, as well as against other organisms employed routinely for assaying such activity. One such antibacterial factor, extracted from the North Atlantic red-beard sponge, Microciona prolifera, was named "ectyonin".

It has been reported (44) that sponges were apparently resis-

tant to the action of the multitude of microorganisms that entered their complex system of canals and cavities (spongocoels). The role that antibacterial fluids extracted from sponges might play in this resistance is not presently known.

Phylum COELENTERATA

Only limited evidence is available for immune-like phenomena in this group of animals; however, one study by Phillips and Yardley reported the induction of a substance resembling antibody in the sea anemone, Anthopleura elegantissima. The rise of this substance followed injections of bovine serum albumin (68). Interpretation of these results is difficult from the equivocal data presented and additional work will be necessary before the immune capabilities of this coelenterate can be evaluated.

Phylum ECHINODERMATA

The echinoderms (e.g. seastar, sea urchin) share with the mollusks, annelids and arthropods the distinction of reaching the highest organization of the invertebrates. Of all invertebrates, echinoderms are placed nearest to the chordates because of a vertebrate-like mesodermal endoskeleton and a similar pattern of embryonic development (39). In light of this close relationship, the echinoderms would seem likely candidates for humoral immune mechanisms.

Hilgard and Phillips have reported the sea urchin, Strongylocentrotus purporatus, capable of responding selectively to injections of human and bovine serum albumin (40). However, accelerated clearance or uptake of these foreign proteins could not be demonstrated.

In another study utilizing sea urchins of the genus Strongylocentrotus (45), Johnson found that coelomocytes of the sea urchin (in vitro) were capable of lysing gram-negative bacteria, while introduction of gram-positive organisms resulted in marked phagocytosis. Apparently, bactericidal substances specific for gram-negative bacteria are released by the coelomocytes. Whether or not the same reaction occurs in vivo remains to be demonstrated.

These studies have not convincingly shown vertebrate-type antibodies operative in this group. In view of the close relationships between echinoderms and chordates, future studies should concentrate on representatives of this phylum.

Phylum SIPUNCULIDA

Many investigators have found that the marine sipunculid worms display a type of humoral immunity. Induced responses were investigated by Bang (7) and Evans et al. (26). Natural bactericidins have been reported by Rabin and Bang (70), Krassner (50), Cushing et al. (20), and Johnson and Chapman (46).

One of the most fruitful studies was by Bang (7) in which he injected marine protozoan parasites into sipunculid worms and found

that the worms produced a heat labile substance within 20 to 48 hours. This substance resulted in lysis of the parasites within 15 minutes after contact. Lytic substance was maintained for 5 to 8 days after which it was lost. The lytic substance was shown to reappear upon secondary injection but no increase in titer was detected. Additional experiments have shown that the lysin is not present in the coelomic fluid or plasma of the normal sipunculids. Although the lytic substance appeared in response to the injection of protozoa, it was shown that normal crab blood or massive inocula of bacteria would also provoke the reaction. No attempt was made to determine the nature of the lysin.

Recent investigations by Evans et al. with D. zostericolum showed this animal capable of an induced bactericidal response (26). Coelomic fluid from normal controls (non-immunized) had no bactericidal activity. Following injections of a gram-negative bacteria (EMB-1), 60% of the animals had developed bactericidal titers at the end of two months. Secondary injections of the same bacteria resulted in enhanced bactericidal titers in those animals showing a primary response.

Rabin and Bang (70) studied the antibacterial activity of the coelomic fluid of the marine sipunculid worm Golfingia gouldii against a marine vibrio in vitro. Fluids withdrawn from individual worms were mixed with suspensions of bacteria. After 3 hours' incubation of this mixture at 25°C, 66% of the individual fluids

showed antibacterial activity.

Bacteria were inactivated within 30 minutes after mixing with coelomic fluids which had this capacity, and the activity reached its peak by 5 hours. If bacteria and fluids were mixed and incubated at 11°C for 24 hours, 95% of individual fluids showed antibacterial activity. Coelomic fluids from which cells had been removed were tested for antibacterial activity; 72% of cell-free fluids from individual worms were positive. Prior inoculation of the worms with bacteria had little effect on *in vitro* antibacterial activity.

Krassner (50) reported a substance released from the cells of the coelomic fluid of the sipunculid worm, *Phascolosoma gouldii*, which killed several different marine bacteria. However, injection of bacteria did not induce higher bactericidal titers.

The coelomic fluid of the sipunculid worm, *Dendrostomum zostericolum*, has been recently shown by Cushing *et al.* (20) to rapidly immobilize the marine dinoflagellate *Cyrodinium dorsum*, and other similar mobile organisms. Prior injections of the dinoflagellate did not increase the activity of the immobilizing substances.

Johnson and Chapman (46) reported a natural bactericidin present in the coelomic fluid of this same species of marine worm. Whole coelomic fluid was strongly depressive or fatal to all gram-negative organisms tested. No effect was observed on the one gram-

positive bacterium included in the study. Cell-free coelomic fluid was less depressive or fatal in its effects suggesting the active principle to be released from cells or cell-bound.

Studies by Triplett et al. demonstrated naturally-occurring agglutinins for human erythrocytes in Dendrostomum zostericolum. However, attempts to stimulate specific agglutinins, lysins, and precipitins were not successful (88). Additional investigations involving D. zostericolum by Weinheimer et al. demonstrated that the coelomic fluid could lyse and agglutinate a wide variety of vertebrate erythrocytes (98). The role of lysine and agglutinin as an immune mechanism is open to speculation.

From the results of the studies presented it is apparent that certain members of phylum sipunculida are capable of both natural and inducible immune-like responses. Future investigations should focus on the complete characterization of such responses so that a valid comparison to similar vertebrate immune mechanisms can be made.

Phylum MOLLUSCA

Members of the phylum mollusca have received special attention due to their economic importance to man. A number of comprehensive reviews by Stauber (78), Cheng (14), Tripp (90), and Feng (28) discuss defense reactions and other mechanisms common to this group of animals.

Reports of nonspecific humoral factors with antibiotic-like

action have been made, but are not numerous. Lysozyme has been demonstrated in the hemolymph of the oyster Crassostrea virginica by McDade and Tripp (55). It was thought initially that this lysozyme played a significant part in the oyster's defense mechanism. However, further detailed study (56) showed this hemolymph lysozyme to be an artifact resulting from a "spillover" of enzyme secreted into mucus layers on epithelial surfaces.

A variety of extracts from marine mollusks such as the abalone, clam, conch, and sea snail have been shown by Li et al. (52) to have antibacterial and antiviral activity both in vitro and in vivo. Characterization of these extracts was not undertaken.

Schmeer (74) reported Mercenene, a substance extracted from the clam, Mercenaria mercenaria, to possess antitumor activity. This material was thought to be a polypeptide; however, its mode of action has not yet been demonstrated.

In addition to these nonspecific factors there have been suggestions of immune type of responses in mollusks. Studies by Tripp (89) employing the snail, Australorbis glabratus, have shown this animal to possess a sophisticated recognition mechanism able to determine "self" from "not self". Implanted homologous tissue elicited no response; however, when tissue from another snail of a different genus was implanted, the "foreign" tissue was quickly encapsulted and deteriorated.

Using this same species of snail, Michelson demonstrated an

acquired type of immunity (61). Animals receiving injections of the digenetic trematode Schistosoma mansoni produced an immobilizing substance within two weeks after injection and normal controls did not show immobilizing activity. The active substance could be absorbed by living or killed trematodes. Substances with similar immobilizing power were found in tissue extracts of uninfected Bulinus truncatus and Helisoma caribalum as well.

Hemolymphs from a variety of mollusks have been shown to contain natural hemagglutinins for vertebrate erythrocytes (58). Tyler described many naturally-occurring hemagglutinins found in body and seminal fluids of these animals. The only study of mechanism of action of such substances utilized an oyster hemagglutinin and suggested that the protein reacted with ABO (H) haptens of human cells, but at different sites on cells of other species (92). In the oyster, Crassostrea virginica, studies by Acton et al. showed that calcium tended to stabilize the protein hemagglutinin. Under certain conditions it was degraded to protein subunits with a molecular weight of approximately 20,000. Peptide mapping, amino acid analysis, carbohydrate analysis, and limited sequence data suggested that this material was not comparable to mammalian immunoglobulin light chains; however, more extensive sequence data must be obtained before any definite conclusion can be reached (1).

Acton and Evans (2) have recently shown that the oyster will clear secondary injections of T2 coliphage more rapidly than

primary injections. The results, showing an enhanced secondary clearance of virus, most likely have a cellular basis since a virus-neutralizing humoral factor was not detected.

Attempts by Weinheimer et al. to demonstrate a bactericidal response in the oyster were not successful. Gram-negative bacteria isolated from tissue homogenates were used to assay for bactericidal activity in normal hemolymph and in hemolymph from oysters previously injected with vaccines prepared from the gram-negative tissue isolates (99).

Phylum ANNELIDA

At present, there is no convincing evidence for humoral immune responses in the annelids. An attempt by Cooper et al. to demonstrate bactericidal response in the earthworm using its normal flora as both vaccine and assay organism was unsuccessful despite the large number of animals assayed (18). However, in earlier studies, Cooper showed the earthworm capable of graft rejection. In a series of experiments, Cooper was able to demonstrate the acceptance of auto-grafts, but xenografts exchanged between two different species of worms were destroyed (17). Rejection of second-set xenografts was observed to occur at an accelerated rate over the first-set grafts. Additional studies using Lumbricus terrestris showed this animal to have the capability of distinguishing among allo-, auto- and xeno-genic tissue antigens (17). The studies of Cooper suggest that

earthworms are capable of cellular, but not a humoral immune response.

Phylum ARTHROPODA

Class Insecta. A review of the literature on invertebrate immunity reveals that more work has been done with insects than any other class of animals. Diseases of insects are mainly generalized, bacteremic conditions, and the great majority are caused by gram-negative coccobacilli (12). Gram-positive cocci have been isolated from a small number of infections of insects; e.g. a Streptococcus from a disease of caterpillars of the gypsymoth; a Staphylococcus from locusts; and a Staphylococcus in one of the rare examples of a disease of adult horseflies (95).

Some of the outstanding work concerning humoral immunity in insects was completed by Briggs (11). Briggs used the larvae of 11 species of Lepidoptera as experimental insects. Live and attenuated suspensions of bacteria (pathogenic and non-pathogenic for insects) were used as particulate antigens, and solutions of ovalbumin were used as soluble antigens. Standard serological techniques, such as agglutinin, precipitin and complement-fixation tests were found not to be adaptable for demonstration of natural or actively acquired immune properties in the hemolymph of lepidopterous larvae. The hemolymph of larvae injected with various live and attenuated bacterial suspensions showed the rise of an extremely heatstable antibacterial principle, demonstrated in vitro, a few hours after injec-

tion. The acquired antibacterial property in the larval hemolymph was shown not to be entirely specific in its action, was retained throughout larval life, resisted exposure to acid and alkali, but was lost when exposed to the action of pepsin. Thus, this thermostable antibacterial substance resembles in some respects the B-lysins, leukins or plakins found in some vertebrate serum.

This work by Briggs established the fact that some insects have the faculty to acquire a demonstrable immunological response. However, the results of Briggs suggest that an entirely new concept must be advanced with reference to the type of "antibodies" formed, the nature of the active principle in these acquired immunological responses, and the significance of these responses.

Duncan (21) showed that a bactericidin was present in the gut-contents of several adult insects and arachnids. The bactericidal action was greater and more rapid at 30°C than at room temperature. Activity of dried material was unimpaired for at least six months. It was thermostable and resisted tryptic digestion. There is no doubt that the active principle was found in the stomach, but its cellular source and biochemical identity require further investigation.

Frings et al. (31) reported that the blood of the large milkweed bug, Oncopeltus fasciatus, contained an antibacterial agent active in vitro against Staphylococcus aureus and one strain of Bacillus subtilis.

The bactericidin was water soluble, stable to boiling for 30 minutes, destroyed by autoclaving, and was active at a dilution of at least 1/10,000. The active principle was said not to be a protein. Further work on the bactericidin of O. fasciatus was carried out recently by Gingrich (33). He found that blood from one insect was roughly equivalent to 1 Oxford unit of penicillin when tested in vitro with S. aureus. The antibacterial substance was water soluble, insoluble in ether and ethyl alcohol, and was not precipitated by boiling.

Bernheimer et al. (9) used caterpillar larvae to test the ability of insects to produce antibodies to substances known to be antigenic in higher animals. In one experiment they injected larvae with human erythrocytes and found that the hemolymph of some of the larvae did acquire agglutinins for the erythrocytes. Bernheimer concluded that caterpillar larvae did not form circulating antibodies upon stimulation with foreign antigen with exception of the case mentioned above.

An extensive amount of research has been conducted by Stephens using the blood of the larval wax moth, Galleria mellonella (80-84). Stephens reported that bactericidal activity was inducible in the normal wax moth larvae. Normal wax moth larval blood was not bactericidal for Pseudomonas aeruginosa, but upon injection of 0.01 ml vaccine the blood became bactericidal. This immune state was detectable 18-24 hours after injection with the vaccine and

lasted until about the third day.

In another report by Stephens (79) in which the bactericidal activity of hemolymph from normal insects was surveyed, it was found that insects generally showed no activity against bacteria that were pathogens, but exhibited varying degrees of bactericidal activity against non-pathogens.

It thus appears from the work of Briggs (11), Gingrich (33), and Stephens (79-84) that weak, non-specific antibacterial or bactericidal substances may be found in the blood of insects, and these substances can be quantitatively enhances by injection of specific or non-specific antigens. All of these investigators concluded that antibodies, as they are known in vertebrates, do not exist in insect blood.

In a more recent study by Kamon (48) the response of locusts to scorpion venom was tested. The specificity of the immune response of Locusta migrathria migratoriades was tested as follows: saline, china ink, charcoal, amylase and bovine serum albumin were injected. Twenty-four hours after the injection of each substance, the locusts were challenged with 2-3 times the LD_{50} dose of venom of Leiurus quinquestriatus.

No immunity was observed in locusts injected with saline, china ink or charcoal. A significant immune response was obtained in locusts challenged after injection with amylase and bovine serum albumin. The response appeared to depend on the number of injections

rather than on the quantity of the substance injected.

Bettini (10) recently detected an acquired immune response of the house fly to injected spider venom. In this study, groups of house flies were injected with a low dose of crude venom of the spider Latrodectus mactans tredecimguttatus. The flies were reinjected at different time intervals with a challenge dose of venom. Within 24 hours inoculated house flies developed a low but significant degree of immunity against the venom. This immunity appeared to increase between 24 and 48 hours and to last for 96 hours. Latrodectus venom thus represented the second toxin (the first being scorpion venom) showing a protective immunological response in insects. Musca domestica (house fly) is the first species of the order Diptera to show protective immunity following inoculation with a sublethal dose of antigen.

In insects there seems to be a little evidence for antibodies similar to those found in vertebrates; however, there is evidence that insect blood contains inducible substances, but these factors are incompletely understood (38). Whatever may be the physiological mechanisms by which insects acquire resistance to bacterial infection, the phenomenon bears a striking resemblance to the acquired resistance in vertebrates that goes by such names as "non-specific immunity", "pro-immunity" and "interference immunity" (95).

Class Merostomata. The only survivor of this ancient and extinct class is often referred to as a "living fossil" and is

termed Limulus polyphemus or the "horseshoe crab".

In examining the hemolymph of the horseshoe crab, Cohen et al. found heteroagglutinins to a variety of vertebrate erythrocytes (16). Bang observed that the hemolymph from this animal formed a gel-like mass when combined with living bacteria or bacterial toxins (4). In other studies, Noguchi reported the formation of an inducible hemolysin in the hemolymph of the horseshoe crab following injections of fish erythrocytes. The hemolytic activity was heat labile and not present in normal controls (65).

Perhaps the most revealing study was that of Marchalonis and Edelman (60) in which they isolated and characterized the naturally-occurring hemagglutinin from the horseshoe crab, Limulus polyphemus. The hemagglutinating activity migrated electrophoretically with mobilities of alpha and beta globulins, and could be enhanced by the addition of calcium ions. Physicochemical studies revealed the purified agglutinin to have a molecular weight of approximately 400,000 and an $S^{o}_{20,w}$ value of 13.5s. The molecule consisted of subunits of about 22,500 molecular weight held together through non-covalent bonds. Amino acid composition analysis, peptide maps and immunological studies showed the agglutinin to be different from mammalian immunoglobulins. Electron microscopic data indicated a ring-shaped structure consisting of eighteen subunits (29). Despite the obvious differences between this hemagglutinin molecule and vertebrate immunoglobulins, these data provide a basis for comparison

with other molecules implicated in invertebrate immune phenomena.

Class Crustacea. Early work by Tyler and Metz (93) demonstrated that the hemolymph of the spiny lobster, Panulirus interruptus, contained at least ten heteroagglutinins for sperm or blood cells of various vertebrate and invertebrate species. The heteroagglutinins were found to be protein in nature and by means of electrophoresis were shown to be distinct from hemocyanin.

In a study involving a small number of animals, Taylor et al. (86) inoculated T1 bacteriophage into the hemocoel of the shore crab, Carcinus maenas. Two crabs cleared the inoculum completely, one within 42 days and the other within 70 days postinjection. The level in a third crab dropped as well, but the animal died before total clearance was achieved. The two survivors received a second inoculation of phage. Secondary clearance was more rapid than primary in both instances. These studies have been confirmed by additional data from a larger number of animals (63). These results contrast with those reported by Cushing (20). In his review, Cushing states that he and McNeely found that bacteriophage T4 persisted for long periods of time in the California spiny lobster and two species of crabs (not specified).

Teague et al. (87) examined the crayfish Cambarus virulis for an enhanced clearance of Ø X 174 bacteriophage. The experiment was concluded when the animals failed to clear phage after 88 days. Because of Taylor's findings (86), it seems possible that termination

of the study was premature and the length of time the animals were under observation should have been extended.

