THE ISOLATION, PURIFICATION AND CHARACTERIZATION OF HEMAGGLUTININ AND ANTI-TUMOR ACTIVITIES FROM *AMPHITRITE ORNATA*, A MARINE POLYCHAETOUS ANNELID

by

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ABSTRACT

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SEYMOUR J. GARTE

Advisor: Professor Charlotte S. Russell

Extracts of the marine polychaetous annelid Amphitrite ornata were found to possess hemagglutinating activity against rat, rabbit, chicken and human erythrocytes, and anti-tumor activity toward Ehrlich ascites tumor in mice. Fractionation of extracts by gel filtration on Sephadex G-100 gave three active fractions of molecular weights 30,000, 54,000 and $\geq$100,000 daltons. The 30,000 dalton fraction (fraction B) was purified 72-fold by ammonium sulfate precipitation, gel filtration and preparative disc gel electrophoresis. The purified hemagglutinin was homogeneous on disc gel electrophoresis at several pH values, and gave one sharp boundary in sedimentation velocity ultracentrifugation.

The three hemagglutinin fractions were characterized with respect to several biological, chemical and physical properties. The three fractions showed parallel specificity toward vertebrate erythrocytes; rat red blood cells (RBC) gave the highest titers. The agglutinin was active against

A, B, O, and AB blood groups, but exhibited 4-fold higher activity toward group A erythrocytes. The Amphitrite hemagglutinin activity was inhibited only by N-acetyl-D-galactosamine, the terminal sugar residue of the group A immunodeterminant. The hemagglutinin activity from all three fractions was insensitive to dialysis or treatment with EDTA. The activity was not affected by digestion with trypsin or pronase, but was destroyed by phenolic extraction. Analytical disc gel electrophoresis showed one protein band in each fraction (with high anodal mobility at pH 8.5) which was not affected by the proteolytic enzymes, but was removed by phenol. Preparative disc gel electrophoresis proved this band to contain the hemagglutinin activity. The activity was heat stable up to 85° for 30 minutes, and showed maximum activity at pH 7-9. Below pH 7 the activity reversibly decreased, and at pH 4 was irreversibly destroyed.

The high molecular weight hemagglutinin fraction (A1) could be dissociated to give the low molecular weight fraction B by 6M urea. The dissociation was not reversible after the removal of urea by dialysis.

The purified hemagglutinin (from fraction B) was chemically characterized. It is a glycoprotein containing 13% carbohydrate. The molecular weight of the molecule as determined by gel filtration, SDS disc gel electrophoresis, approach to equilibrium sedimentation and amino acid analysis was 32,000. The four methods gave good agreement. Amino acid analysis

showed a preponderance of acidic amino acids. Other unusual features of the amino acid composition were relatively large amounts of cysteine (14) and proline (18), and the small number of basic amino acid residues. The carbohydrate composition included mannose, galactose, glucosamine, and sialic acid. Both amino acid and carbohydrate analyses were in good agreement with published values for other invertebrate hemagglutinins.

In order to test for a possible correlation between anti-tumor and hemagglutinin activities in extracts of Amphitrite ornata, the purified hemagglutinin was assayed for anti-tumor activity. The purified hemagglutinin was active (approximately 10-fold greater activity than crude extracts) against Ehrlich ascites tumor in vivo. None of the Amphitrite hemagglutinin fractions exhibited any agglutinating activity toward Ehrlich ascites cells in vitro.

The Amphitrite hemagglutinin was compared with hemagglutinins from other invertebrate species in terms of structure and biological activity. The Amphitrite hemagglutinin is the first hemagglutinin isolated from a polychaetous annelid, the first annelid agglutinin to be purified and characterized chemically, and is the first example of an invertebrate hemagglutinin with anti-tumor activity.

Dedicated to my wife

Michal

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

PBS - Phosphate buffered saline, 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.0

RBC - Red blood cells

TRIS - 2-amino-2-(hydroxymethyl)-1,3 propanediol

TEMED - N,N,N',N',-tetramethylethylenediamine

BIS - N,N -dimethylbisacrylamide

SDS - Sodium dodecyl sulfate

TPCV - Total packed cell volume (for Ehrlich ascites)

T/C - Test/control

TCA - Trichloroacetic acid

ABS - Absorbance

NM - Nanometers

TMS - Trimethylsilyl

MCE - Mercaptoethanol

INTRODUCTION

The ability of an organism to distinguish its own tissues and cells from all other "foreign" materials is crucial to the defense of the organism. In vertebrates, the mechanism by which extraneous substances are recognized, and then neutralized or removed is the immune system. A critical step in the process of immunity is the binding of the invading agent, or antigen, by defense molecules produced by the host: the antibodies. If the antigen is a cell, such as bacteria or the erythrocytes of another species, addition of the specific antibody to a suspension of cells may cause them to clump together. This phenomenon, called agglutination, is one of several well-known antigen-antibody reactions, which also include lysis (for cells), precipitation and complement fixation (for soluble antigens) (1,2).

In order to bind to cells the agglutinin (a macromolecule) must interact with specific sites on the cell surface. These binding sites are usually carbohydrate, such as N-Acetyl-D-galactosamine on human group A erythrocytes (3). The specificity of agglutinins toward cells is therefore due to the chemical nature of the cell surface.

Antibodies are not the only molecules which may act as agglutinins. Sperm molecules are agglutinated by fertilisin, a protein molecule extracted from sea urchin eggs (4). Many types of viruses will agglutinate red blood cells (hemagglu-

tination). A clinically important instance of hemagglutination is that caused by intra-species blood group antigens, or isoagglutinins, such as the human group A, B and O substances. Of growing interest and significance are the hemagglutinin molecules which have been isolated from certain plants and invertebrates, organisms that do not possess a vertebrate type immune response system.