Studies by Schwab et al. revealed that normal hemolymph from the crayfish Parachaeraps bicarinatus contained bactericidal activity for several strains of gram-negative and one strain of gram-positive bacteria (75). The bactericidin was partially purified and found to be associated with less than 2% of the total hemolymph proteins, with a molecular weight of approximately 10,000. No attempt was made to enhance activity by injections of bacteria. McKay et al., using the same species of crayfish, found the hemolymph to contain hemagglutinins for a number of vertebrate erythrocytes (58). It was their contention that since these hemagglutinins "mimic" mammalian antibodies by acting as opsonins, they might represent primitive or precursor immunoglobulins. Subsequent studies by McKay and Jenkin (57) have shown this crayfish capable of developing resistance to a pathogenic bacterium following immunization. The form of the response in terms of the animal's resistance to infection was found to be similar to that observed in vertebrates. However, the immunity was relatively non-specific in that several vaccines from gram-negative bacteria were able to increase the resistance of the crayfish to subsequent Pseudomonas infection. The onset of the response was rapid and was first observed at one day, reaching a maximum by 2 to 3 days.

Evans and coworkers reported that the spiny lobster, Panulirus

argus, synthesized a nondialyzable bactericidin in response to injections of living or killed bacteria (25). The active substance was present in the hemolymph and usually detectable within 12 hours. In the primary response, it reached a peak within 24-48 hours. The most effective antigen was EMB-1, a gram-negative rod isolated from the normal gut flora of a healthy lobster. P. argus was found to respond to secondary and tertiary injections of EMB-1 in a manner reminiscent of specific anamnesis in vertebrate immunoglobulin synthesis (27). Persistence of activity for many weeks without additional stimulation suggested a type of immunological memory.

The bactericidin was found to be active against only gram-negative bacteria. The response was not entirely specific since the bactericidin induced by injections of EMB-1 was also reactive against Salmonella typhosa and vice versa (25). Additional studies demonstrated that saline solution, formalin or gram-positive organisms would not cause a significant rise in bactericidal titer (100). Inactivation by heating at 65°C for 20 minutes was observed, however, ethylenediamine tetraacetate (EDTA), dialysis, carageenin, and freezing had no effect. The bactericidin could be absorbed by homologous and heterologous bacteria (25).

More recently, a bactericidal response to EMB-1 has been demonstrated in the California spiny lobster, Panulirus interruptus. In this species, bactericidal activity reached a peak at 7 days after the primary injection and persisted for 60 days at high levels

reminiscent of antibody synthesis in higher vertebrates. As in the case of P. argus, specificity was only partial (24).

Acton et al. (3) reported a naturally-occurring bactericidin in the hemolymph of the American lobster, Homarus americanus. All animals employed in the study exhibited pre-existing bactericidal titers which could be enhanced by injections of the EMB-1 organism.

From the literature cited (summarized in Table I), it is apparent that members of the invertebrate phyla are capable of protecting themselves against potentially harmful foreign substances. This protection is afforded by various cellular responses such as phagocytosis, encapsulation, segregation, and nodule formation as well as by naturally-occurring agglutinins and inducible humoral substances in the hemolymph. From the examples mentioned, it is obvious that the properties of natural agglutinins and induced humoral substances of the invertebrates differ from those of vertebrate antibodies. However, present evidence does not preclude the possibility that the invertebrate molecules are structurally related to vertebrate antibodies; indeed they may represent primitive or precursor forms of immunoglobulins. Only detailed structural studies of these natural and inducible invertebrate proteins will provide the necessary data to answer this question.

TABLE I

IMMUNE MECHANISMS OF INVERTEBRATES

PHYLUM	SPECIES	COMMON NAME	AGGLUTININ ACTIVITY	ANTIBACTERIAL ACTIVITY	OTHER ACTIVITY
PORIFERA	Microciona prolifera	red-beard sponge	0	+	0
COELENTERATA	Anthopleura elegantissima	Sea-anemone	0	0	Antibody-like
ECHINODERMATA	Strongylocentrotus purporatus	Sea-urchin	0	+	
SIPUNCULOIDA	Sipunculus nudus	marine-worm	+	0	Lysin
	Golfingia gouldii	"	0	+	0
	Dendrostromum zostericolum	"	+	+	Hemolysin
	Phascolosoma gouldii	"	0	+	0
MOLLUSCA	Crassostrea virginica	oyster	+	0	Enhanced phage clearance
	Mercenaria mercenaria	marine clam	0	0	Antitumor
	Australorbis glabratus	snail	0	+	"Self-not self"
ANNELIDA	Lumbricus terrestris	earthworm	+	0	graft rejection

TABLE I (Con't)

PHYLUM	SPECIES	COMMON NAME	AGGLUTININ ACTIVITY	ANTIBACTERIAL ACTIVITY	OTHER ACTIVITY
ARTHROPODA					
Insecta	Lepidopterus (sp.)	-	0	+	0
	Oncopeltus fasciatus	Milkweed bug	0	+	0
	Galleria mellonella	Wax moth	0	+	0
	Locusta migrathria migratoriades	Locust	0	0	Antibody-like
	Musca domestica	house fly	0	0	"Protective immunity"
Merostomata	Limulus polyphemus	horseshoe crab	+	+	Hemolytic
Crustacea	Panulirus interruptus	West coast spiny lobster	+	+	Heteroagglutinin
	Carcinus maenas	shore crab	0	0	Phage clearance
	Cambarus virulis	crayfish	0	0	0
	Maia maia	spider crab	+	0	0
	Parachaeraps bicarinatus	crayfish	+	+	"Protective immunity"
	Panulirus argus	West indian spiny lobster	+	+	Hemolytic
	Homarus americanus	American lobster	0	+	0

Symbols: + = activity present; 0 = activity absent

CHAPTER II

EXPERIMENTAL PROCEDURES

Materials

Animals: Mature specimens of the West Indian spiny lobster, Panulirus argus, were collected in the waters surrounding Bimini, Bahamas, and maintained in hardware cloth cages in the tidal seawater lagoon (26° to 28°C) at the Lerner Marine Laboratory, American Museum of Natural History, at Bimini.

Hemolymph collection: Hemolymph was collected from the pericardial sinus of live lobsters by inserting an 18-gauge needle between the cephalathorox and abdomen. Lobsters bled in this manner yielded 15-30 ml of hemolymph which was collected directly into saturated sodium citrate to prevent clotting (1 vol. of saturated sodium citrate/10 vol. of hemolymph). This hemolymph was centrifuged in the cold at 1,500 X gravity within 30 minutes to remove the cells. Hemolymph to be used immediately was maintained in melting ice baths. Other samples collected for use at a later date were frozen and stored at -25°C to minimize loss of activity.

Erythrocytes: Sheep, calf, rabbit, dog, horse, turkey, and chicken erythrocytes were obtained preserved in Alsevers solution from Colorado Serum Company, Denver, Colorado. Human A,B and O erythrocytes were obtained from local donors. All erythrocytes were

washed free of plasma and suspended in a standard isotonic NaCl-Veronal buffer, pH 7.3, of ionic strength 0.147, containing 0.00015 M Ca^{++}, 0.001 M Mg^{++} and 0.1% gelatin. The cells were standardized spectrophotometrically as outlined by Kabat and Mayer (47). In some instances the erythrocytes were suspended in 0.85% sodium chloride solution containing 0.001 M Mg^{++} and 0.00015 M Ca^{++} (Me^{++} sodium chloride solution) as 1% and 5% (v/v) cell suspensions.

Chemicals: Guanidine hydrochloride (ultra pure, Mann Research Laboratories) was clarified by passage of 6 M solution through a Millipore membrane filter (0.45μ). This resulted in a solution with an optical density at 280 mμ of less than 0.05. Refractive index measurements were performed on a Bausch and Lomb refractometer from which concentrations and densities were determined as described by Kielley and Harrington (49).

Sephadex G-200 (Pharmacia) was prepared for chromatography by boiling for 2 hours in distilled water. After cooling, the fine particles were removed and the gel equilibrated with the buffer to be used over 1 to 2 days before column preparation.

Sepharose 6-B (Pharmacia), obtained as swollen beads in distilled water, was washed with 0.01 M Tris (Hydroxymethyl) aminomethane buffer, adjusted to pH 7.4 with HCl, containing 0.14 M NaCl, 0.001 M Mg^{++} and 0.00015 M Ca^{++} (Tris buffer). The gel was allowed to equilibrate overnight with the buffer before the column was poured. Diethylaminoethyl (DEAE) Sephadex A-50 (Pharmacia) was allowed to

swell in distilled water and then washed repeatedly with the desired buffer on a fritted glass funnel until equilibration.

Trypsin was purchased from the Worthington Biochemical Corporation of Freehold, New Jersey. The trypsin was reacted with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) to inactivate chymotryptic activity, and was stored in solution in 0.001 N HCl at a concentration of 10 mg per ml (101).

Reagent grade Tris was obtained from Sigma. The tetrasodium salt of EDTA was obtained from Nutritional Biochemicals. Acrylamide gelling agents and catalyst were obtained from Fisher. Iodacetamide, 2-mercaptoethanol, pyridine, and acetic anhydride were obtained from Eastman Organic Chemical. Before use, iodacetamide was twice recrystallized from hot water. N-Bromosuccinimide and 5-dimethylamino-1-naphthalenesulfonyl chloride (Danzyl reagent) were purchased from K and K Laboratories, Incorporated, Plainview, New Jersey. 5, 5 Dithiobis-nitrobenzoic acid (DTNB) reagent was obtained from Aldrich Chemical Company. Acetylation tubes were obtained from Regis Chemical Company, Rockford, Illinois. Carbohydrates used as standards in the carbohydrate analysis were obtained from Calbiochem. All other materials used were reagent grade according to American Chemical Society standards.

Methods

Hemolytic activity assays: The natural hemolytic activity of

individual animals was evaluated by combining serum dilutions with a constant amount of sheep erythrocytes standardized spectrophotometrically as outlined by Kabat and Mayer (47). Each tube contained a dilution of serum, 0.25 ml of sheep erythrocyte suspension containing 1.0 X 10^8 erythrocytes/ml, and buffer to give a final volume of 3.75 ml. A standard isotonic NaCl-Vernonal buffer, pH 7.3, of ionic strength of 0.147, containing 0.00015 M Ca^{++}, 0.001 M Mg^{++}, and 0.1% gelatin was used for all dilutions. The degree of hemolysis was determined by using a Beckman-DB spectrophotometer at a wavelength of 413 mμ. At the conclusion of each assay the reaction tubes were centrifuged to remove unlysed cells and the percentage hemolysis was calculated by reading the OD of free hemoglobin and correcting for hemolymph color and cell blank. Both positive and negative controls were performed for each assay. The positive control consisted of 0.25 ml of sheep erythrocytes in 3.5 ml of distilled water which provided a 100% hemolysis reference point. The negative control, used to detect any indiscriminate cell lysis, consisted of a similar amount of red cells added to 3.5 ml of standard isotonic buffer. All tubes were incubated at 37°C for 90 minutes unless otherwise noted.

Hemagglutinin assays: Hemagglutinin activity was measured by tube agglutination tests using human A type Rh+ erythrocytes as described by Kabat and Mayer (47). In all assays, twofold dilutions of hemolymph were prepared, incubated at 25°C for 2 hours and left

overnight at 4°C before reading. The last tube having visible agglutination was the dilution recorded as the titer.

Hemagglutinin purification procedure: Whole lobster hemolymph was first subjected to isoelectric precipitation according to the method outlined by Tyler (94). Hemolymph was exhaustively dialyzed in the cold versus distilled water, followed by dialysis against 0.01 M acetate buffer pH 4.8. The resulting "pink" precipitate was centrifuged in the cold and the blue hemocyanin supernatant decanted off. The precipitate was then redissolved in cold saline solution and the precipitation procedure repeated twice under the same conditions. Subsequent tests showed both hemagglutinin and hemolysin activity to be associated with the precipitate fraction and the supernatant hemocyanin fraction to be completely inactive. The precipitate was dissolved in, and dialyzed against, cold barbital buffer pH 8.6, ionic strength 0.05 (barbital buffer) overnight. Preparative Pevikon (Mercer Chemical Corporation) block electrophoresis was performed as described by Müller-Eberhard and Osterland (62). The dissolved precipitate was subjected to electrophoresis on a block 60 X 14 cm in barbital buffer for 36 hours at 4°C with a potential gradient of 8.3 volts/cm. Following electrophoresis, the block was sectioned into 1.5 cm segments and the solute eluted by displacement filtration. The eluates were then analyzed for protein content by the Folin-Ciocalteu method described by Lowry et al. (53). The fractions showing hemagglutinin activity were

pooled and concentrated by ultrafiltration utilizing Diaflow XM-100 membranes (Amicon Corporation) and dialyzed at 5°C against tris buffer. This material was subjected to gel filtration at 10°C utilizing upward-flowing Sepharose 6-B columns (2.5 X 110 cm, bed volume 490 ml, flow rate 12 ml per hour) equilibrated with tris buffer. Fractions of 5 ml each were collected and monitored by absorption at 280 mμ and assayed for activity. The fractions having hemagglutinin activity were pooled, concentrated, and recycled through Sepharose 6-B.

Electrophoresis: Polyacrylamide gel disc electrophoresis was adopted from the procedure of Maizel (59). A solution containing 5% acrylamide, 0.14% N, N-bismethylenacrylamide, 0.1 M sodium phosphate (pH 7.2 with NaOH), 0.1% sodium lauryl sulfate, and 0.5 M urea was prepared in distilled water. Polymerization was catalyzed by adding 1 ml of fresnly prepared 10% ammonium persulfate and 30 microliters of N,N,N',N'-tetramethylethylenediamine to 60 ml of solution. Gel columns (7 cm long and 6 mm in diameter) were filled with the final solution, layered with distilled water, and allowed to gel for one hour. The buffer chamber contained 0.1 M sodium phosphate (pH 7.2) and 0.1% sodium lauryl sulfate. Three milliamperes per tube were applied for 4 hours. Gels were stained with 1% Amido Black in 7% acetic acid. Destaining was carried out by several changes of 40% methanol in 10% acetic acid. In some instances, analytical gel electrophoresis was performed in 7.5% polyacrylamide

gels using Tris-glycine buffer, pH 8.9 (5.16 g Tris and 3.48 g glycine/liter) as described by Habeeb (36). Zone electrophoresis was carried out on a Beckman Model R-101 Microzone Cell using a cellulose acetate support medium. The cellulose acetate strips were run of pH 8.6 in barbital buffer 0.075 ionic strength. Proteins were stained using Ponceau-S. Immunoelectrophoresis was performed on an LKB Immunophor apparatus using 1% of Difco's purified Noble agar, veronal buffer, pH 8.6, ionic strength 0.1, on microscope slides. Runs were for 1-2 hours at 2.5 v/cm.

Preparation of antisera: Rabbit antisera to whole lobster hemolymph were prepared by intramuscular and foot pad injections in Freund's complete adjuvant (Difco) at 0, 1, 3 and 6 weeks. The antisera obtained at 6 weeks after the last stimulation were used in these studies.

Reduction and alkylation: Reduction was performed by dissolving purified material in 7 M guanidine hydrochloride, adjusted to pH 7.6 by the addition of 0.5 M Tris-HCl, followed by the addition of 2-mercaptoethanol to a final concentration of 0.2 M. The mixture was incubated at 37°C for 4 hours. Alkylation was achieved by adding a slight molar excess of iodoacetamide and incubating at room temperature for 15 minutes. Reduced and alkylated material was applied to a Sephadex G-200 upward-flowing column (2.5 X 120 cm) equilibrated with 5 M guanidine hydrochloride as described by Small and Lamm (51) with flow rates of 10 ml per hour. After monitoring

the column effluent at 280 mμ, pooled fractions were exhaustively dialyzed against distilled water and lyophilized. Mild reduction was carried out on proteins dissolved in Tris buffer. The concentration was adjusted to 0.1 M in mercaptoethanol and allowed to stand 2 hours at room temperature. Alkylation was achieved by dialysis against 0.02 M iodoacetamide.

Physical measurements: Sedimentation velocity measurements were performed in a Spinco Model E analytical ultracentrifuge at 20°C with both single and double-sector 12 mm cells, schlieren optics, phase plate angle of 60° and a speed of 56,100 rpm. Sedimentation coefficients were calculated according to procedures described by Schachman (73). Diffusion measurements were made in the same centrifuge at 20°C and a speed of 3,617 rpm with a synthetic boundary cell as described by Ehrenberg (22). Diffusion coefficients were calculated by the "maximum ordinate" method (22).

Sedimentation and diffusion experiments were performed in 0.076 M sodium phosphate buffer, pH 7.5. All samples were exhaustively dialyzed against the buffer before analysis. Measurements were performed at different protein concentrations and corrected to the reference state of water at 20°C. Values at $S^{\circ}_{20,w}$ were extrapolated to zero concentration using the method of least-squares to obtain $S^{\circ}_{20,w}$ and $D^{\circ}_{20,w}$. The patterns were measured on a Nikon Microcomparator.

The partial specific volume ($\bar{v}$) was calculated from the amino

acid and carbohydrate compositions by the method described by Cahn and Edsall (13) and was found to be 0.711.

Carbohydrate determinations: Neutral and amino sugars were identified and quantitated by the gas chromatographic method of Niedermeier (64). Samples were dried to constant weight in a vacuum desiccator at 30°C, dissolved in water made acid with HCl, and digested with pepsin at an enzyme:protein ratio of 5 µg/mg overnight at 37°C. An aliquot of the digest was placed in each of 3 acetylation tubes, an equal volume of 2 N HCl added and the mixtures hydrolyzed at 100°C for 1, 4 and 10 hours. After hydrolysis, the tubes were cooled and 0.25 µM of arabinose added as an internal standard for the neutral sugars; 0.5 µM galactosamine was used as the internal standard for glucosamine determinations. The solutions were mixed and neutralized with Dowex 1 bicarbonate. After removal of the resin, the solutions were cooled to 0-2°C and 0.5 ml of cold 0.22 M sodium borohydride solution added. Reduction was carried out overnight in the cold after which the sodium borohydride was decomposed by the addition of HCl. The mixtures were lyophilized and methanol added to convert the boric acid to the volatile trimethyl borate. The methanol was removed under reduced pressure at room temperature.

The sugars were then acetylated in acetylation tubes by adding 0.2 ml of a 1:1 solution of pyridine and acetic anhydride and reacting 15 minutes in a boiling water bath. The solutions were then centrifuged and 2 to 10 µl of the supernatant injected into a

Hewlett-Packard Model 404 gas chromatograph equipped with dual flame ionization detectors, and six foot V-shaped, ¼" glass columns packed with 1% ECNSS-M (ethylene succinate cyanoethyl silicone) on 60/80 mesh Gas Chrom. Q (Applied Science Laboratory, State College, Pennsylvania). An Infotronics Model CRA 104 electronic integrator was used to quantitate the results. All analyses were performed in triplicate.

Sialic acid was determined by the thiobarbituric acid method of Warren (96). Samples were hydrolyzed in 0.05 N H_2SO_4 at 80°C for 30 minutes.

Amino acid analysis: Analyses were performed on a Beckman Model 120C automatic amino acid analyzer utilizing a 55 cm column for separation of neutral and acidic amino acids and an 8 cm column for basic amino acids as described by Hubbard (42). Samples were hydrolyzed in duplicate with constant boiling HCl under a nitrogen atmosphere at 106°C for 20 hours. After hydrolysis, HCl was removed in a vacuum over NaOH. The values reported are not corrected for destruction during acid hydrolysis. Tryptophan was determined spectrophotometrically by the N-bromosuccinimide method of Spande and Witkop (77).

Peptide maps: The peptide mapping technique was that described by Bennett (8). Protein samples (10 mg per ml) were digested with trypsin at 37°C for 4 hours in 0.1 M NH_4HCO_3 (pH 8.2). Approximately 1.0 to 1.5 mg samples of the tryptic digest were spotted on Whatman

No. 3 MM chromatographic paper and subjected to descending chromatography in the organic phase of butanol:acetic acid: water (4:1:5) for 20 hours. Electrophoresis was performed in the second dimension in pyridine-acetate pH 3.6 buffer, at 3,200 volts for 65 minutes at 22°C in a Gilson Medical Electronic Model DW electrophorator. Peptides were detected by staining with ninhydrin-collidine.

NH_2-terminal analysis: Qualitative determination of NH_2-terminal amino acids was performed using the dansyl reagent (35). The dansyl derivatives were determined by polyamide layer chromatography according to Woods and Wang (102).

CHAPTER III

RESULTS

Lobster Hemolytic System

Determination of Optimal Assay Conditions. Freshly collected lobster hemolymph possessed a natural hemolysin which exhibited activity for sheep erythrocytes. Hemolytic titers were found to vary according to the concentration of sheep red blood cells used as an indicator. Results of controlled assays employing various erythrocyte concentrations revealed that 1.0×10^8 erythrocytes/ml gave optimal sensitivity in this hemolytic system.

Dose-Response. A typical dose-response curve that resulted from the addition of increasing amounts of lobster hemolymph to a constant amount of sheep erythrocytes is illustrated in Figure 1. The range of the curve extends from 7% lysis at a final hemolymph dilution of 1:75 to 62% lysis at a 1:6 dilution. In this system, large increases in the amount of hemolymph would be required for total lysis. The 50% end point in this hemolytic titration was reached at a final dilution of 1:14 of the lobster hemolymph. Attempts to increase the degree of lysis, and thus increase the sensitivity of the assay system, by addition of rabbit hemolysin were not successful.

Temperature Effects. As indicated in Table II, the activity of

Figure 1

Dose-response curve: Percentage of hemolysis of sheep erythrocytes plotted as a function of the volume of lobster hemolymph

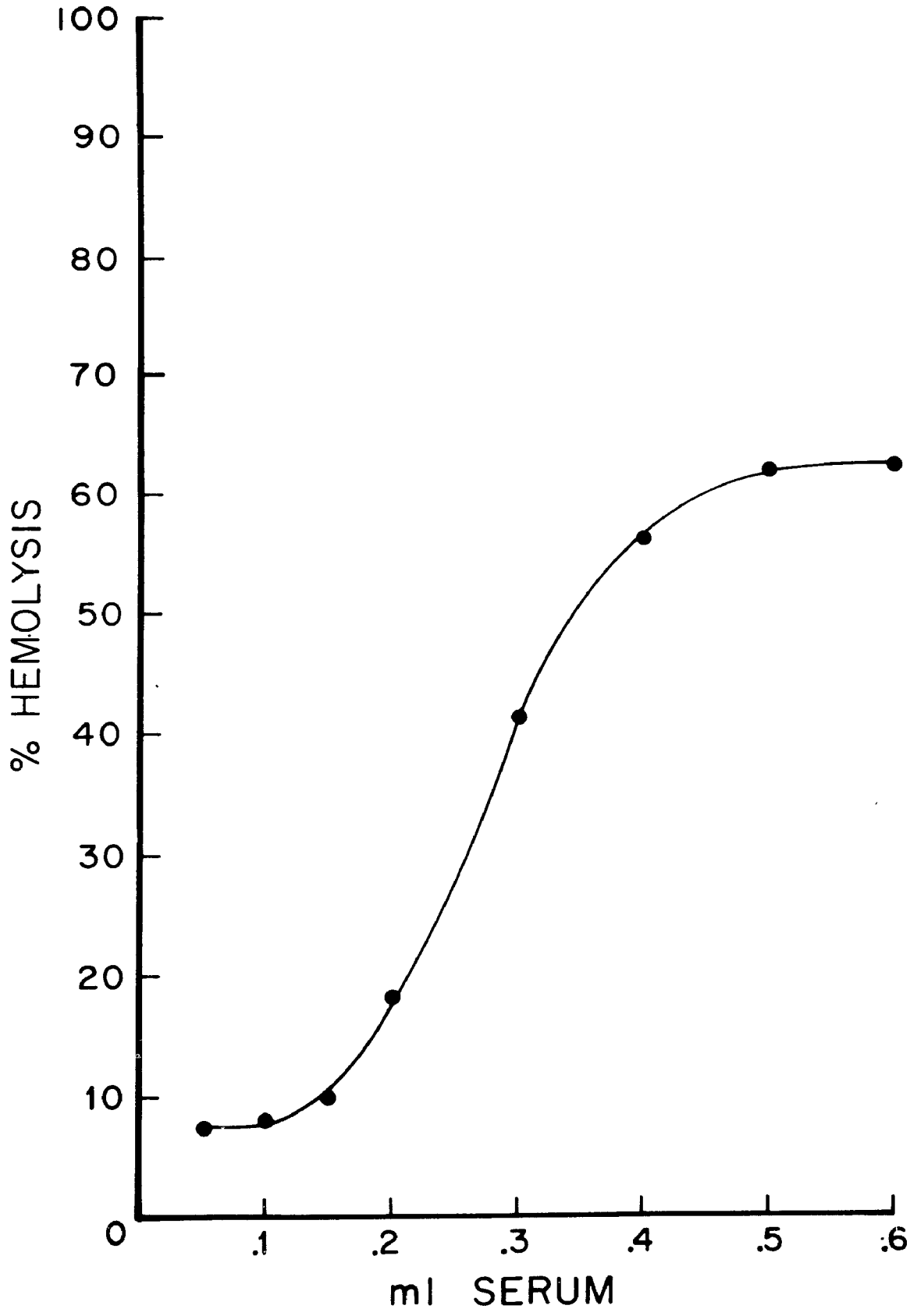

100
90
80
70
60
50
40
30
20
10
0
% HEMOLYSIS
.1
.2
.3
.4
.5
.6
ml SERUM

TABLE II

Lysis of sheep erythrocytes by normal lobster hemolymph[a] at various temperatures after incubation (18 hr)

Temperature (°C)	Hemolysis (%)
0	0
4	0
25	100.0
37	100.0

[a]One-half ml of a 1:7.5 dilution of hemolymph was used.

this naturally-occurring lobster hemolysin was temperature-dependent. Incubation of hemolymph with a constant amount of sheep erythrocytes at 0 and 4°C for an 18-hour period resulted in no detectable hemolysis. Incubation of a similar mixture at both room temperature and 37°C for the same period of time gave 100% lysis. This inhibition of lytic activity at lower temperatures suggests the involvement of an enzymatic type reaction in this hemolytic system. When the mixture of sheep erythrocytes and hemolymph that exhibited no hemolysis at 0°C was kept at this temperature, washed three times with cold isotonic buffer to remove the hemolymph, and then incubated at 37°C for 90 minutes in a standard assay system, 100% lysis occurred suggesting that the hemolysin had been absorbed onto the red cell surface.

Inactivation Studies. Heating of the lobster hemolymph to 52°C for 20 minutes resulted in complete loss of lytic activity. Other inactivation procedures involved the use of EDTA (0.05 M) which also successfully inhibited the hemolysin. The hemolytic system remained after dialysis for 7 days provided that Ca^{2+} and Mg^{2+} were replenished. Absorption procedures using packed human and sheep cell stroma resulted in loss of hemolytic activity suggesting that the hemolysin was absorbed from the hemolymph by these techniques. Such procedures removed hemolytic activity even when conducted at 0°C suggesting that the hemolysin could attach to the red cell membrane but, as shown by the next experiment, no

lysis occurred at this temperature in a standard assay system.

Kinetic Analysis. The characteristic kinetics of this natural hemolytic system are illustrated in Figure 2. A modification of the method described in Kabat and Mayer (47) was used for these experiments. Samples incubated at 37°C were removed at various time intervals and placed immediately into a 0°C water bath to halt the reaction. The samples were centrifuged and the degree of lysis was determined. As shown by the resulting curve, hemolysis began at zero time and did not reach 100 per cent even after 3 hours of incubation. It would appear that the optimal time for maximum (70%) lysis under these conditions is between 150 and 180 minutes.

Lobster Hemagglutinin

Range of Activity. Many investigators have demonstrated that many invertebrates possess natural hemagglutinins against several species of vertebrate erythrocytes (58, 92, 98). As shown in Table III, spiny lobster hemolymph is capable of agglutinating a wide range of vertebrate red blood cells to varying degrees. Because of the reactivity of human Group A erythrocytes, (HuA), they were used for all subsequent titrations of hemagglutinating activity in spiny lobster hemolymph.

Inactivation Studies. In order to test the heat stability of the lobster hemagglutinin, samples of hemolymph were exposed to various constant temperatures for 20 minutes. Results shown in

Figure 2

Kinetics of hemolysis of sheep erythroytes by lobster hemolymph at 37°C

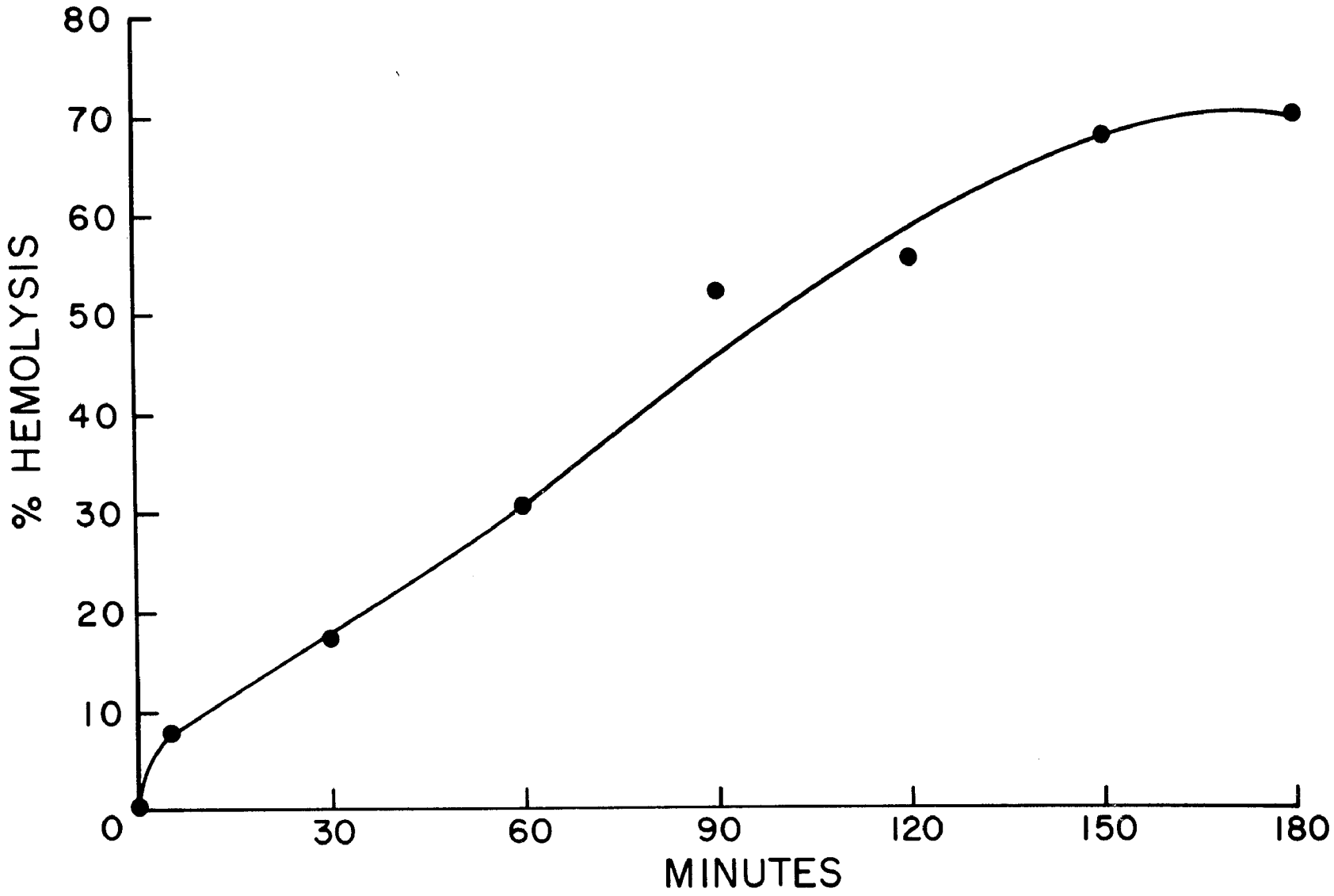

80
70
60
50
40
30
20
10
0
% HEMOLYSIS
30
60
90
120
150
180
MINUTES

TABLE III

Range of lobster hemolymph hemagglutinating activity

Source of erythrocytes[a]	Hemagglutinin Titer^{-1}
Human A	1024
Human B	1024
Human O	1024
Sheep	256
Calf	128
Rabbit	512
Dog	1024
Horse	64
Turkey	128
Chicken	32

[a]Erythrocytes suspended in Me^{++} sodium chloride solution at a 1% (v/v) cell suspension.

Table IV indicate some diminution of activity at 48°C and complete inactivation at 60°C.

Other inactivation procedures involved the use of the chelating agent EDTA. Dialysis of lobster hemolymph overnight versus 0.85% sodium chloride solution containing 0.05 M EDTA resulted in complete loss of hemagglutinin activity. Subsequent attempts at reactivation of the hemagglutinin by the addition of equimolar amounts of various divalent cations revealed that Ca^{++} was by far the most effective in restoring activity. However, no enhancement of activity was observed by the addition of excess Ca^{++} ions to the assay system as reported by Marchalonis and Edelman for the hemagglutinin from the horseshoe crab, Limulus polyphemus (60).

Absorption of lobster hemolymph with whole human A erythrocytes or with human A erythrocyte stroma at 4°C resulted in loss of hemagglutinin activity for all the erythrocytes shown in Table II.

Purification of Lobster Hemagglutinin. Initial attempts to purify the component or components of lobster hemolymph responsible for hemagglutinating activity involved the use of gel filtration. Whole lobster hemolymph (5.0 ml) was applied to an upward-flowing Sephadex G-200 column. This sample had an initial hemagglutinin titer of 1:512. The protein elution pattern and hemagglutinin activity is shown in Figure 3. As illustrated, hemagglutinin activity was associated with the first peak with a portion of the active material apparently being excluded from the column indicating a

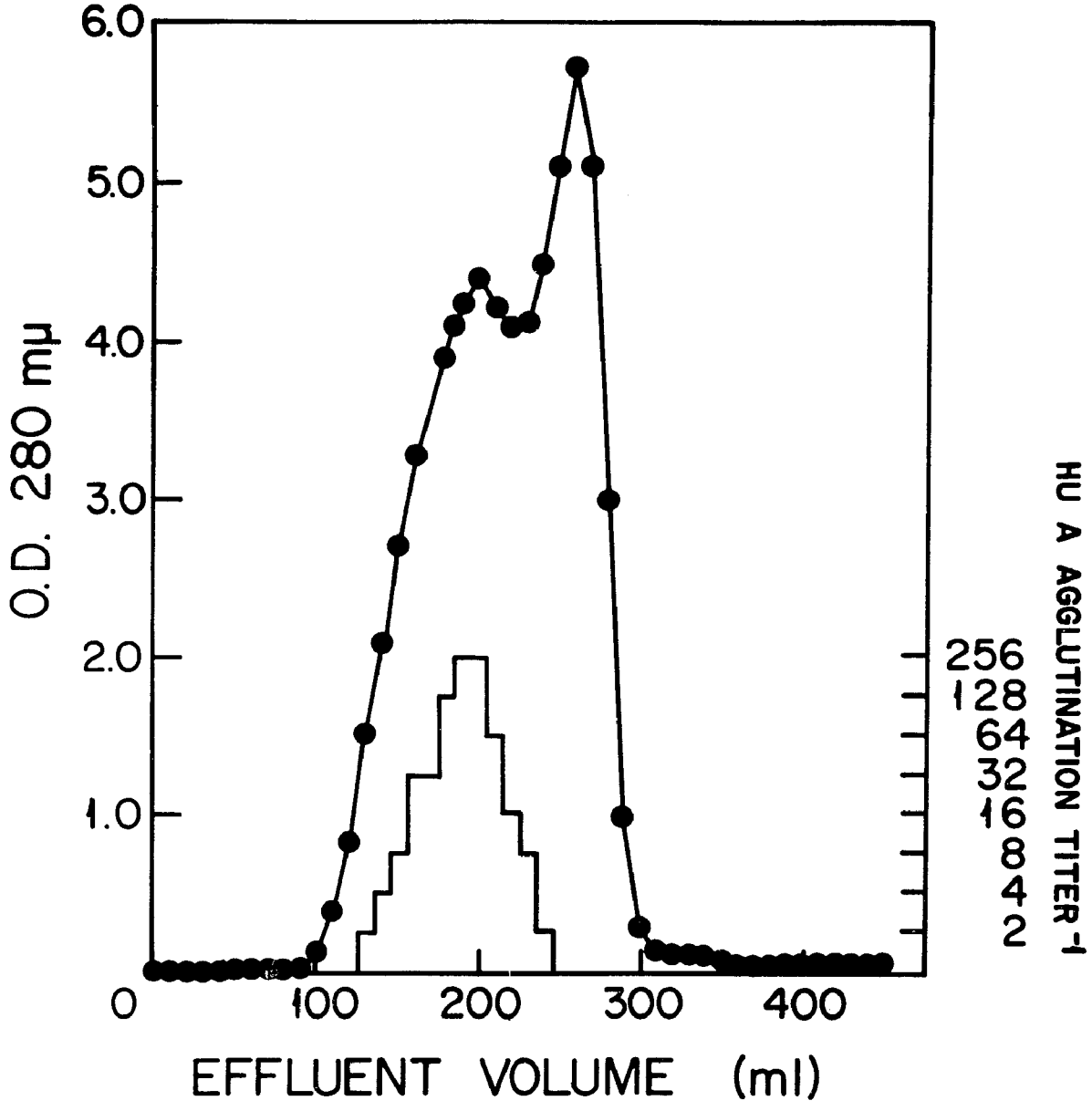
TABLE IV

Heat inactivation of lobster hemagglutinating (HA) activity

Temperature °C (20 minutes)	Percentage of HA activity compared to untreated hemolymph
36	100
40	100
44	100
48	86
52	86
56	14
60	0

Figure 3

Elution pattern of whole lobster hemolymph from a Sephadex G-200 column (2.5 x 100 cm) equilibrated with 0.14 M NaCl, 0.01 M Tris, 0.001 M Mg^{++} 0.00015 M Ca^{++} buffer adjusted to pH 7.4 with HCl. ——●——●——, Absorbancy at 280 mμ; _____, human A erythrocyte agglutinin titer.