Hemagglutinins from Plants

The ability of certain plant extracts to agglutinate mammalian erythrocytes has been known since 1888 (5). The hemagglutinins from plants, called lectins, have been isolated from a wide variety (over 800 species) of legumes. (See review by Sharon and Lis (6)). A number of lectins have been purified and crystallized, including Concanavalin A (from jack beans) which has been extensively studied. The amino acid sequence and quarternary structure of this molecule has been determined by Edelman and co-workers (7), and detailed information of its binding function has been provided by a number of laboratories (8). The lectins are predominantly glycoproteins (with the notable exception of Concanavalin A which is a protein) with the carbohydrate moiety contributing from 2 to 10% of the molecule. The molecular weights of the lectins are varied, although most are in the range 100,000-200,000 daltons. Many of these molecules consist of dissociable subunits of various size, and require a divalent cation, usually Ca^{++} for

activity (see Sharon and Lis (6) for examples).

In addition to agglutinating erythrocytes of several vertebrate species with varying specificity, lectins will also bind to certain specific saccharides. In general, a particular lectin will agglutinate the erythrocytes of a particular species if the saccharide specific for that lectin is present on the erythrocyte surface. The affinity of lectins for particular saccharides may be determined by testing a number of saccharides for competitive inhibition of cell agglutination. Information about the chemical makeup of the cell surface may be obtained using these agglutination inhibition tests (see below).

It has recently been discovered that certain lectins, notably Wheat Germ Agglutinin, and ConcanavalinA, have higher agglutinin activity toward mouse lymphocyte tumor and transformed culture cells than toward normal cells (6). Ehrlich ascites cells are agglutinated <u>in vitro</u> by soy bean agglutinin, which is specific for N-Acetyl-D-galactosamine (9).

The many interesting and important biological properties of the lectins have made them the subject for a great deal of research. They have been used as probes for the study of cell surface structure in normal and transformed cells, of protein-saccharide interactions and of problems of immunological mechanisms, such as the stimulation of lymphocyte mitosis (mitogenesis). There is no evidence for the role if any that these molecules may play in nature, although some hypotheses have been

advanced (6).

Invertebrate Hemagglutinins

Hemagglutinins have been isolated from many invertebrate species including horseshoe crab (Limulus polyphemus), oyster (Crassostrea virginica), snail (Helix pomatia), lobster (Homarus americanus), earthworm (Lumbricus terrestris), crayfish (Procambarus clarkii), sea hare (Aplysia californica), starfish (Asterias forbesi), ascidian (Styela plicata), cockroach (Periplaneta americana), spiny lobster (Panulirus argus) and blue crab (Calinectes sapidus), especially within the past decade. Table 1 is a summary of characteristics and properties of some hemagglutinins isolated from invertebrates. The focus of most research on invertebrate agglutinins has been on the role they may play in the defense of the host organism, and on the evolution of the vertebrate immune response. Since invertebrates do not produce antibodies, these hemagglutinins have been studied as possible evolutionary precursors to antibodies. The evidence from all laboratories indicates that the invertebrate hemagglutinins are structurally and functionally distinct from vertebrate antibodies, although they may function in defense by aiding phagocytosis as opsonins (10). The invertebrate hemagglutinins resemble the plant lectins in that they are generally large proteins or glycoproteins, often consisting of non-covalently bound subunits. They exhibit agglutinating specificity toward different vertebrate erythro-

TABLE 1

INVERTEBRATE HEMAGGLUTININS

Species	M.W.	Subunit and Method	Erythrocyte and Other Cell Specificity	Sugar Inhibition	Chemical Nature	Purification or Isolation Method	Reference
Crassostrea virginica (oyster)	33.4S	20,000 daltons 5M guanidinium chloride	Sheep		Glycoprotein 13% carbohydrate mannose, galactose, glucosamine	G-200, Sepharose 4B	39, 40
			Trout Rabbit Human			$(NH_4)_2SO_4$	41
Helix pomatia (snail)	100,000		Human Blood Group A	Gal-NAc[1]	Glycoprotein 7% carbohydrate mannose, galactose	Affinity Chromatography to Group A	13
Aplysia californica (sea hare)	150,000		Chicken, pig, pseudomonas, bacteria		Protein	Serum	11
Lumbricus terrestris (earthworm)			Chicken horse, sheep, rabbit			Coelemic fluid unpurified	15
Homarus americanus (lobster)	11.3S	55,000 6M urea	Mouse, horse, hamster, human	Gal-NAc[1] Mann-NAc[2] Glucosamine Sialic acid	Glycoprotein	Pevikon electrophoresis Sepharose 6B DEAE-Seph	10
Panulirus argus (spiny lobster)	400,000 10.3S	68,500			Glycoprotein		75
Procambarus clarkii (crayfish)	150,000		Chicken pseudomonas micrococcus rabbit		Protein	Hemolymph	12
Limulus polyphemus (horseshoe crab)	400,000 13.5S	18 units 23,000 daltons 8M urea	Horse rabbit, chicken, sheep	Sialic acid	Protein	Ultracent, starch electrophoresis, G-200	14
	12.6S	25,000 daltons 8M urea	Sheep, rabbit, horse		Protein	Ultracent, agar electrophoresis	37
	335,000 13.6S	SDS 19,000		Sialic acid	Glycoprotein	G-50, DEAE Sephadex, Sepharose 6B	45
Callinectes sapidus (blue crab)	150,000		Rabbit human, chicken		Protein	Serum	79

TABLE 1
(continued)

Species	M.W.	Subunit and Method	Erythrocyte and Other Cell Specificity	Inhibition	Chemical Nature	Purification or Isolation Method	Reference
Periplaneta americana (cockroach)			Sheep		Glycoprotein (Lipid)	Ethanol solubility fractionation	44
Asterias forbesi (starfish)	6.5S	25,000 13,000 daltons 6M urea	Sheep, rabbit, hare		Protein	Concentration Agar electrophoresis Sucrose density gradient	37
Styela plicata (ascidian)	150,000		Rabbit, mouse		Polysaccharide		45