6.0
5.0
4.0
3.0
2.0
1.0
0
O.D. 280 mμ
100
200
300
400
EFFLUENT VOLUME (ml)
256
128
64
32
16
8
4
2
HU A AGGLUTINATION TITER⁻¹

probable molecular weight greater than 200,000. The blue respiratory pigment, hemocyanin, was visually apparent in the active excluded peak. As a result, all subsequent purification attempts were preceded by an isoelectric precipitation procedure first described by Tyler for isolation of hemolymph constituents from another species of spiny lobster (94). Following his procedure, dialysis of whole lobster hemolymph against acetate buffer (0.01M pH 4.8) overnight at 4°C resulted in the formation of a pink precipitate, leaving the majority of blue hemocyanin in the supernate. The pink precipitate was removed, washed three times in cold acetate buffer, and dissolved in Tris buffer. The remaining supernate, containing most of the hemocyanin was concentrated to its original volume, and dialyzed against Tris buffer in the cold. Hemagglutination assays of these two fractions revealed that all activity was in the precipitated fraction. Following this isoelectric precipitation procedure, the specific activity of the hemagglutinin increased approximately ten fold.

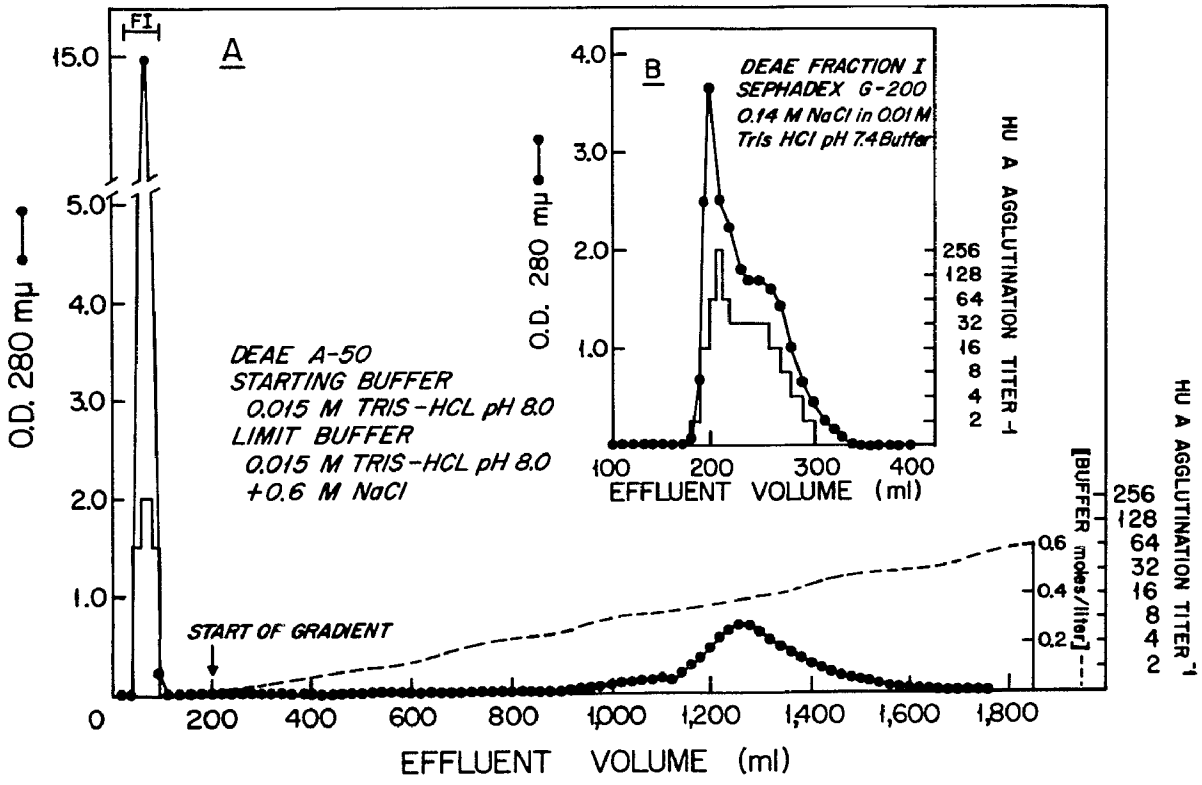
In an attempt to resolve the component or components in the precipitate responsible for hemagglutinin activity, ion-exchange chromatography was employed. Figure 4A shows the elution profile of the redissolved precipitate from a DEAE Sephadex A-50 column equilibrated with 0.015 M Tris-HCl pH 8.0. Human A erythrocyte (HuA) agglutinin activity was found to be associated with the first peak eluted (FI) which was pooled as indicated, concentrated, and

Figure 4

A. Fractionation of whole lobster hemolymph on a DEAE A-50 anion exchange column (2.5 x 45 cm). A molarity gradient with 0.015 M Tris-HCl, pH 8.0 as the starting buffer and 0.015 M Tris-HCl + 0.6 M NaCl, pH 8.0 as the limit buffer was used. FI indicates the fraction pooled for gel filtration on Sephadex G-200.

B. Gel filtration of FI on a Sephadex G-200 column (2.5 x 100 cm) equilibrated with 0.14 M NaCl, 0.01 M Tris, 0.001 M Mg^{++}, 0.00015 M Ca^{++} buffer adjusted to pH 7.4 with HCl.

—●—●—, Absorbancy at 280 mμ; ——, human A erythrocyte agglutinin titer.


FI
A
B
DEAE A-50
STARTING BUFFER
0.015 M TRIS-HCL pH 8.0
LIMIT BUFFER
0.015 M TRIS-HCL pH 8.0
+0.6 M NaCl
START OF GRADIENT
O.D. 280 mμ
EFFLUENT VOLUME (ml)
DEAE FRACTION I
SEPHADEX G-200
0.14 M NaCl in 0.01 M
Tris HCl pH 7.4 Buffer
HU A AGGLUTINATION TITER -1
[BUFFER moles/liter]
HU A AGGLUTINATION TITER -1

filtered through on Sephadex G-200 (Figure 4B). The elution profile suggested a minimum of three molecular weight species, all having hemagglutinin activity.

A similar situation was observed when the DEAE step was omitted. Figure 5 shows the results of applying the dissolved precipitate directly onto a G-200 column equilibrated with Tris buffer. Here again multiple molecular weight components were resolved and the hemagglutinin activity paralleled protein concentration.

The failure of gel filtration and ion-exchange chromatography to yield a single component with hemagglutinin activity led to the use of other separation procedures. Figure 6 is a pattern of zone electrophoresis on cellulose acetate of whole lobster hemolymph. A normal human serum control is included for purposes of comparison. As illustrated, approximately 87% of the protein migrated towards to anode as a large peak containing the respiratory pigment hemocyanin. Two other components of lesser anodic mobility were resolved. They contained approximately 12% and 1% of the total hemolymph protein, respectively.

Because of this electrophoretic separation, whole lobster hemolymph was once again precipitated isoelectrically (94) and the active, redissolved precipitate subjected to zone electrophoresis on Pevikon. The protein distribution pattern is shown in Figure 7. As illustrated, the hemagglutinating activity directed against human A erythrocytes was localized in the fraction of lower electrophoretic mobility which

Figure 5

Elution pattern of isoelectric precipitate from lobster hemolymph from a Sephadex G-200 column (2.5 x 100 cm) equilibrated with 0.14 M NaCl, 0.01 M Tris, 0.001 M Mg^{++}, 0.00015 M Ca^{++} buffer adjusted to pH 7.4 with HCl. ——●——●——, Absorbancy at 280 mμ; ——, human A erythrocyte agglutinin titer.

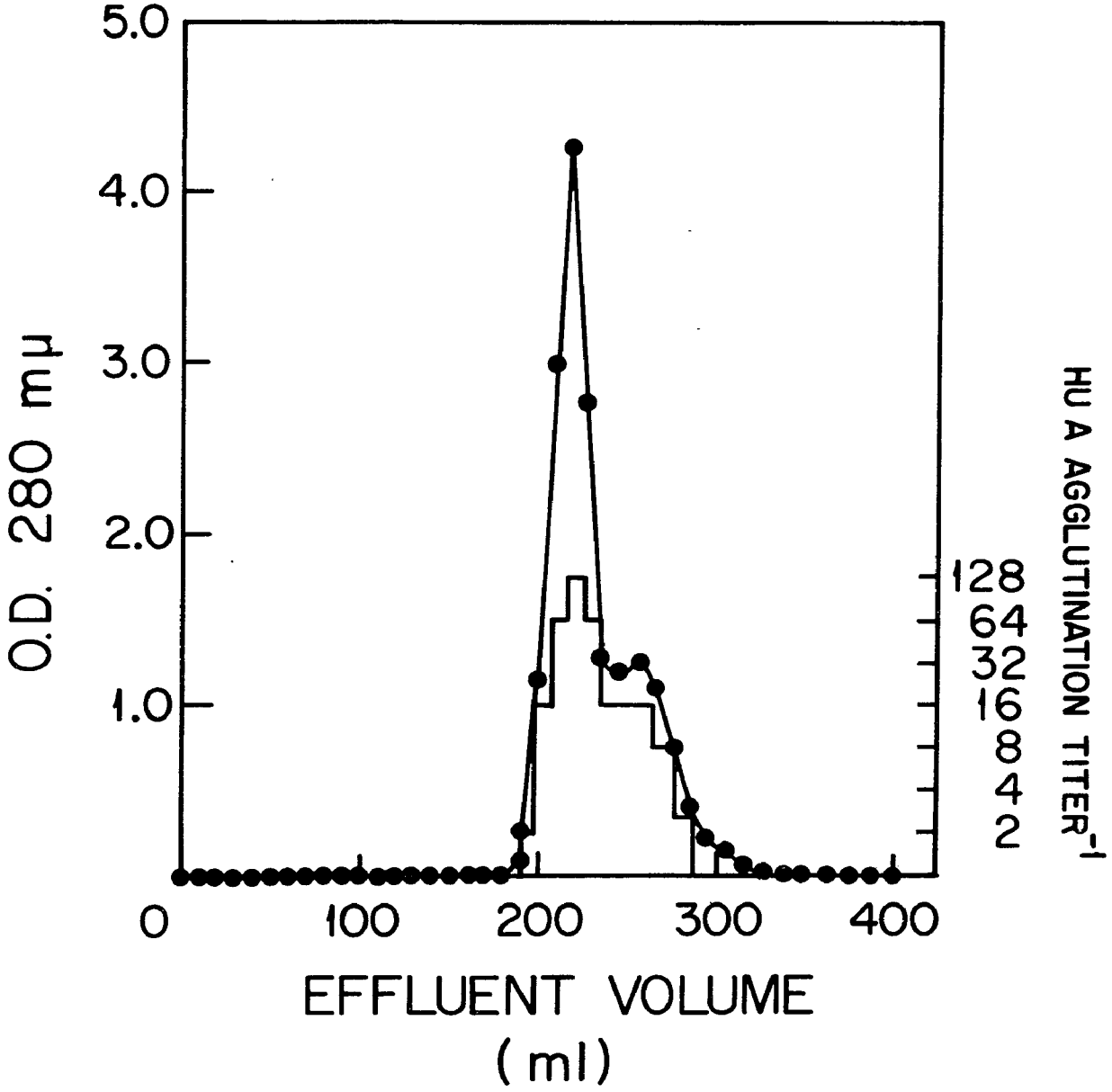

5.0
4.0
3.0
2.0
1.0
O.D. 280 mμ
128
64
32
16
8
4
2
HU A AGGLUTINATION TITER⁻¹
0
100
200
300
400
EFFLUENT VOLUME
(ml)

Figure 6

Cellulose acetate zone electrophoresis of whole lobster hemolymph compared with whole human serum. Thirty micro liters of lobster hemolymph were applied and ten micro liters of human. Samples were run in barbital buffer, pH 8.6, for 20 minutes at 2.50 volts.

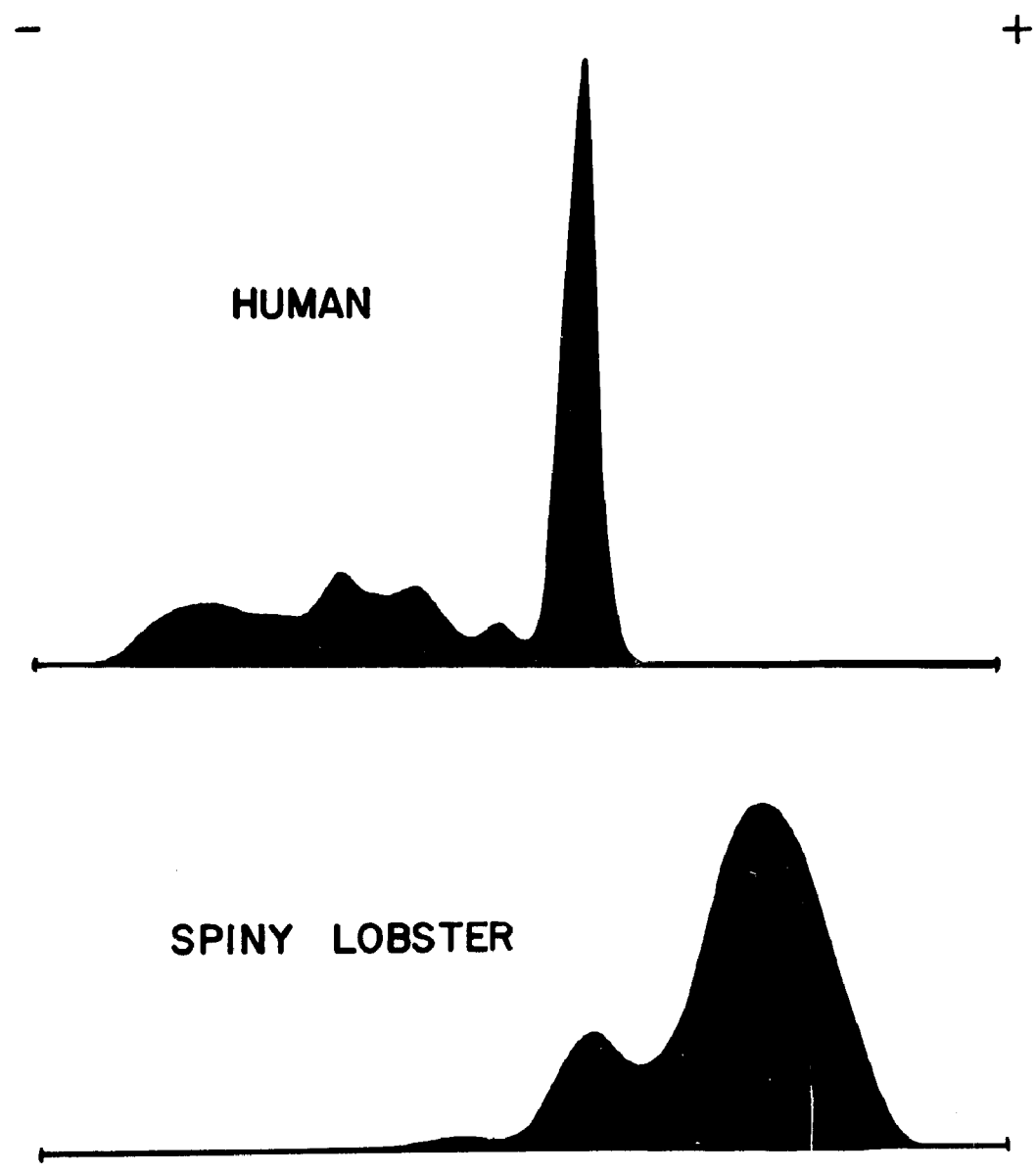
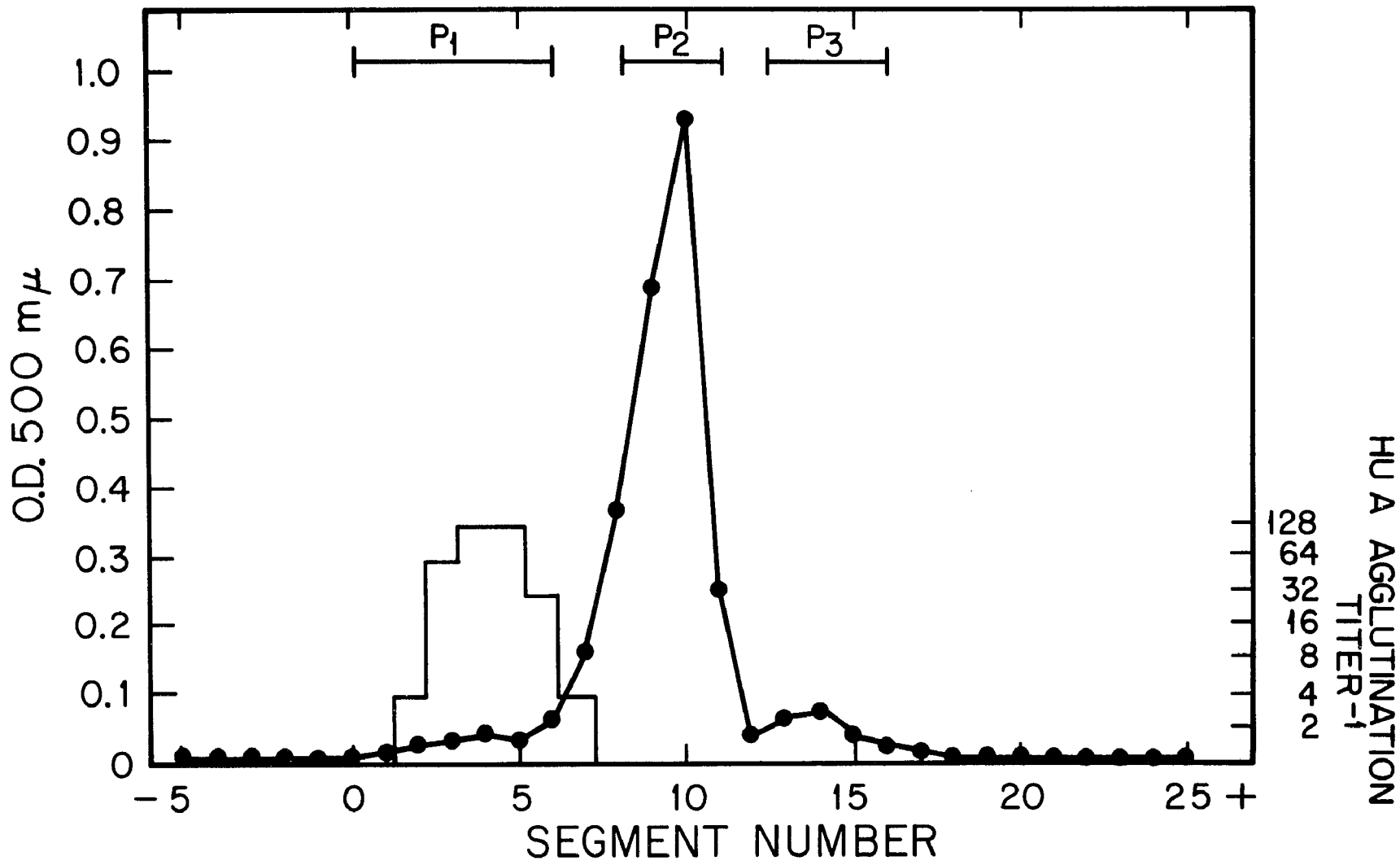