[1] Gal-NAc = N-acetyl-D-galactosamine

[2] Mann-NAc= N-acetyl-D-mannosamine

cytes: Hall and Rowlands (10) have recently demonstrated the presence of at least two distinct hemagglutinins with different erythrocyte specificities in the lobster. The hemagglutinins from sea hare and crayfish will agglutinate bacteria as well as erythrocytes (11, 12). The hemagglutinins from snail, lobster, and horseshoe crab are inhibited by specific saccharides (10, 13, 14). The hemagglutinins from horseshoe crab, oyster, starfish, and lobster are dependent on Ca^{++} ion for activity, while those from sea hare and ascidian are independent of Ca^{++} concentration. Sea hare, blue crab and crayfish hemagglutinins, although proteins, are insensitive to trypsin or pronase digestion. Most of the invertebrate hemagglutinins show a microheterogeneous pattern of protein bands in polyacrylamide gel electrophoresis. The invertebrate hemagglutinin subunits retain hemagglutinating activity. Hemolymph titers for most invertebrate hemagglutinins are quite high (in the range 256-1024 for crude hemolymph) depending on the erythrocytes used, with the exception of the hemagglutinins from earthworms and sipunculids which show titers in the 1-8 range (15, 42).

The isolation and study of hemagglutinating macromolecules from plants and invertebrates is of growing importance and significance in several areas of immunology and biochemistry, including the question of their in vivo and evolutionary roles. These molecules have also provided an excellent tool for the study of cell surface structures, particularly those

of mammalian erythrocytes and human blood groups (16). Springer et al. (17) used two agglutinins (from Vicia graminea and eel) to study the relationships and structures of erythrocyte surface components from human, ox, and sheep. Cell surface carbohydrates may be determined by the use of a large variety of saccharide-specific lectins (18, 19). The finding that transformed cells are often agglutinated better than normal cells implies an alteration in cell surface structure accompanying transformation (20). The relevance of this aspect of agglutinin research to cancer research is apparent, and has prompted a major surge of interest in the field by workers studying the biochemistry of cancer.

The hemagglutinins from plants and invertebrates may also be useful in the study of protein-carbohydrate interactions and binding, although more detailed knowledge of the chemical structures of the hemagglutinins is needed. Many of the hemagglutinins possess other biological activities, such as the induction of blast transformation in lymphocytes (mitogenesis), induction of insulin release from pancreatic islet cells (19), and toxicity (6).

Anti-Tumor Agents from Marine Invertebrates

Anti-tumor agents have been isolated from a variety of natural sources including microorganisms, plants, and animals. The anti-tumor antibiotics from microorganisms (daunomycin, mytomycin C, etc.) and many of the plant drugs are small mole-

cular weight molecules whose chemical structures are often known (21-24). Exceptions include an anti-tumor mucoprotein isolated from mushrooms (25). Among animals, marine invertebrates have been the best source of anti-tumor agents, although extracts of butterflies (26) and of some marine vertebrates (27) have anti-neoplastic activity. Schmeer (28) found a potent anti-tumor substance in common clams, Mercenaria mercenaria, active against Sarcoma 180 and Krebs 2 carcinoma in mice. This material, called "mercenene" has a molecular weight below 1000 (29) and is found predominantly in the clam liver (30). An extract of sea cucumber with anti-viral and anti-bacterial activities called "Holothurin" is active against Krebs 2 Ascites tumor in mice (31). Pettit and co-workers (27) have found anti-tumor activity in 23 marine species from 6 phyla. In a similar study by Sigel et al (32) 20 species from 7 marine phyla had activity against Leukemia P388 in mice. Extracts of 4 out of 6 randomly chosen species of marine Coelenterates showed activity against Ehrlich ascites tumor in mice (33). The activity from one of these species, Stoichactis kenti, has been purified and partially characterized (34). The material called "Stoichactin," is acid-stable, has a molecular weight around 5000, and is insensitive to pronase. It also has hemolytic activity. The same group has found anti-tumor activity in the marine polychaetous annelids Lanice conchilega, and Reteterebella queenslandia (35). The active material from the latter organism has a molecular weight

greater than 30,000, a cathodal mobility in disc gel electrophoresis at acid pH, and is possibly protein in nature (36).

It is noteworthy that marine invertebrates have served as sources for both anti-tumor and hemagglutinin molecules. As yet, however, there is little convergence of these two lines of research. Of the hemagglutinins, only those from certain plants have been reported to have anti-tumor activity, and many of these, like Concanavalin A are toxic (6). It is interesting that one of the species found to have anti-tumor activity by Sigel's group (32), horseshoe crab (Limulus polyphemus), is also one of the best known sources of invertebrate hemagglutinins (14, 37) although there is no mention in the literature of any correlation between the two activities. Another species studied in more detail by Sigel, the tunicate Ecteinascidia turbinata, has been reported not to have any "lectin-like" properties (38).

Purpose and Summary of Present Work

The annelid species investigated by Tabrah et al (35) are indigenous to Hawaii. Russell and Stock (unpublished data) found anti-tumor activity against Ehrlich ascites tumor in mice in extracts of two Northeast coast species of marine polychaetous annelids, Nereis, and Amphitrite ornata (see Results section for details). During the subsequent partial purification of the anti-tumor agent from Amphitrite ornata, active fractions were found to have hemagglutinin activity

toward erythrocytes of several species. Hemagglutinin activity was also found in Nereis extracts. The Amphitrite hemagglutinin has been purified to homogeneity with respect to disc gel electrophoresis and sedimentation velocity ultracentrifugation. Its biological properties (including anti-tumor activity) and physical and chemical characteristics have been studied and compared with those of other invertebrate hemagglutinins.

This dissertation describes the isolation, purification, and biological and chemical characterization of the hemagglutinin from the marine polychaetous annelid Amphitrite ornata, a new invertebrate hemagglutinin, and presents evidence for a correlation between the hemagglutinin and anti-tumor activities found in extracts of this organism.