−
+
HUMAN
SPINY LOBSTER

Figure 7

Fractionation of isoelectric precipitate from lobster hemolymph by zone electrophoresis on Pevikon. Blocks (60 x 14 cm) were run in barbital buffer, pH 8.6, for 36 hours at 4°C with a potential gradient of 8.3 volts/cm. O, Origin; (+), anode; (-), cathode. —●—●—, Absorbancy of the Folin reaction at 500 mμ; ——, human A erythrocyte agglutinin titer. The areas under the horizontal cross bars indicates the volumes pooled (P1, P2, P3).


P1
P2
P3
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
O.D. 500 mμ
−5
0
5
10
15
20
25 +
SEGMENT NUMBER
128
64
32
16
8
4
2
HU A AGGLUTINATION TITER-1

constituted approximately 9% of the precipitate protein applied and only 1% of the total hemolymph protein. The fractions were pooled as indicated, concentrated, and subjected to Ouchterlony analysis. Results shown in Figure 8 indicate the fast migrating hemocyanin fraction to be non-identical with the P2 fraction. However, P2 is shown to contain obvious hemocyanin contamination. P1, though broad and diffuse, appears antigenically identical to the P2 fraction, but definitely unrelated to the P3 hemocyanin. P1 is seen to contain at least two additional components present in lower concentrations.

The pool (P1) containing hemagglutinating activity was subjected to further purification on Sepharose 6-B in Tris buffer (Figure 9A). The hemagglutinating activity was found to be associated with the main peak of material which was pooled as indicated. Several runs on 6-B were pooled, concentrated and recycled on the same column. An example of the elution pattern of the pool is shown in Figure 9B. The peak was fairly symmetrical and had hemagglutinating activity. Active fractions were pooled as indicated. The specific activity (hemagglutination titer per mg protein) of this pool was about twenty times that of the whole hemolymph. When the pool from Figure 9B was analyzed in the ultracentrifuge (Figure 10B), at a concentration of 3.2 mg per ml, a relatively homogeneous peak was obtained which had a sedimentation coefficient of 10S. Figure 10A illustrates the centrifuge pattern of whole lobster hemolymph. There were three components sedimenting at 4.9S, 7.4S, and 10.2S. The broad concen-

Figure 8

Immunodiffusion showing antigenic relationships of the pools, P1-P3, made following zone electrophoresis of the lobster isoelectric precipitate on Pevikon (see Figure 7). Center well contains rabbit antiserum against whole lobster hemolymph.

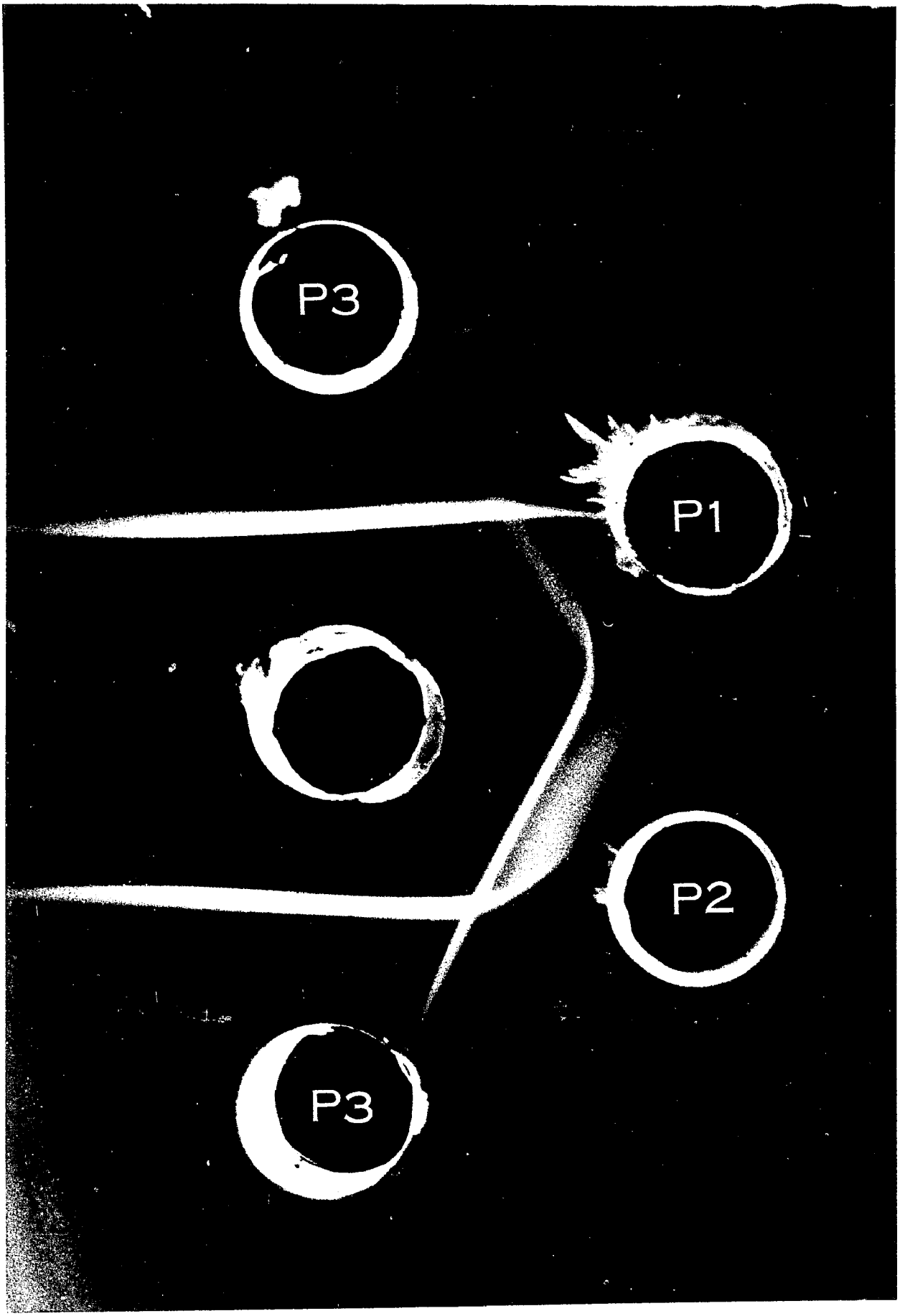

P3
P1
P2
P3

Figure 9

A. Elution pattern of material from Pevikon block showing hemagglutinating activity (Fig. 7, P1) from a Sepharose 6-B column (2.5 x 100 cm) equilibrated with 0.14 M NaCl, 0.01 M Tris, 0.001 M Mg^{++}, 0.00015 M Ca^{++} buffer adjusted to pH 7.4 with HCl. The area under the horizontal cross bar indicates the volume pooled.

B. Pool from A recycled. The area under the horizontal cross bar indicates the volume pooled and will be referred to as purified lobster hemagglutinin.

—●—●—, Absorbancy at 280 mμ; ——, human A erythrocyte agglutinin titer.

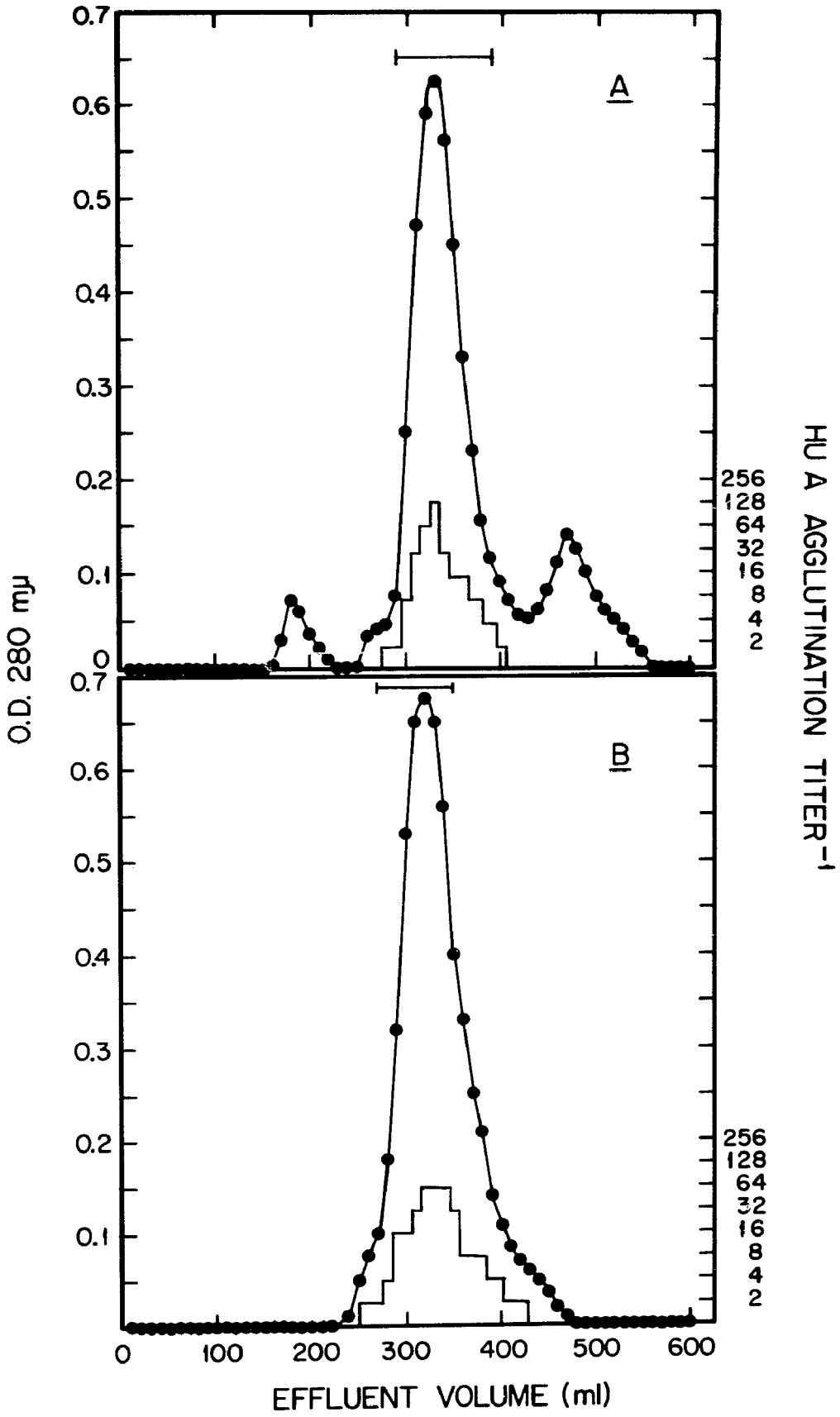
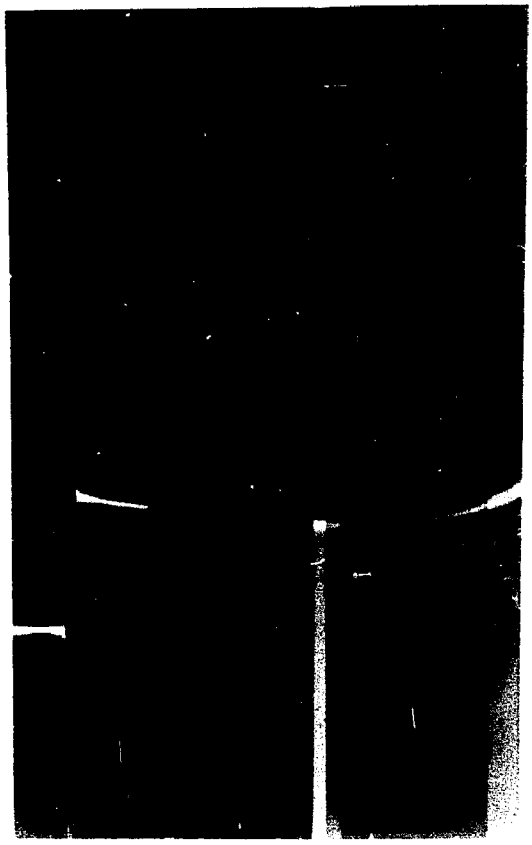

A
B
O.D. 280 mμ
HU A AGGLUTINATION TITER⁻¹
EFFLUENT VOLUME (ml)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
256
128
64
32
16
8
4
2
0
100
200
300
400
500
600

Figure 10

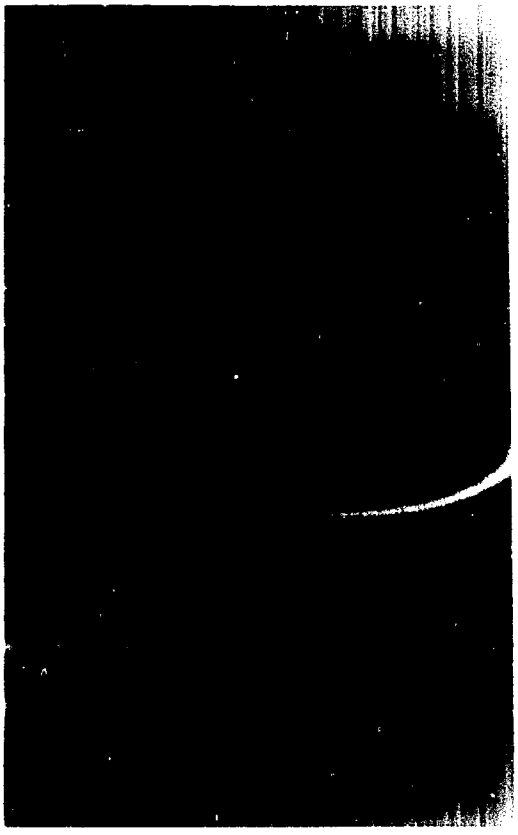
A. Ultracentrifuge pattern of whole lobster hemolymph in 0.076 M sodium phosphate buffer pH 7.5. The photograph was taken 48 minutes after reaching a speed of 56,100 rpm at a phase plate angle of 60° and temperature of 20°.

B. Ultracentrifuge pattern of purified lobster hemagglutinin (Fig. 8B). Photograph taken at 32 minutes. All other conditions same as A. Protein concentration is 3.2 mg per ml.

Sedimentation proceeds from left to right.



A



B

tration band to the right in this frame represented the hemocyanin portion of the hemolymph. The pool from Figure 9B and whole lobster hemolymph were compared by immunoelectrophoresis using rabbit antiserum to whole lobster hemolymph. Figure 11 shows that the Figure 9B pool (PLH) moved as a single component with moderate mobility as compared to the whole hemolymph (WLH).

Thus, the Figure 9B pool had hemagglutinating activity and consisted of a single component. The 9B pool will be referred to as "purified lobster hemagglutinin". All material used in subsequent experiments was prepared in the same manner and consisted of a single component.