EXPERIMENTAL SECTION

Materials

Chemicals and Reagents

All chemicals used were reagent grade obtained from standard sources (Fisher, Mallinckrodt, Schwarz-Mann). Formamide was purchased from Pfalz and Bauer (New York). Amphitrite ornata specimens were purchased from the Marine Biological Laboratory, Woods Hole, Massachusetts. Nereis was purchased from a local bait store. Chicken, rabbit, guinea pig, and sheep erythrocytes were purchased in a type 3 alseviers suspension from Pel-Freez (Rogers, Arkansas). Chicken cells were also purchased from Microbiological Associates (Bethesda, Maryland). Rat erythrocytes, obtained from live Wistar rats, were the gift of Chien Kuo Yeh of City College. Human blood groups A, AB, B, and O were the gift of Mary Walker of the New York Blood Center.

Chemicals used in the preparation of polyacrylamide gels were purchased from Canalco, Rockville, Maryland, or from Eastman, Rochester, New York. "Stains-all" or 1-ethyl-2-(3-(1-ethylnaphtho (1,2d)-thiazolin-2-ylidene)-2 methylpropenyl) naphtho (1,2d) thiazolium bromide was purchased from Eastman, Rochester, New York. Schiff reagent and anthrone were purchased from Fisher, Fair Lawn, New Jersey. Saccharides were obtained from Sigma, St. Louis, Missouri. Sephadex G-100 was purchased from Pharmacia, Piscataway, New Jersey. Aldolase,

ovalbumin, chymotrypsinogen, ribonuclease A, and Blue Dextran were purchased as a calibration kit from Pharmacia. Trypsin, insolubilized on polyacrylamide was obtained from Sigma, and pronase was obtained in the form of an insolubilized "Enzite" column from Miles, Elkart, Indiana. Deoxyribonuclease was purchased from Sigma.

Instrumentation and Apparatus

Annelids were homogenized in a Waring blender. Centrifugation at 5° C. was performed in a Beckman J-21 refrigerated centrifuge using either the JA-20 or JA-14 rotors. A Buchi rotary flash evaporator was used for flash evaporation. Freeze-drying of samples was done on a Virtis lyophilizer. Spectra were recorded on a Perkin-Elmer 402 U.V.-VIS. spectrophotometer. Absorbance measurements for protein, carbohydrate, and nucleic acid determinations were taken on a Zeiss Spectrophotometer M4QIII. This instrument, fitted with a Vicon linear gel-scanner was used for gel-scanning absorbance measurements. An LKB Ultrorac 7000 fraction collector with an LKB Uvicord II detector set at 280 nm. was used for automatic fraction collection and U.V. absorbance measurements for gel filtration respectively. Ultrafiltration was performed in one of three Amicon ultrafiltration cell models: 12 (10 ml.), 52 (50 ml.), or 402 (400 ml.). Amicon Diaflo ultrafiltration membranes PM-10, UM-10 or UM-2 were used. Disc gel, and SDS polyacrylamide gel electrophoresis were

performed using a Shandon gel electrophoresis apparatus with a Vokam power supply. Samples in the 0.5-5.0 mg. range were weighed on a Cahn G-2 electrobalance. Dialya-por dialysis tubing, 14.6 mm. diameter, was used for dialysis. Hemagglutination assays were done with Cooke Engineering Microtiter U plates, or Corning 25 µl. disposable micropipettes. Amino acid analyses were performed on a Beckman model 118 amino acid analyser. The pH of buffers and solutions was measured and adjusted with a Corning model 12 pH meter. Gas-liquid chromatography was performed on a Perkin-Elmer 881 Gas Chromatograph. Analytical ultracentrifugation was done on a Beckman Model E Analytical Ultracentrifuge. Analysis of Ultracentrifuge photographs was done with a Gaertner Microcomparator.

Methods

Preparation of Extracts

The extraction procedure was modified from that of Tabrah et al. (35). Amphitrite or Nereis worms weighing an average of 8 grams each, were homogenized in a Waring blender at 5°C for 10 minutes with 55 ml. of cold 30% ethanol (aq.) per gram of worm. The homogenate was then stirred at 5°C for 18 hours with an additional 83 ml. 30% ethanol per gram of worm. Most batches contained approximately 60 grams in approximately 8 liters. After stirring, the extract was centrifuged at 10,000 RPM in the JA-14 rotor at 5°C for 20 minutes to remove insoluble debris. The supernatant was concentrated by flash evaporation at 40°C. The extract was brought to a final volume of 350 ml. and centrifuged again. The residue was discarded. The supernatant was ultrafiltered using a UM-10 membrane (76 mm. diameter) which had been soaked in two changes of water for 1 hour. The extract was ultrafiltered under 35 psi nitrogen with stirring in an ice bath. Water was added to the ultrafiltration cell as ultrafiltration proceeded. Approximately 400-500 ml. of ultrafiltrate were collected and discarded. The retentate (150-200 ml.) was flash evaporated to near dryness at 40°C in a 1 liter round-bottomed flask. This was then frozen and lyophilized, at 1-3 torr. The dry retentate was transferred to a tared vial, and stored frozen over anhydrous $CaSO_4$. A summary of the extrac-

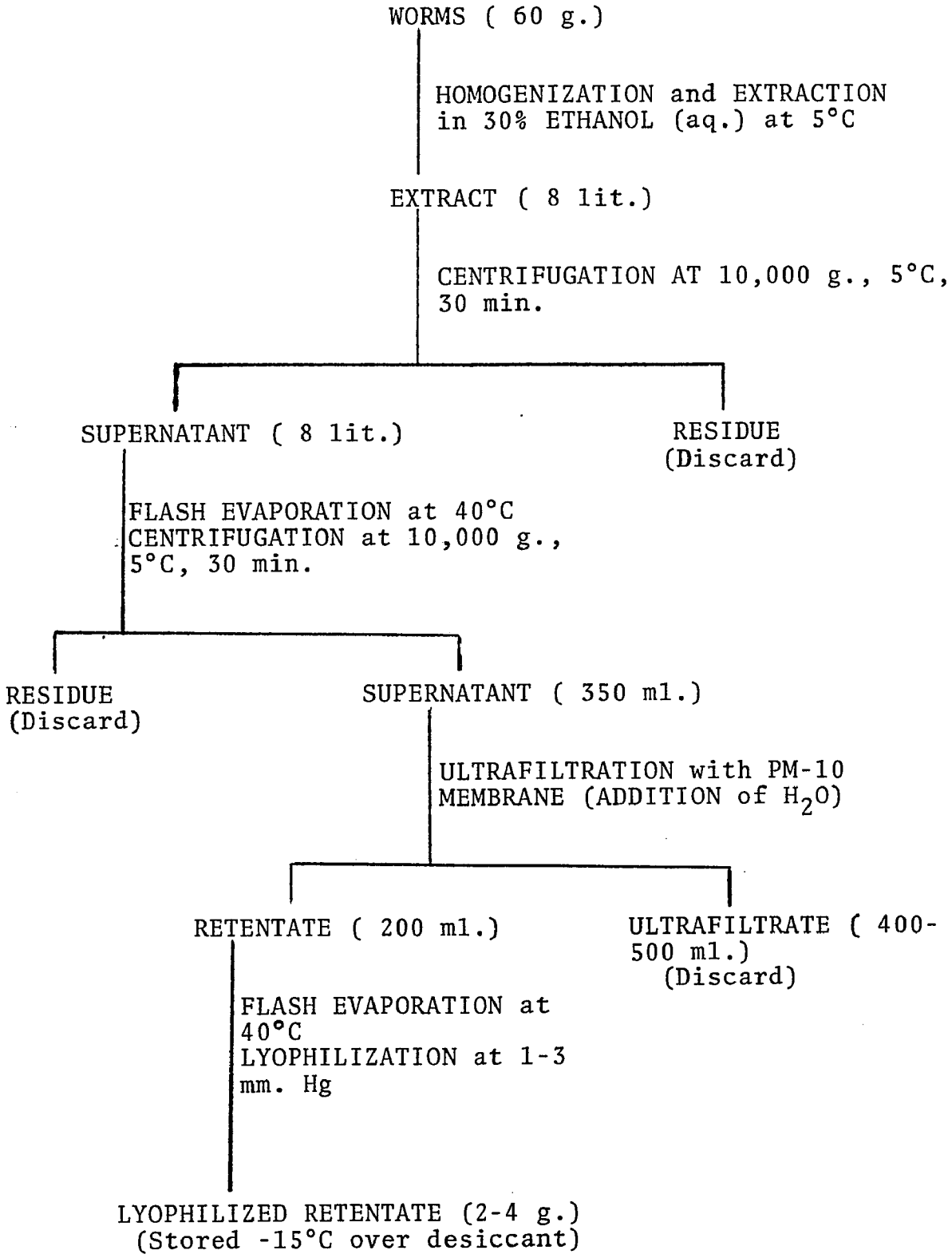
tion procedure is given in Figure 1.

Hemagglutination Activity Assays

Blood obtained from commercial sources or from live animals was centrifuged in an International table-top centrifuge, washed with 3 volumes of saline, and filtered through cheesecloth to remove clots. The cells were then washed three times with saline. After the final wash, the RBC were spun down in a graduated centrifuge tube. The volume of packed cells was determined, and saline or PBS was added to make a 5% RBC suspension for use in microtiter well assays and sedimentation velocity assays. The packed cells were used directly in capillary tube assays.

Capillary Tube Assay. A 25 µl. capillary micropipette was filled by capillary action with 20 µl. of a test solution. For titrations, serial dilutions of the sample were previously prepared with PBS. Approximately 5 µl. of packed RBC was drawn into the capillary, which was then inverted, fixed at a slight slant from the vertical in a piece of plasticine. The RBC at the top of the capillary settled down through the sample solution to the bottom of the tube. In positive hemagglutination tests, dots of clumped cells were readily observable against a white background in the tube after about 30-40 minutes of settling. In negative or control tubes (with 20 µl. PBS) this phenomenon was absent. If hemolysis had occurred, the capillary showed a diffuse red

FIGURE 1


WORMS (60 g.)
HOMOGENIZATION and EXTRACTION in 30% ETHANOL (aq.) at 5°C
EXTRACT (8 lit.)
CENTRIFUGATION AT 10,000 g., 5°C, 30 min.
SUPERNATANT (8 lit.)
RESIDUE (Discard)
FLASH EVAPORATION at 40°C
CENTRIFUGATION at 10,000 g., 5°C, 30 min.
RESIDUE (Discard)
SUPERNATANT (350 ml.)
ULTRAFILTRATION with PM-10 MEMBRANE (ADDITION of H_2O)
RETENTATE (200 ml.)
ULTRAFILTRATE (400-500 ml.) (Discard)
FLASH EVAPORATION at 40°C
LYOPHILIZATION at 1-3 mm. Hg
LYOPHILIZED RETENTATE (2-4 g.) (Stored -15°C over desiccant)

color from liberated hemoglobin.