Physical studies of the purified Lobster Hemagglutinin. Figure 12 presents the sedimentation coefficients ($S_{20,w}$) as a function of protein concentrations. A partial specific volume of 0.711 calculated from the amino acid and carbohydrate composition was used for the correction of S_{obs} to the standard state of $S_{20,w}$. The sedimentation constant of the purified hemagglutinin was determined to be 10.2_5s at infinite dilution. The values for the diffusion coefficient ($D_{20,w}$) plotted as function of protein concentration are seen in Figure 13. The diffusion of the molecule does not demonstrate concentration dependency.

The molecular weight of the purified hemagglutinin was calculated from the sedimentation and diffusion coefficients at infinite dilution by use of the Svedberg equation. Using the calculated

Figure 11

Immunoelectrophoresis of whole lobster hemolymph (WLH) and purified lobster hemagglutinin (PLH, concentration 4.0 mg/ml). Precipitin arcs were developed using rabbit antiserum against whole lobster hemolymph (AWLH).

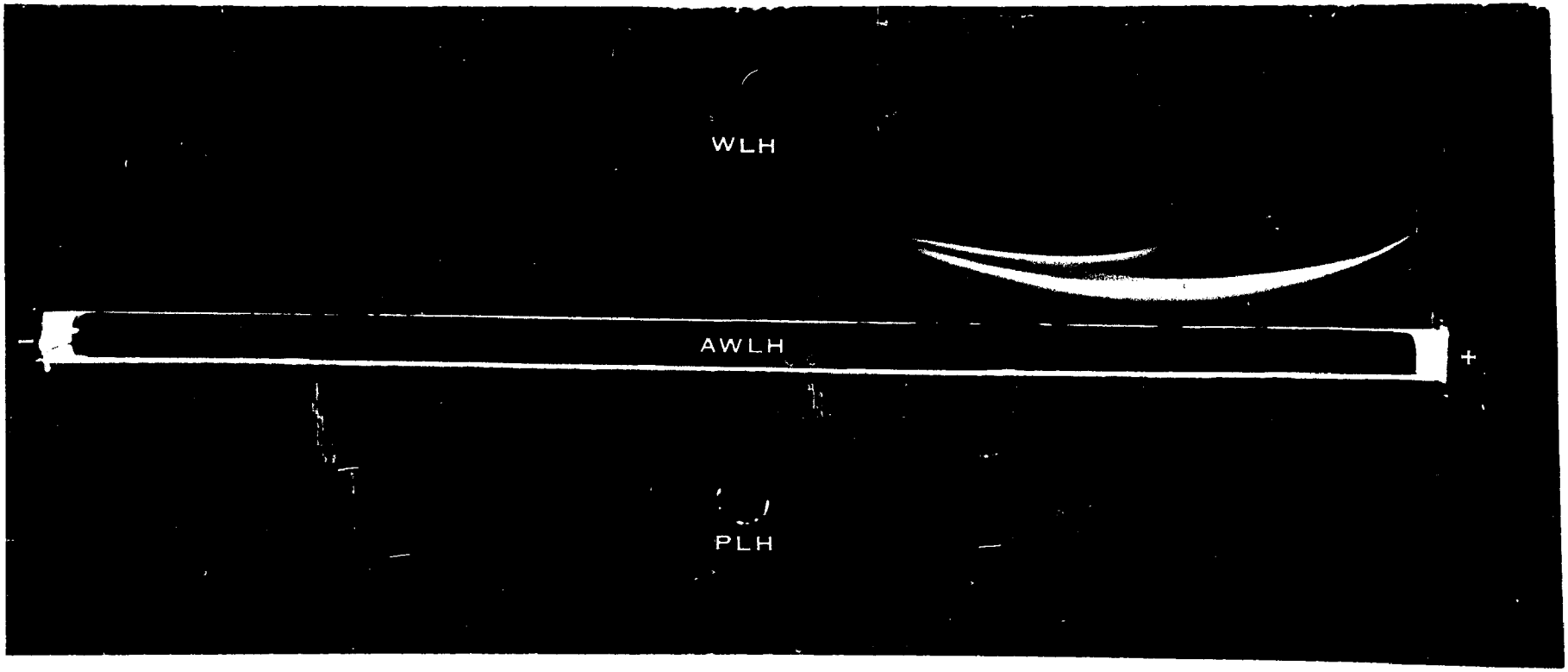

WLH
AWLH
+
PLH

Figure 12

Determination of sedimentation coefficient ($S^{o}_{20,w}$) of purified lobster hemagglutinin from a plot of $S_{20,w}$ values versus protein concentration. Measurements were performed in 0.076 M sodium phosphate buffer pH 7.5. The extrapolated value of $S_{20,w}$ at infinite dilution is indicated in parenthesis. The line is a least-squares fit of the experimental points.

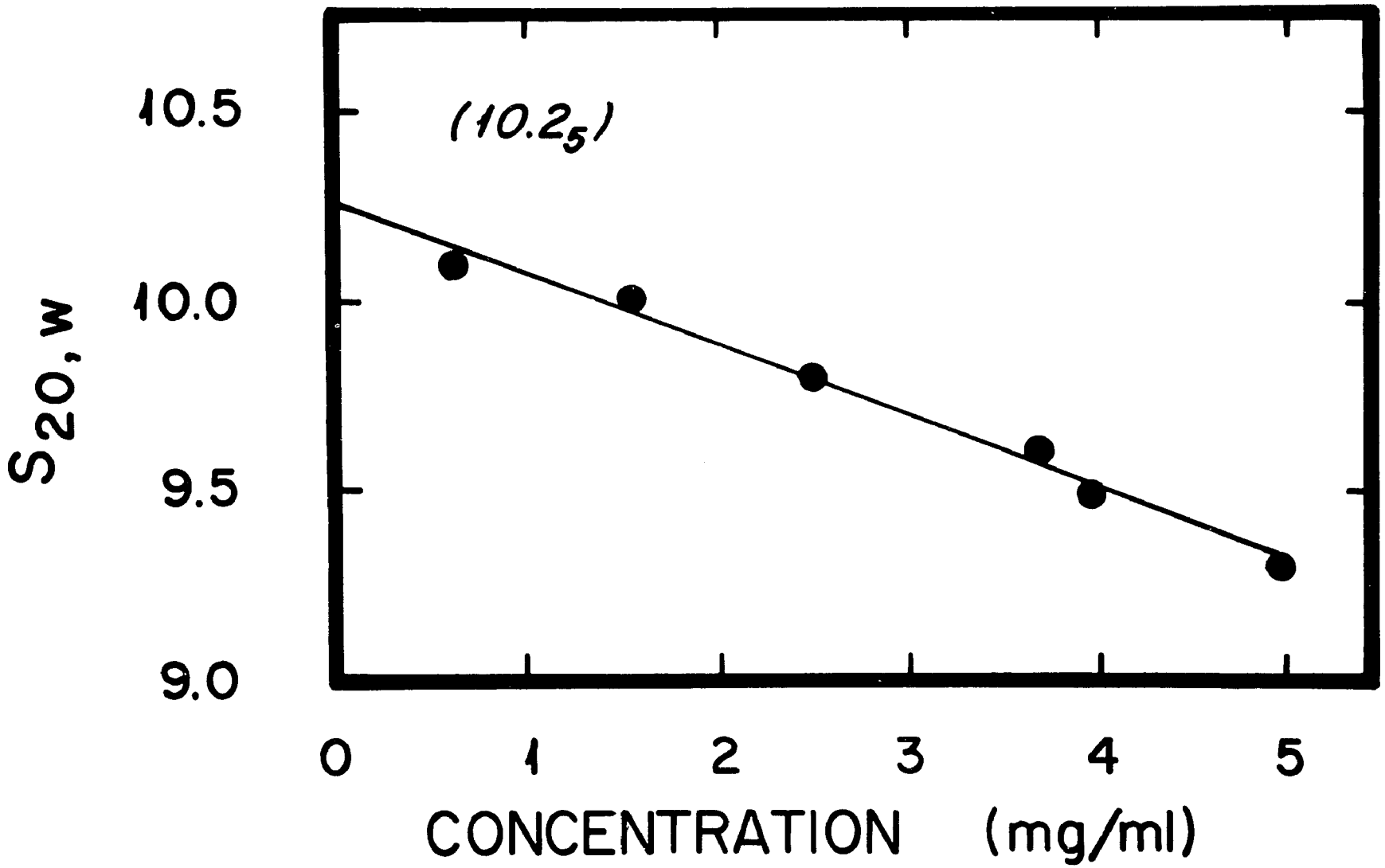
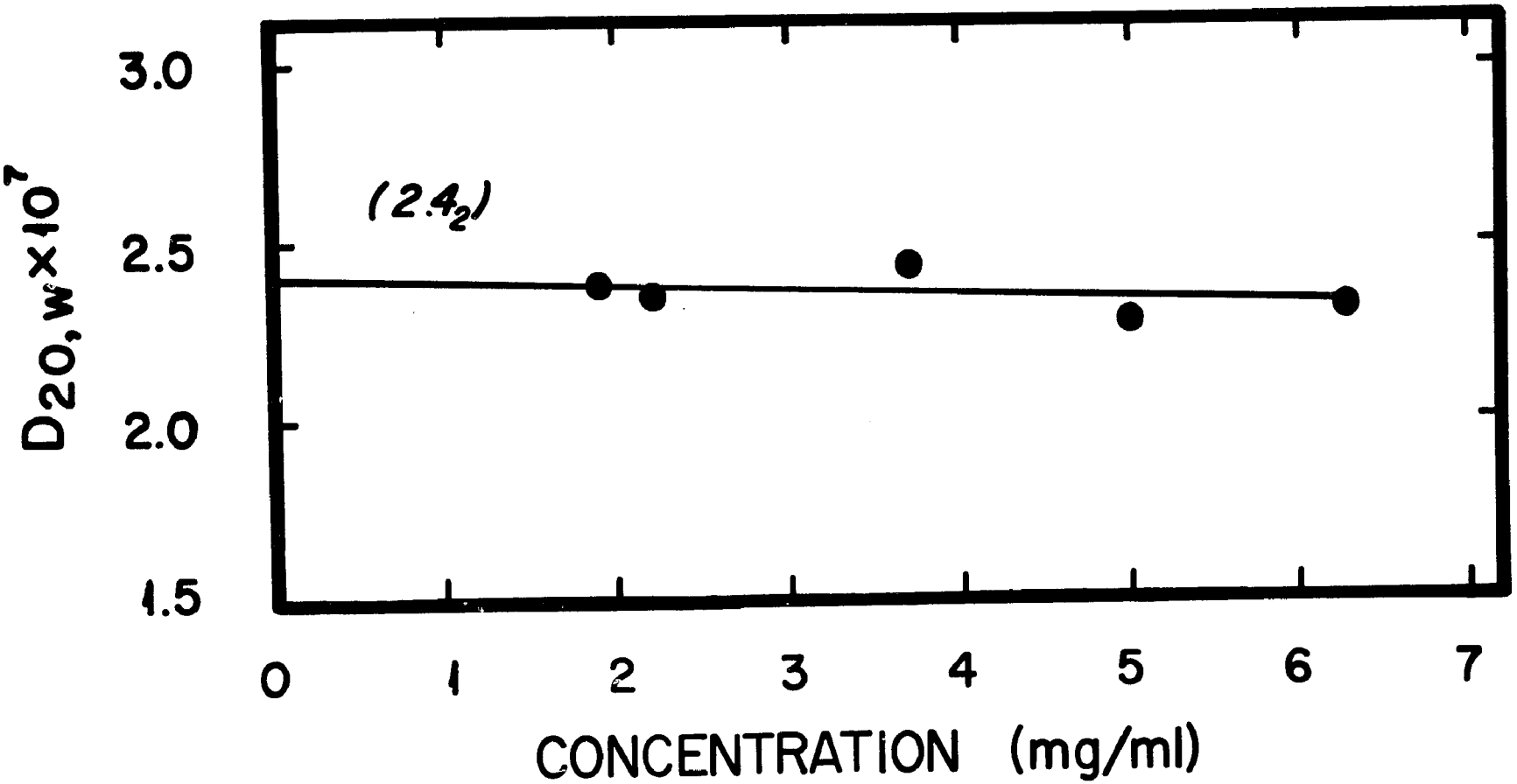

(10.2s)
10.5
10.0
9.5
9.0
S20,w
0
1
2
3
4
5
CONCENTRATION (mg/ml)

Figure 13

Determination of diffusion coefficient ($D^{o}_{20,w}$) of purified lobster hemagglutinin from a plot of $D_{20,w}$ values versus protein concentration. Diffusion measurements were made with a synthetic-boundary cell in 0.076 M phosphate buffer pH 7.5. The $D^{o}_{20,w}$ value in parentheses was obtained by extrapolation of the line determined by a least-squares fit of the experimental points.


3.0
2.5
2.0
1.5
$D_{20,w} \times 10^7$
(2.4_2)
0
1
2
3
4
5
6
7
CONCENTRATION (mg/ml)

partial specific volume of 0.711, a molecular weight of 400,000 was obtained.

Chemical Studies of the Purified Lobster Hemagglutinin. Table V shows the carbohydrate composition of the purified hemagglutinin. The values represent the averages of three determinations and the moles of carbohydrate were calculated on the basis of the 400,000 molecular weight.

The amino acid composition data for the purified hemagglutinin are given in Table VI. These data are averages of duplicate determinations. The lobster hemagglutinin had a high content of both aspartic and glutamic acid which is a common feature of vertebrate immunoglobulins.

Figure 14 shows a tracing of a tryptic peptide map of the purified hemagglutinin. There were forty-five strongly positive ninhydrin-stained spots (spots circled with solid lines) and eleven lightly positive ninhydrin-stained spots (spots circled with dotted lines). Pauly stain for histidine and tyrosine revealed a total of fourteen spots (cross-hatched spots). There was always a dense origin spot on the maps and streaking occurred in the electrophoretic dimension suggesting an indigestible core or large peptides which were poorly stained.

A tryptic hydrolysate of the purified lobster hemagglutinin following reduction and alkylation in 7 M guanidine-HCl was also mapped and showed peptide spots equivalent in number and position

TABLE V

Carbohydrate composition of purified lobster hemagglutinin

	Mannose	Fucose	Galactose	Glucosamine	Sialic acid	Total carbohydrate
M/MP[a]	64	2	14	20	3	-
CHO[b]	2.89	0.09	0.63	0.79	0.23	4.63

[a]Moles carbohydrate/400,000 molecular weight protein

[b]Per cent carbohydrate

TABLE VI

Amino acid composition of purified lobster hemagglutinin

Amino acid	Observed residues per molecule[a]	Mole integer
Aspartic acid	354.00	354
Threonine	183.76	184
Serine.	417.92	418
Glutamic acid	444.81	445
Proline	247.71	248
Glycine	299.95	300
Alanine	237.91	238
Valine.	227.53	228
Methionine.	49.52	50
Isoleucine.	138.20	138
Leucine	281.73	282
Tyrosine.	116.62	117
Phenylalanine	103.15	103
Lysine.	210.00	210
Histidine	105.52	106
Arginine.	208.31	208
Tryptophan[b]	7.83	8

[a]Observed residues per mole were calculated on the basis of an observed molecular weight of 398,400 less the weight due to carbohydrate (18,500)

[b]Determined spectrophotometrically by the method of Sponde and Witkop (77).

to those of the native molecule illustrated in Figure 14. Amino acid analyses of this reduced and alkylated preparation revealed both cysteine and s-carboxymethylcystine present in the preparation indicating either incomplete alkylation or insufficient reduction so that complete alkylation was not achieved. These results were true for two separate preparations.

Quantitative determination of the NH_2-terminal amino acid of the reduced and alkylated purified lobster hemagglutinin was performed using the dansyl reagent. The dansyl derivatives were determined by polyamide layer chromatography and disclosed a single end group, phenylalanine.

Investigation of Subunit Structure. Attempts were made to generate the subunit of lowest molecular weight by means of gel filtration in dissociating solvents so that further analyses could be made. Figure 15A illustrates the protein elution pattern of purified lobster hemagglutinin from an upward-flowing Sephadex G-200 column previously calibrated with molecules of known molecular weights and equilibrated with 5 M guanidine-HCl, pH 5.0. As shown, the majority of material was eluted from the column in the excluded volume indicating that dissociation of hemagglutinin into subunits of less than 200,000 molecular weight did not occur.

In an attempt to facilitate breakdown, the purified hemagglutinin was reduced and alkylated in 5 M guanidine-HCl prior to fractionation under the same column conditions just described. A

Figure 14

Tracing of the tryptic peptide map of purified lobster hemagglutinin. Chromatography was in the horizontal dimension and electrophoresis was in the vertical dimension with the positive electrode on the origin side. Solid lined circles are peptides which stain darkly with ninhydrin. Dotted lined circles are those peptides which stain faintly with ninhydrin. Cross-hatched circles represent Pauly-positive peptides. Gr and ol are peptides staining gray and olive, respectively, with ninhydrin color dip.


GR
OL
TRYPSIN

typical elution profile is shown in Figure 15B. Note that the elution volumes of the protein peaks in Figure 15A and B were almost identical indicating that reduction and alkylation did not result in dissociation of the molecule. Further attempts to dissociate the intact hemagglutinin molecule into subunits involved gel filtration on upward-flowing Sephadex G-200 columns equilibrated with acetic-formic acid pH 2.2 (Figure 15C) and 6 M urea, 1 M propionic acid (Figure 15D). As illustrated by the respective protein elution profiles, the majority of the material applied was excluded from the columns, again suggesting that dissociation into low molecular weight subunits (<200,000 mol wt.) had not been achieved.

Further investigation into the subunit nature of the purified hemagglutinin involved sedimentation velocity experiments in various solvents. These experiments are summarized in Table VII. Purified hemagglutinin had a sedimentation coefficient in pH 7.5 phosphate buffer of 10S. Following partial reduction and alkylation in the same buffer, minor dissociation into a 8.1S component was observed. Exposure to low pH resulted in a greater degree of dissociation as evidenced by the 4.3 sedimentation coefficient obtained in 1.0 M acetic-formic acid buffer. Guanidine-HCl (5.0 M, pH 7.5) appeared to be the most efficient dissociating agent resulting in sedimentation rates for the whole molecule and a reduced and alkylated preparation of 3.1S and 3.4S, respectively.