Microtiter Well Assay. Microtiter well assays were performed on microtiter plates containing one-hundred twenty 200 µl. U-shaped wells per plate. Into each well 100 µl. of test solution and 25 µl. of 5% RBC suspension were placed using an automatic 25 µl. Oxford micropipetter and a 50 µl. Hamilton syringe. The 100 µl. of test solution contained various dilutions of sample in PBS. For titrations, the sample was diluted in 2-fold serial dilutions. A typical titration, for example, would consist of 100 µl. sample + 25 µl. RBC in the first well, 50 µl. sample + 50 µl. PBS + 25 µl. RBC in the second well, 25 µl. sample + 75 µl. PBS + 25 µl. RBC in the next well, etc. All titrations were run in duplicate. Control wells containing 100 µl. PBS + 25 µl. RBC were tested on the same plate. The plates were covered with sealers, shaken for 3 minutes on a Tektator Plate Shaker and incubated for 1 hour at room temperature. The plates were then read by tilting against a white background. In wells with controls or negative samples, the RBC at the bottom of the well flowed freely upon tilting. In positive hemagglutination tests, the RBC remained clumped at the center of the well. Hemolysis was detected by the diffusion of hemoglobin throughout the well. The plates were stored at 5°C overnight and read again in the following day.

Sedimentation Velocity Assay. The sedimentation velocity method of Kabat and Mayer (46) was used in the initial finding

of hemagglutinin activity in Amphitrite extracts. In this method, 5 ml. of a 5% RBC suspension was mixed with 2 ml. of test solution in a centrifuge tube. After intervals of 30 and 60 minutes, the volume of sedimented cells was compared with that in controls containing 2 ml. PBS. Larger volumes (faster sedimentation) in test samples indicated positive hemagglutination activity.

Hemagglutination activity was calculated for all three assay methods by titration. Positive samples were diluted with PBS until agglutination was no longer evident. The reciprocal of the highest dilution (e.g., 1:8) of sample which still gave positive agglutination is the titer (e.g., 8). Units of agglutinin activity were expressed as titers. Units of activity, divided by the total weight of protein in mg. was equal to the hemagglutinin specific activity.

Because of their greater sensitivity and economy of sample use compared to the sedimentation velocity assay, the capillary and microtiter well assays were used in all purification and characterization studies.

Polyacrylamide Gel Electrophoresis

The method of Davis (47) was followed for the preparation of 12% acrylamide gels for analytical disc gel electrophoresis. All solutions were stored at 4°C with the exception of ammonium persulfate which was freshly prepared for each run. Separating gels were prepared by mixing 1 ml. separating gel buffer (3M TRIS, 0.5M HCl, 0.46% TEMED, pH 8.9), 2 ml. acrylamide

solution (4.25M acrylamide, 0.05M BIS, 0.455mM $K_3Fe(CN)_6$) 4 ml. ammonium persulfate (1.0mM) and 1 ml. H_2O in a 10 ml. graduate cylinder. The solution was immediately transferred to 4-8 glass tubes (4.5 mm.id x 60 mm.), and allowed to polymerize at room temperature for at least 30 min. Stacking gels were prepared by mixing 1 ml. stacking gel buffer (0.5M TRIS, 0.5M HCl pH 6.7), 2 ml. acrylamide solution (1.42M acrylamide, 0.15M BIS), 1 ml. riboflavin (0.106mM) and 4 ml. H_2O. This mixture was layered on top of the separating gel, and photopolymerized by a fluorescent light for at least 20 min.

Samples of 0.1 - 0.2 ml. containing a drop of glycerine were applied to the top of the stacking gel. Electrophoresis was carried out in TRIS-glycine buffer (0.05M TRIS, 0.38M Glycine pH 8.5) for 30 min. at 5 ma. (constant current) per gel. Gels were removed from tubes and stained by one of three methods. Gels were stained for protein in 0.1% Coomassie Blue in 7% acetic acid for 2 hours. The stain was then poured off, and gels were destained in 7% acetic acid for 2-3 days. For nucleic acid staining the method of Dahlberg et al (48) was followed. A stock solution of 1% "Stains-all" in formamide was diluted 1:20 with formamide before use. Gels were stained in this solution in the dark overnight, then were washed with water and destained for 1-2 hours in water, in the dark. DNA appeared blue and RNA appeared purple with this stain. This stain also gave a purple color for sialic acid (see Results, p.). Gels were stained for carbohydrate by

the periodic acid-Schiff method of Kratoski and Weimer (49). Gels were soaked sequentially at room temperature with stirring in 80 ml. each of 0.5% periodic acid for 2 hours, 0.5% sodium arsenite in 5% acetic acid for 40 min., 0.1% sodium arsenite in 5% acetic acid for 20 min. twice, 7% acetic acid for 30 min., then transferred to 10 ml. Schiff reagent for 30 min. Carbohydrate appeared as pink bands.

Gel scans were recorded by scanning the length of the gel at 650 nm. for "Stains-all" and Coomassie Blue-stained gels and at 550 nm. for periodic acid-Schiff stained gels. Analytical disc gel electrophoresis was performed at pH values of 6.5, 7.0, 8.0, and 8.5 by adjusting the pH of the separating gel buffer with HCl to the desired value.

Preparative disc gel electrophoresis was performed as above in larger tubes (8 mm. id x 85 mm.) with samples of 0.5-1.0 ml. After electrophoresis gels were sliced at 2 mm. intervals, the slices were crushed, and soaked in 1 ml. saline at 4° overnight. The eluate was then filtered on Whatman No. 1 paper (pre-wetted with saline) and dialysed against saline. One gel from each preparative run was stained with Coomassie Blue to correlate protein bands with the elution activity profile.

Sodium dodecyl sulfate (SDS) gels were prepared according to the method of Weber and Osborn (50). A mixture of 15 ml. gel buffer, (0.01M sodium phosphate, 0.1% SDS, pH 7.0), 13.5 ml. 10% acrylamide solution, 0.045 ml. TEMED, and 1.5 ml. ammonium

persulfate (15 mg./ml. freshly prepared), was poured into glass tubes (5 x 100 mm.). Samples for SDS gel electrophoresis were dialysed against distilled water, and lyophilized. The dry material was dissolved in 0.01M phosphate buffer, 1% SDS, pH 7.0 to a concentration of 1 mg./ml., and incubated for 2 hours at 37°C. When mercaptoethanol was used as a sulfhydryl reducing agent, the sample buffer contained 1% mercaptoethanol. Standard proteins (1 mg./ml. of each) used were: ribonuclease A, ovalbumin, chymotrypsinogen, aldolase, and bovine serum albumin. A mixture of protein standards was run on one gel as a sample consisting of 5 drops of each standard, 10 drops gel buffer, 1 drop glycerine, 1 drop bromphenol blue. Electrophoresis was carried out with gel buffer diluted 1:1 with water, at 8 ma./gel for approximately 3 hours, or until the tracking dye had reached the bottom of the gel. Gels were stained in Coomassie Blue for 2 hours, then destained electrophoretically in an Ames destainer in 7.5% acetic acid, 5% methanol (aq.) for 20-30 minutes. A plot of mobility or R_f (distance from origin to band/distance from origin to bromphenol blue) of the standard proteins vs. log molecular weight was used to determine the molecular weight of unknown samples.

Protein Determination

Quantitative estimates of protein concentrations were determined by the method of Lowry et al (51). Standard solu-

tions of bovine serum albumin were prepared in concentrations of 100, 80, 60, 40, 20, and 0 μg./ml. in saline. A mixture of 0.5 ml. 1% $CuSO_4 \cdot 5H_2O$ (aq.), 0.5 ml. 2% sodium potassium tartrate, and 50 ml. 2% Na_2CO_3 in 1N NaOH was prepared. Three ml. of this reagent was added to 0.6 ml. standard protein solution or unknown sample. After 10 minutes at room temperature, 0.3 ml. of phenol reagent (Fisher) diluted 1:1 with water was added. After an additional 20 minutes, the absorbance at 650 nm. of each tube was measured. The protein concentration of the unknown sample was determined by comparison with the standard curve of absorbance vs. concentration.

Amino Acid Analysis

A weighed sample of purified hemagglutinin (0.75 mg.) was dissolved in 2 ml. 6N HCl. The sample was transferred to a 10 ml. ampoule, which was frozen in dry ice-acetone, and sealed under vacuum (1-3 TORR) with an oxygen flame. The ampoule was incubated at 110°C for 24 or 48 hours. The ampoule was then broken open, covered with filter paper, and stored in a vacuum desiccator over anhydrous $CaSO_4$ and NaOH. After 1 day the dried residue was dissolved in 1 ml. water and again dried in the desiccator. The hydrolysed sample was treated twice in this manner to remove HCl. Finally, the dried sample was dissolved in 2 ml. sodium citrate buffer pH 2.2. Samples of 250 μl. were applied to long and short columns of the Beckman Model 118 amino acid analyser. The short column

(9 mm. i.d. x 80 mm.) containing PA - 35 resin was eluted with a buffer of 0.35M sodium citrate pH 5.26. The long column (9 mm. i.d. x 560 mm.), containing AA-15 resin, was eluted with 0.2M sodium citrate buffer pH 3.25 for 103 minutes, then with 0.2M sodium citrate buffer pH 4.25 for 105 minutes. An automatic timing program controlled the buffer change. Flow rates for both columns were 60 ml./hr. An expanded scale (0-0.2 O.D.) was used for both standard and sample runs. A standard mixture (250 µl.) of amino acids (0.1 µmole/ml. of each of 20 amino acids) was run before the sample run. Areas under peaks were calculated by measuring and multiplying the height and width at half height (H x W). The umoles of each amino acid were calculated by the formula:

EQ. 1

$$\mu\text{mole amino acid in sample} = \frac{\text{Peak area (H x W) sample}}{\text{Peak area (H x W) standard}} \times 0.1 \frac{\mu\text{mole}}{\text{ml.}} \times 2 \text{ ml.}$$

Values for serine, threonine and tyrosine were obtained by extrapolation to zero time hydrolysis.

Tryptophan Determination. Tryptophan was determined by the spectrophotometric method of Spande and Witkop (52). A sample of purified hemagglutinin (0.28 mg.) was dissolved in 0.63 ml. 8M urea adjusted to pH 4 with acetic acid. The absorbance of this solution at 280 nm. was determined in the Perkin-Elmer 402 spectrophotometer. To the cuvette 10 µl. of 10 mM N-Bromosuccinimide (aq.) was added with stirring for 5 minutes. The absorbance at 280 nm. was again read. This procedure was repeated until no further decrease in the ab-

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sorbance at 280 nm. was noted. The number of Tryptophan residues was calculated by the formula:

EQ. 2

$$N = \frac{\text{O.D. x 1.31 x 32,000 x Vol. sample (ml.)}}{\text{Wt. sample (mg.) x 5500}}$$

where N = number of tryptophan residues, 1.31 is a correction factor, 32,000 is the molecular weight of the purified hemagglutinin, and 5500 is the molar extinction coefficient.

Carbohydrate Determinations

Quantitative estimates of carbohydrate concentration were determined by the anthrone method of Scott (53). Anthrone reagent was prepared by dissolving 2 g. anthrone (Fisher) in 1 liter concentrated H_2SO_4. Standard solutions of 100, 80, 60, 40, 20, and 0 µg. galactose per ml. water were prepared. Unknown samples were dissolved in water to a concentration of 100 µg./ml. Two ml. anthrone reagent was added to 1 ml. of unknown or standard sugar solution in test tubes. The tubes were heated in a boiling water bath for 3 minutes, cooled, and absorbances were measured at 624 nm. The standard curve of absorbance vs. concentration was used to determine the concentration and percent composition of carbohydrate in the unknown sample.

Sialic Acid Determination. A sample of purified hemagglutinin (0.804 mg.) was dissolved in 0.40 ml. water. To this was added 0.4 ml. 0.1M sodium acetate buffer, pH 5, and 0.2 ml. of a 0.02 mg./ml. aqueous solution of neuraminidase (Worthington).