Additional attempts to determine the lowest molecular weight

Figure 15

Sephadex G-200 gel filtration in dissociating solvents. A. Purified hemagglutinin eluted from column equilibrated with guanidine-HCl, pH 5.0. B. Purified hemagglutinin reduced and alkylated in 7.0 M guanidine-HCl and eluted from same column as above. C. Elution profile of purified hemagglutinin eluted from column equilibrated with acetic-formic acid, pH 2.2. D. Elution profile of purified hemagglutinin eluted from column equilibrated with 6 M urea, 1 M propionic acid. ——●——●——, Absorbancy at 280 mμ.

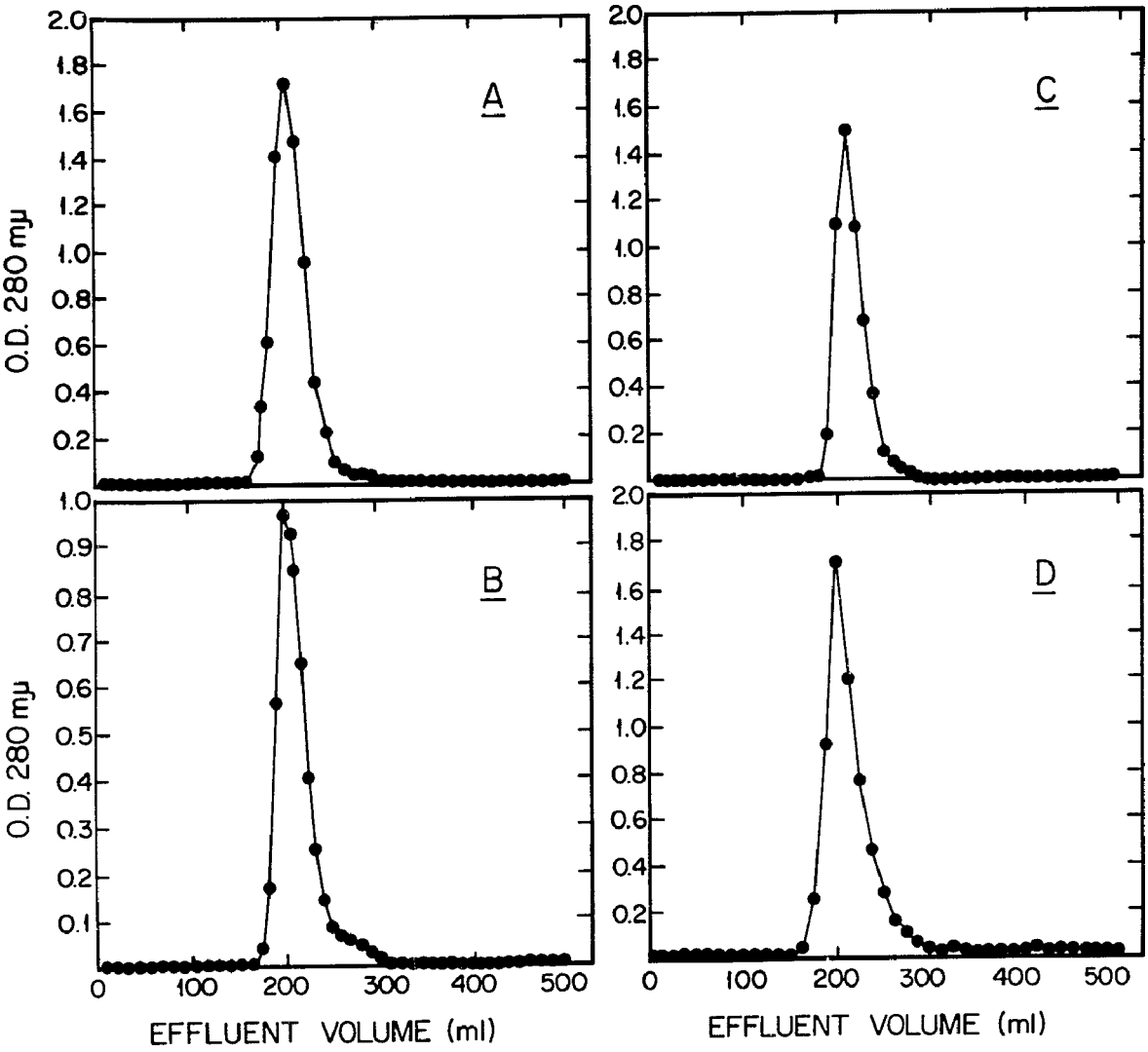

A
B
C
D
O.D. 280 mμ
O.D. 280 mμ
EFFLUENT VOLUME (ml)
EFFLUENT VOLUME (ml)
0
100
200
300
400
500

TABLE VII

Sedimentation Behavior of Purified Lobster Hemagglutinin in Various Solvents[a]

Solvent	pH	$S_{20,w}$ Major Component (s)
Sodium Phosphate 0.076 M	7.5	10.0
Sodium Phosphate 0.076 M (following partial reduction and alkylation)	7.5	8.1
Acetic-Formic acid 1.0 M	2.2	4.3
Guanidine-HCl 5.0 M	7.5	3.1
Guanidine-HCl 5.0 M (following total reduction and alkylation)	7.5	3.4

[a]Total protein concentration in each case was approximately 3.0 mg/ml.

hemagglutinin subunit involved the use of disc acrylamide gel electrophoresis in the dissociating solvent, urea. Purified material was dialyzed overnight versus 10 M urea and subjected to disc acrylamide electrophoresis in gels containing a final urea concentration of 5 M. When compared to an untreated urea preparation, multiple banding was observed indicating that dissociation into lower molecular weight components had occurred. In an attempt to determine the extent of this dissociation, a method for determining molecular weights on disc gels involving the use of known standards was employed. Samples of purified lobster hemagglutinin and standards of known molecular weight were subjected to acrylamide gel electrophoresis in urea. Electrophoretic mobilities of the resultant bands were calculated according to the method of Weber and Osborn (97). Results of these determinations revealed that, compared to the known standards, the lowest molecular weight species resolved from the purified hemagglutinin was approximately 68,500.

CHAPTER IV

DISCUSSION

Many invertebrate species have been shown to possess natural hemagglutinins and hemolysins which in some instances were determined to be associated with proteins (16, 91, 94). The present study was designed to explore the natural hemolysin and hemagglutinin from the spiny lobster, Panulirus argus, and to compare the results obtained to vertebrate antibody systems with similar activities.

The initial experiments reported involved a naturally-occurring hemolysin present in the hemolymph of the spiny lobster. Manipulations involving red cell type, concentration, and quantity were performed in an attempt to achieve optimal indicator conditions in this hemolytic system. A sheep red blood cell concentration of 1.0×10^8 erythrocytes/ml gave the greatest percentage of lysis in the assay used. Attempts were also made to increase titer by varying the ionic strength of the buffer, but were not successful. In each titration the final reaction volume was 3.75 ml. The sigmoidal dose-response curve of the reaction between sheep erythrocytes and increasing amounts of lobster hemolymph indicated that a multiple step mechanism is involved in this hemolytic system.

Recalling the inability of the lysin to hemolyze cells at $0^{\circ}C$,

it may be postulated that the lytic system consists of a single protein species that is first absorbed onto the surface of the red cell, and then proceeds, by one or more subsequent steps, to lyse the cell. If the dose-response curve of the spiny lobster is compared to similar data using sensitized sheep erythrocytes and guinea pig complement, definite similarities are seen to exist indicating that the multiple step phenomenon characteristic of the guinea pig system is also common to the lobster hemolysin.

The results of the kinetics experiment (Fig. 2) were also described by a sigmoid curve. In this plot of the kinetics of hemolysis of sheep erythrocytes by lobster hemolysin, it is interesting that 100 per cent lysis was not achieved even after several hours of incubation. A similar situation occurred when human complement and sensitized sheep erythrocytes were reacted together (47). These findings were in contrast to the guinea pig complement system in which a leveling off of hemolysis was observed after a 50-minute period of incubation in a standard assay system. Again, unlike the guinea pig system whose kinetics exhibited an initial lag period due in part to the need for activation of the first component of this system (C1), the lobster lysin showed no such lag, suggesting a simpler system requiring no activation step.

One of the more interesting facts about the lobster hemolytic system was its inability to react at the lower temperature of $0^{o}C$ and $4^{o}C$ over extended periods of time. In this regard, the lysin

of the spiny lobster differed from the lamprey lysin reported by Gewurz et al. (32), which exhibited increased activity at lower temperatures. As noted previously, incubation of lobster hemolymph and sheep erythrocytes under conditions of lowered temperatures for an 18-hour interval resulted in no detectable lysis. Results such as these suggest that the actual mechanism of cell lysis might proceed by means of an enzymatic type reaction similar to one or more of the reaction steps found in the classical hemolytic complement system.

Subsequent studies involved the isolation and characterization of the naturally-occurring hemagglutinin found in the hemolymph so that its structure could be compared with that of vertebrate immunoglobulins. The lobster hemagglutinin was found to be reactive against a variety of vertebrate erythrocyte species, but showed elevated titers to human red blood cell types. For this reason, and because of their easy accessibility, human A cells were used for all titrations of hemagglutinating activity.

Inactivation studies revealed the hemagglutinin to be destroyed after incubation at 60°C for 20 minutes, while the more labile hemolysin was inactivated at 52°C in the same period. This difference in heat lability of the two activities could indicate that independent molecular species are responsible for each of the observed activities. On the other hand, if mechanisms akin to the complement-antibody interactions of vertebrate sera are involved,

the hemolytic activity may depend on the hemagglutinin ("antibody") and a complement-like system which is inactivated at 52°C like its vertebrate counterpart (47).

It is interesting to note that both hemolytic and hemagglutinin activities were lost following dialysis versus EDTA, and that both could be reactivated by the addition of divalent cations. Ca^{++} was the most efficient in restoring hemagglutinin activity. However, no enhancement of this activity was observed by the addition of excess Ca^{++} to the assay system as described for the hemagglutinin from the horseshoe crab (60). Absorption of lobster hemolymph with whole human erythrocytes or human red cell stroma resulted in loss of hemagglutinin activity with a concomitant loss of hemolytic activity. It should be pointed out, however, that due to the extremely labile nature of the hemolytic system, loss of this activity following exposure to human erythrocytes may have been the result of the mechanical manipulations involved in the absorption procedure itself.

Efforts to define the structural nature of this invertebrate hemagglutinin involved the isolation and purification of the protein species responsible for the observed agglutination activity. Initial attempts to purify the active principle from whole lobster hemolymph by means of gel filtration were not successful. Ultracentrifuge analysis (Figure 10A) revealed lobster hemolymph to contain three different components based on sedimentation velocity.

The major component was the respiratory pigment hemocyanin sedimenting at 10.2S with minor components of 7.4 and 4.9S. The inability to obtain pure fractions following gel filtration of the whole hemolymph was attributed to the reported tendency of invertebrate hemocyanins to dissociate into smaller subunits when subjected to changes in pH and salt concentration (69). Removal of the majority of the hemocyanin was accomplished by using an isoelectric precipitation technique first described by Tyler (94) in characterizing heteroagglutinins from another species of lobster, Panulirus interruptus. Due to the close proximity of the sedimentation rates of the components of lobster hemolymph, preparative ultracentrifugation, as outlined by Marchalonis for the removal of hemocyanin from Limulus hemolymph (60), was not employed. Once the hemocyanin fraction had been removed, subsequent purification was achieved by a combination of Pevikon block electrophoresis (Figure 7), and a series of gel filtrations on Sepharose 6-B (Figure 9).

The purified lobster hemagglutinin was found to be a protein of relatively low electrophoretic mobility (Figure 6) which comprised less than one per cent of the total hemolymph protein. The hemagglutinin molecule was judged to be free of contaminating proteins as determined by analytical ultracentrifugation (Figure 10B) and immunoelectrophoresis (Figure 11). The intact molecule sedimented with $S^{o}_{20,w}$ = 10.2S and had a $D^{o}_{20,w}$ = 2.4. Although the hemagglutinin could be isolated in pure form, aggregation was found to

occur with time as evidenced by the front slope of the peak shown in the ultracentrifuge pattern (Figure 10B). Therefore the molecular weight of the intact molecule was determined by a combination of sedimentation and diffusion data. As pointed out by Creeth and Pain (19), the transport method, in contrast to the equilibrium method, is almost unaffected by high molecular weight aggregates. The molecular weight of the intact hemagglutinin molecule, using a $\bar{v}$ of 0.711 calculated from a combination of amino acid and carbohydrage data, was determined to be approximately 400,000.

Carbohydrate analysis (Table V) revealed a total carbohydrate content of 4.6% which approximates the concentration of the total carbohydrate of 7% reported by Hammarstrom and Kabat (37) for a hemagglutinin isolated from the snail, Helix pomatia, and 6.5% total carbohydrate reported by Acton, et al. for a hemagglutinin isolated from the oyster, Crassostrea virginica (2).

The amino acid composition of the purified hemagglutinin (Table VI) and peptide maps of tryptic hydrolysates (Figure 14) of the protein differed from those of immunoglobulin chains. Amino acid analysis of the purified material following attempted total reduction and alkylation in 7.0 M guanidine-HCl revealed the presence of both cysteine and s-carboxymethylcystine. This suggested an extremely compact molecular configuration which prevented complete reduction and subsequent alkylation. Following tryptic hydrolysis, peptide maps of this material revealed spots of equivalent number and posi-

tion to those obtained with the intact hemagglutinin molecule. When the amino acid composition of the reduced and alkylated material was calculated on the basis of the subunit molecular weight of 68,500, obtained by disc gel electrophoresis, the number of tryptic peptides observed on peptide mapping agreed quite well with the number predicted on the basis of lysine and arginine content.

From the sedimentation velocity data (Table VII) it appears that the subunits are not linked via covalent bonds, such as disulfide bonds, because the sedimentation rates of the molecule were essentially the same before and after reduction and alkylation. Susuki and Deutsch (85) have determined the sedimentation coefficient of human IgM heavy chain (app. 70,000 mol. wt.) to be about 3S in 6 M guanidine-HCl. Thus, the subunit molecular weight of 68,500 as determined by disc acrylamide gel electrophoretic methods is in fair agreement with the sedimentation rate of the hemagglutinin molecule of approximately 3S in 5.0 M guanidine-HCl.

Results of the subunit studies (Figure 15 and Table VII) indicate that extremely strong non-covalent interactions such as hydrophobic interactions, electrostatic interactions and hydrogen bonds are responsible for stabilization of the intact hemagglutinin molecule. On this basis one might postulate a hypothetical structural model for the lobster hemagglutinin. The intact molecule (molecular weight approximately 400,000) might be represented as consisting of six subunits of 68,000 molecular weight each, held together by

extremely strong non-covalent interactions as described above.

The fact that the hemagglutinin subunit had a molecular weight comparable to mammalian immunoglobulin heavy chains and showed some similarity to heavy chains in amino acid and total carbohydrate compositions is not conclusive evidence that the spiny lobster hemagglutinin and vertebrate antibodies are related evolutionary developments. Until comparisons are made between the lobster hemagglutinin and vertebrate immunoglobulins at the primary structural level, the possibility remains that some relationship exists between these two protein species.

CHAPTER V

SUMMARY

The purpose of the present investigation was to determine whether the natural hemagglutinin and hemolysin observed in a representative crustacean, the spiny lobster Panulirus argus, might represent primitive or precursor molecules related to vertebrate immune systems.

Investigation of the naturally-occurring hemolytic system in the spiny lobster showed activity for sheep erythrocytes. The resulting sigmoid shaped dose-response curve of the reaction between sheep cells and increasing amounts of lobster hemolymph indicated that a multiple step mechanism was involved similar to those of mammalian hemolytic systems. Kinetic experiments involving the lysin differed from those characteristic of the classical guinea pig complement system. The lobster hemolytic system was heat labile, inactivated by EDTA, and temperature-dependent; the latter indicating the existence of an enzymatic-type reaction. Attempts to potentiate lytic activity by addition of rabbit hemolysin were not successful.

Subsequent investigation of the natural hemagglutinin found in lobster hemolymph revealed a wide range of reactivity. This hemagglutinin was also heat labile, and inactivated by EDTA. Further studies of this hemagglutinin centered about the elucidation of its physicochemical properties so that comparisons could be made with

the hemagglutinin molecules of other invertebrates and the immunoglobulins of vertebrate species.

The spiny lobster hemagglutinin was found to be a protein of relatively low electrophoretic mobility which comprised less than 1% of the total hemolymph protein. The purified molecule sedimented with an $S^{o}_{20,w}$ = 10.2S and had a $D^{o}_{20,w}$ = 2.4. The molecular weight of the intact molecule was calculated from the sedimentation and diffusion data by use of the Svedberg equation. Using the calculated partial specific volume of 0.711, a molecular weight of 400,000 was obtained. Carbohydrate analysis revealed a total carbohydrate content of approximately 5%. This value was similar to carbohydrate determinations reported for other invertebrate hemagglutinins. Quantitative determination of the NH_2-terminal amino acid revealed a single end group, phenylalanine. From the amino acid composition data, and peptide maps of tryptic hydrolysates of the purified hemagglutinin, no direct correlation with vertebrate immunoglobulin chains could be made. Disc acylamide gel electrophoresis in urea, as well as ultracentrifugation and column chromatography in dissociating solvents, was employed to determine the lowest molecular weight subunit of the hemagglutinin. The molecule consisted of subunits held together by extremely strong non-covalent interactions. From sedimentation velocity data and results of size determination on disc gels, the subunit was determined to have a molecular weight of approximately 68,500. Attempts at further dissociation were not

successful. On this basis, the hemagglutinin molecule most probably has a structure consisting of six subunits of 68,500 molecular weight each held together by non-covalent interactions.

These data do not demonstrate obvious structural similarities to vertebrate immunoglobulins. The fact that the hemagglutinin subunit had a molecular weight comparable to vertebrate immunoglobulin heavy chain and the hemagglutinin molecule showed some similarity of such chains from the data on amino acid and total carbohydrate compositions is not conclusive proof that these proteins are related, despite a similarity of function. Until comparisons are made between this hemagglutinin molecule and immunoglobulin chains of vertebrate species at the level of primary structure, the possibility remains that the lobster hemagglutinin might be a precursor of the vertebrate immunoglobulins.

CHAPTER VI

LIST OF REFERENCES

1. Acton, R. T., Bennett, J. C., Evans, E. E., and Schrohenloher, R. E. Physical and Chemical Characterization of an Oyster Hemagglutinin. J. Biol. Chem. 244:4128-4135, 1969.

2. Acton, R. T. and Evans, E. E. Bacteriophage clearance in the oyster (Crassostrea virginica). J. Bacteriol. 95:1260-1266, 1968.

3. Acton, R. T., Weinheimer, P. F. and Evans, E. E. A bactericidal system in the lobster, Homarus americanus. J. Invert. Path. 13:463-464, 1969.

4. Bang, F. B. A bacterial disease of Limulus polyphemus. Bull. Johns Hopkins Hosp. 98:325-351, 1956.

5. Bang, F. B. Reaction to injury in the oyster Crassostrea virginica. Biol. Bull., Woods Hole. 121:57-68, 1961.

6. Bang, F. B. Serological aspects of immunity in invertebrates. Nature, 196:88-89, 1962.

7. Bang, F. B. Serologic response in a marine worm, Sipunculus nudus. J. Immunol. 96:960-972, 1966.

8. Bennett, J. C. Paper chromatography and electrophoresis: Special procedure for peptide maps. In Methods in Enzymology (Edited by Hirs, C.H.W.) Academic Press, New York Vol. 11, Chapter 36, pp. 330-339, 1967.

9. Bernheimer, A. W., Caspari, E., and Kaiser, A. D. Studies on antibody formation in caterpillars. J. Exp. Zool. 119:23-35, 1952.

10. Bettini, S. Acquired immune response of the house fly, Musca domestica (linnaeus), to injected venom of the spider Latrodectus mactans tredecimguttatus (Rossi). J. Invert. Path. 7:378-383, 1965.

11. Briggs, J. D. Humoral immunity in Lepidopterous larvae.

J. Exp. Zool. 138:155-188, 1958.

12. Briggs, J. D. Immunological Responses. In The Physiology of Insects (Edited by M. Rockstein). Acad. Press., New York, Vol. 3, p. 259-283, 1964.

13. Cahn, E. J. and Edsall, J. T. Determinations of Partial Specific Volume. E. J. Cahn and J. T. Edsall, eds., "Proteins, amino acids, and peptides", Reinhold Publishing Corp., New York, p. 370, 1943.

14. Cheng, T. C. Marine molluscs as hosts for synbioses. Advan. Marine Biol. 5:1-424, 1967.

15. Cheng, T. C. Immunity in mollusca with special reference to reactions to transplants. Transplant. Proc. II:226-230, 1970.

16. Cohen, E., Rose, A. W. and Wissler, F. C. Heteroagglutinins of the horseshoe crab, Limulus polyphemus. Life Sci. 4:2009-2015, 1962.

17. Cooper, E. L. Transplantation immunity in annelids. I. Rejection of xenografts exchanged between Lumbricus terrestris and Eisenia foetido. Transplantation. 6:322-337, 1968.

18. Cooper, E. L., Acton, R. T., Weinheimer, P. F., and Evans, E. E. Lack of a bactericidal response in the earthworm Lumbricus terrestris after immunization with bacterial antigens. J. of Invert. Pathol. 14:402-406, 1969.

19. Creeth, J. M. and Pain, R. H. in J. A. V. Bulter and H. E. Huxley, eds., "Progress in Biophysics and Molecular Biology", Pergamon Press, New York, p. 217, 1967.

20. Cushing, J. E., McNeely, J. L., and Tripp, M. R. Comparative immunology of sipunculid coelomic fluid. J. Invert. Path. 14:4-12, 1969.

21. Duncan, J. T. On a bactericidal principle present in the alimentary canal of insects and arachnids. J. Parasitology. 18:238-252, 1926.

22. Ehrenberg, A. Determination of Molecular Weights and Diffusion Coefficients in the Ultracentrifuge. Acta Chem. Scand. 11:1257-1270, 1957.

23. Evans, E. E., Acton, R. T., Bennett, J. C., and Weinheimer,

P. F. Evolution of the immune response. In Peptides and Protides of the Biological Fluids. Proceedings of the 17th Colloquium, Bruges, Belgium. p. 29-38, 1970.

24. Evans, E. E., Cushing, J. E., Sawyer, S., Weinheimer, P. F., Acton, R. T. and McNeely, J. L. Induced bactericidal response in the California spiny lobster Panalirus interruptus. Proc. Soc. Exp. Biol. Med. 132:111-114, 1969.

25. Evans, E. E., Painter, B., Evans, M. L., Weinheimer, P. and Acton, R. T. An induced bactericidin in the spiny lobster, Panulirus argus. Proc. Soc. Exp. Biol. Med. 128:394-398, 1968.

26. Evans, E. E., Weinheimer, P. F., Acton, R. T. and Cushing, J. E. Induced bactericidal response in a sipunculid worm. Nature 223:695, 1969.

27. Evans, E. E., Weinheimer, P. F., Painter, B., Acton, R. T. and Evans, M.L. Secondary and tertiary responses of the induced bactericidin from the West Indian spiny lobster, Panulirus argus. J. Bacteriol. 98:943-946, 1969.

28. Feng, S. Y. Responses of molluscs to foreign bodies, with special reference to the oyster. Fed. Proc. 26:1685-1692, 1967.

29. Fernández-Morán, H., Marchalonis, J. J. and Edelman, G. M. Electron microscopy of a hemagglutinin from Limulus polyphemus. J. Mol. Biol. 32:467-469, 1968.

30. Finstad, J. and Good, R. A. The evolution of the immune response. III. Immunologic responses in the lamprey. J. Exp. Med. 120:1151-1168, 1964.

31. Frings, H., Goldberg, E. and Arentzen, J. C. Antibacterial action of the blood of the large milkweed bug. Science 108:689-690, 1948.

32. Gerwurz, H., Finstad, J., Muschel, L. H. and Good, R. A. In "Phylogeny of Immunity", Univ. of Florida Press, Gainesville, Florida, p. 105, 1966.

33. Gingrich, R. E. Acquired humoral immune responses of the large milkweed bug, Oncopeltus fasciatus (Dallas), to injected materials. J. Insect Physiol. 10:179-194, 1964.

34. Good, R. A. and Papermaster, B. W. Ontogeny and phylogeny of

adaptive immunity. Advan. Immunol. 4:1-115, 1964.

35. Gray, W. R. and Hartly, B. S. The Structure of a Chymotryptic Peptide from Pseudomonas cytochrome C-551. Biochem. J. 89:379-382, 1963.

36. Habeeb, A.F.S.A. Studies on the ε-prototoxin of Clostridium perfringens Type O. Archives of Biochem. and Biophysis. 130:430-438, 1969.

37. Hammarström, S. and Kabat, E. A. Purification and characterization of a blood-group A reactive hemagglutinin from the snail Helix pomatia and a study of its combining site. Biochem. 8:2696-2705, 1969.

38. Heimpel, A. M. and Harshbarger, J. C. Symposium on microbial insecticides V. Immunity in insects. Bact. Rev. 29:397-405,

39. Hickman, C. P., ed., Integrated Principles of Zoology. C. V. Mosby Co. St. Louis, Mo. p. 372-390, 1961.

40. Hilgard, H. R. and Phillips, J. H. Sea urchin response to foreign substances. Science 161:1243-1245, 1968.

41. Hood, L., Gray, W. R., Sanders, G. B. and Dreyer, W. J. Light chain evolution. Cold Spr. Harb. Sym. Quant. Biol. 32:133-146, 1967.

42. Hubbard, R. W. Studies in accelerated amino acid analysis. Biochem. Biophys. Res. Commun. 19:679-685, 1965.

43. Huff, C. G. Immunity in invertebrates. Phys. Rev. 20:68-85, 1940.

44. Jakouska, S. and Nigrelli, R. F. Antimicrobial substances from sponges. Ann. N. W. Acad. Sci. 70:913-916, 1960.

45. Johnson, P. T. The coelomic elements of sea urchins (Strongylocentrotus) III. In Vitro reaction to bacteria. J. Invert. Path. 13:42-62, 1969.

46. Johnson, P. T. and Chapman. F. A. Comparative study on the in vitro response of bacteria to invertebrate body fluids I. Dendrostomum zostericolum, a sipunculid worm. J. of Invert. Pathol. 16:127-138, 1970.

47. Kabat, E. A. and Mayer, M. M. Agglutination. In Experimental Immunochemistry. Charles C. Thomas, Springfield, Chapter 3,

pp. 97-132, 1967.

48. Kamon, E. and Shulov, A. Immune response of locusts to venom of the scorpion. J. Invert. Path. 7:192-198, 1965.

49. Kielley, W. W. and Harrington, W. F. A model for the myosin molecule. Biochem. Biophys. Acta. 41:401-421, 1960.

50. Krassner, S. M. Further studies on an antibacterial factor in the blood of Phascolosoma gouldii. Biol. Bull. 125:382-383, 1963.

51. Lamm, M. E. and Small, P. A., Jr. Polypeptide chain structure of rabbit immunoglobulins. II. γM-immunoglobulin. Biochemistry 5:267-285, 1966.

52. Li, C. P., Prescott, B., Jahues, W. G. and Martins, E. C. Antimicrobial agents from mollusks. Trans. N. Y. Acad. Sci. 24:504-509, 1962.

53. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275, 1951.

54. McDade, J. E. and Tripp, M. R. Mechanisms of agglutination of red blood cells by oyster hemolymph. J. Invert. Path. 9:523-530, 1967.

55. McDade, J. E. and Tripp, M. R. Lysozyme in the hemolymph of the oyster. J. Invert. Path. 9:531-535, 1967.

56. McDade, J. E. and Tripp, M. R. Lysozyme in oyster mantle mucus. J. Invert. Pathol. 9:581-584, 1967.

57. McKay, D. and Jenkin, C. R. Immunity in the invertebrates. II. Adaptive immunity in the crayfish Parachaeraps bicarinatus. Immunology 8:127-137, 1969.

58. McKay, D., Jenkin, C. R. and Rowley, D. Immunity in the invertebrates. I. Studies on the naturally occurring hemagglutinins in the fluid from invertebrates. Aust. J. Exp. Biol. Med. Sci. 47:125-134, 1969.

59. Maizel, J. V., Jr. Acrylamide gel electrophorograms by mechanical fractionation: Radioactive adenovirus proteins. Science 151:988-990, 1966.

60. Marchalonis, J. J. and Edelman, G. M. Isolation and charac-

terization of a hemagglutinin from Limulus polyphemus. J. Mol. Biol. 32:453-465, 1968.

61. Michelson, E. H. Miracidia-immobilizing substances in extracts prepared from snails infected with Schistosoma mansoni. Am. J. Trop. Med. Hyg. 13:36-42, 1964.

62. Müller-Eberhard, H. J. and Osterland, C. K. Preparative Zone Electrophoresis. In C. A. Williams and M. W. Chase, eds., "Methods in Immunology and Immunochemistry", Vol. 2, Academic Press, N. York, p. 57, 1968.

63. Nelstrap, A. E., Taylor, C. and Collard, P. Studies on phagocytosis. III. Antigen clearance studies in invertebrates and poikilothermic vertebrates. Immunol. 14:347-356, 1968.

64. Niedermeier, W. N. Gas Chromatography of Neutral and Amino Sugars in Glycoproteins. J. of Anal. Biochem. In press, 1970.

65. Noguchi, H. On the multiplicity of the serum hemagglutinins of cold-blooded animals. Zentr. Bakteriol. Parasitenk. 33: 353-356, 1903.

66. Papermaster, B. W., Condie, R. M., Finstad, J. and Good, R. A. Evaluation of the immune response. I. The phylogenetic development of adaptive immunologic ressponsiveness in vertebrates. J. Exp. Med. 119:105-109, 1964.

67. Papermaster, B. W., Condie, R. M. and Good, R. A. Immune response in the California hagfish. Nature 196:355-357, 1964.

68. Phillips, J. H. and Yardley, B. J. Detection in invertebrates of inducible, reactive material resembling antibody. Nature 188:728-730, 1960.

69. Pickett, S. M., Riggs, A. F. and Larimer, J. L. Lobster hemocyanin: Properties of the minimum functional subunit and of aggregates. Science. 151:1005-1006, 1966.

70. Rabin, H. and Bang, F. B. Studies on the antibacterial activity of Golfingia gouldii Coelomic fluid. Biol. Bull. 125:388, 1963.

71. Reade, P. C. Phagocytosis in invertebrates. Aust. J. Exp. Biol. Med. Sci. 46:219-229, 1968.

72. Salt, G. Cellular defense mechanisms in insects. Fed. Proc.

26:1671-1674, 1967.

73. Schachman, H. K. Ultracentrifugation in Biochemistry. Academic Press, New York, 1959.

74. Schmeer, M. R. and Huala, C. V. Mercenene: In vitro effects of mollusk extracts on the Sarcoma 180. Science. 118:603-610, 1965.

75. Schwab, G. E., Reeves, P. R. and Turner, K. J. Bactericidal activity of serum of the yabbie Parachaeraps bicarinatus. Brit. J. Exp. Path. 47:266-274, 1966.

76. Smith, R. T., Miescher, P. A. and Good, R. A., eds. "Phylogeny of Immunity", Univ. of Florida Press, Gainesville, Florida, 1966.

77. Spande, T. F. and Witkop, B. Determination of the tryptophan content of proteins with N-bromosuccinimide. In Methods in Enzymology (Edited by Hirs, C.H.W.) Academic Press, N. Y. Vol. 11, Chapter 58, pp. 498-506, 1967.

78. Stauber, L. A. Immunity in invertebrates, with special reference to the oyster. Proc. Natl. Shellfisheries Assoc. 50:7-20, 1961.

79. Stephens, J. M. Immune responses of some insects to some bacterial antigens. Can. J. of Microbiol. 5:203, 1959.

80. Stephens, J. M. Bactericidal activity of the blood of actively immunized wax moth larvae. Can. J. of Microbiology. 8:491-494, 1962.

81. Stephens, J. M. Influence of active immunization on melanization on the blood of the wax moth larvae. Can. J. of Microriol. 8:597-601, 1962.

82. Stephens, J. M. Bactericidal activity of hemolymph of some normal insects. J. of Insect Pathol. 5:61-64, 1963.

83. Stephens, J. M. Protective effects of several immunizing preparations that produce active immunity in Galleria mellonella (Linnaeus). J. of Insect Pathol. 5:129-132, 1963.

84. Stephens, J. M. Effect of active immunization on total hemocyte counts of larvae of Galleria mellonella (Linnaeus). J. of Insect Pathol. 5:152-155, 1963.

85. Suzuki, T. and Deutsch, H. F. Dissociation, Reaggregation, and Subunit Structure Studies of Some Human γM-Globulins. J. Biol. Chem. 242:2725-2738, 1967.

86. Taylor, A. E., Taylor, G. and Collard, P. Secondary immune response to bacteriophage T1 in the shore crab, Carcinus meanus. Nature, London 203:775, 1964.

87. Teague, P. D. and Friou, G. J. Lack of immunological responses by an invertebrate. Comp. Biochem. Physiol. 12:471-478, 1964.

88. Triplett, E. L., Cushing, J. E. and Durall, G. L. Observations on some immune reactions of the sipunculid Dendrostromum zostericolum. Am. Naturalist 92:287-293, 1958.

89. Tripp, M. R. The fate of foreign materials experimentally introduced into the snail Australorbis glabratus. J. Parasitol 47:751-754, 1961.

90. Tripp, M. R. Cellular responses of mollusks. Ann. N. Y. Acad. Sci. Art. 1, 113:467-474, 1963.

91. Tripp, M. R. Hemagglutinin in the blood of the oyster, Crassostrea virginica. J. Invert. Path. 8:478-484, 1966.

92. Tyler, A. Natural heteroagglutinins in the body fluids and seminal fluids of various invertebrates. Biol. Bull. 90:213-219, 1946.

93. Tyler, A. and Metz, C. B. Natural heteroagglutinins in the serum of the spiny lobster, Panulirus interruptus. I. Taxonomic range of activity, electrophoretic and immunizing properties. J. Exp. Zool. 100:387-406, 1945.

94. Tyler, A. and Scheer, B. T. Natural heteroagglutinins in the serum of the spiny lobster, Panulirus interruptus. II. Chemical and antigenic relation to blood proteins. Biol. Bull. 89:193-200, 1945.

95. Wagner, R. R. Acquired resistance to bacterial infection in insects. Bact. Rev. 25:100-110, 1961.

96. Warren, L. The thiobarbiturie acid assay of sialic acids. J. Biol. Chem. 234:1971-1975, 1959.

97. Weber, K. and Osborn, M. The Reliability of Molecular Weight Determinations by Dodecyl Sulfate-polyacrylamide Gel Electrophoresis. J. Biol. Chem. 244:4404-4412, 1969.

98. Weinheimer, P. F., Acton, R. T., Cushing, J. E. and Evans, E. E. Reactions of Sipunculid coelomic fluid with erythrocytes. Life Sciences. 9:145-152, 1970.

99. Weinheimer, P. F., Acton, R. T. and Evans, E. E. Attempt to induce a bacterialcidal response in the oyster. J. Bacteriol. 97:462-463, 1969.

100. Weinheimer, P. F., Acton, R. T., Sawyer, S. and Evans, E. E. Specificity of the induced bactericidin of the West Indian spiny lobster, Panulirus argus. J. Bacteriol. 98:947-948, 1969.

101. Wong, S. S. and Carpenter, F. H. A Compositional Assay for Insulin Applied to a Search for "Proinsulin" J. Biol. Chem. 240:1619-1625, 1965.

102. Woods, K. R. and Wang, K. T. Separation of dansyl-amino acids by polyamide layer chromatography. Biochem. Biophys. Acta. 133:369-370, 1967.