After incubation at 37°C for 1 hour, 0.2 ml. of this solution was assayed for sialic acid by the method of Warren (54). To the neuraminidase-digested material 0.1 ml. of 0.2M sodium periodate in 9M H_3PO_4 was added. After 20 minutes at room temperature, 1 ml. 10% sodium arsenite in 0.5M sodium sulfate, 0.1M H_2SO_4 was added, and the solution was shaken until all brown color had disappeared. Three ml. of 0.6% thiobarbituric acid in 0.5M sodium sulfate was added and the solution was heated in a boiling water bath for 15 min. then transferred to cold water. An equal volume of cyclohexanone was added, the tube was shaken thoroughly and the contents were centrifuged in an International table-top centrifuge. The upper organic layer was transferred to a cuvette, and the absorbance at 549 nm. was determined. The concentration of sialic acid was determined from the absorbance and the molar extinction coefficient of 57,000. The assay was checked on a known concentration of N-acetylneuraminic acid (Sigma).

Gas-Liquid Chromatography of Neutral Sugars. Neutral sugars were determined on a Perkin-Elmer 881 Gas Chromatograph (with a flame ionization detector) by the trimethylsilyl derivative method of Reinhold (55). Purified hemagglutinin (1.02 mg.) and standard sugars (2 mg. each, of glucose, mannose, galactose, and fucose) were heated separately with 1 ml. 0.5M HCl in anhydrous methanol for 16 hours at 65°C. The samples were dried with N_2, then re-suspended in 2 ml. of a commercial silylation reagent "Tri-Sil" (Pierce Chemical Co., Rockford,

Ill.). After 1/2 hour at room temperature, the silylation reagent was evaporated under a stream of nitrogen, and the residue extracted with 1 ml. hexane. The hexane extract was used in injections. Standard solutions were quantitatively diluted with hexane to give peaks at the same attenuation as the unknown peaks.

Chromatography was carried out in a stainless steel column (6' x 1/4") packed with 0.05% OV-17 on textured glass beads (120/140 mesh) purchased from Analabs, North Haven, Ct. Conditions were as follows: Carrier gas (He) flow rate = 60 ml./min., detector temperature = 250°C, injector temperature = 220°C, column temperature was kept at 100°C at the start of each run for 15 minutes, then increased automatically 16°/min. to 250°C. Samples of 1.5-2.5 μl. were injected with a Hamilton syringe. Standard sugars were injected separately, and as a mixture. Purified hemagglutinin hydrolysate was injected alone, and with the sugar standards to confirm the identity of the unknown peaks by enhancement of one peak, or the appearance of a new peak. Quantitative results were obtained by cutting out and weighing the peaks from photostatted copies of the recorder traces, using the known galactose peak as a quantitative standard.

Determination of Amino Sugars. Amino sugars were determined on the Beckman Model 118 amino acid analyser. A sample of purified hemagglutinin (0.633 mg.) was hydrolysed in 2 ml. 6N HCl under vacuum at 105°C for 6 hours. The hydrolysed sample

was dried in a vacuum desiccator, over $CaSO_4$ and NaOH, then re-dissolved in 0.6 ml. citrate buffer pH 2.2 and a sample of 250 µl. was applied to the long column of the amino acid analyser. Column and instrument conditions were the same as for amino acid analysis, except that the column was eluted with 0.2M citrate buffer pH 3.25 for 15 minutes, then with 0.2M citrate buffer pH 4.25 for 200 minutes. A standard solution containing 0.125 µmole/ml. each of glucosamine, galactosamine, and mannosamine was used for the identification and quantitative estimation of hexosamines in the purified hemagglutinin.

Anti-Tumor Activity Assay

Anti-tumor activity assays were performed by Dr. C. Chester Stock and Dr. G. Tarnowski at the Walker Laboratory of the Sloan-Kettering Institute at Rye, New York. Male Swiss Webster albino mice weighing 20-25 g. were used. Ehrlich ascites cells (1×10^7) were injected intraperitoneally. Treatment consisting of 6 intraperitoneal injections of test material (1 - 200 mg./kg. body weight) administered once a day, began one day after tumor inoculation. After 8 days the mice were killed. A value of "total packed cell volume" (TPCV) was determined from the ascites fluid found in the dissected intraperitoneal cavity. Values for TPCV of test and control mice were reported as a ratio: T/C. Values for T/C less than 0.40 indicated statistically significant

activity of the test material for this tumor. The reported values for T/C were converted to percent activity by setting T/C of 0.4 equal to 0% and T/C of 0 equal to 100%. The Relative Activity was obtained by dividing the percent activity by the dosage (mg./kg. body weight/day) of test sample used in the assay. The formula for the Relative Activity is:

EQ. 3

$$\text{Relative Activity} = \frac{(0.40 - T/C)\ (2.5)\ (100)}{\text{Dosage}}$$

Values for Relative Activity were used for approximate qualitative comparisons of anti-tumor activity of different preparations.

EXPERIMENTS

An outline of the experimental purification procedures is presented in Figure 2. Amphitrite hemagglutinin from the ultrafiltration retentate was further purified by two alternate methods. In one approach the retentate was chromatographed directly on Sephadex G-100, giving the three active fractions A1, A2 and B. In the other approach, ammonium sulfate precipitation of the retentate was employed prior to gel filtration. This procedure yielded one active fraction, B, which was equivalent to fraction B from gel filtration of whole retentate. Details of the relationships between these fractions, and of variations in this general experimental procedure are discussed in the Results Section.

Gel Filtration of Amphitrite Retentate

A sample of Amphitrite retentate from ultrafiltration was subjected to gel filtration on Sephadex G-100. The sample contained 600 mg. lyophilized retentate suspended in 15 ml. PBS (40 mg./ml.). Before application to the column, the sample solution was clarified by centrifugation at 20,000 RPM in the Beckman JA-20 rotor at 5°C for 20 minutes. The insoluble residue was lyophilized and stored at -15°C over desiccant, prior to testing for anti-tumor activity (see Table

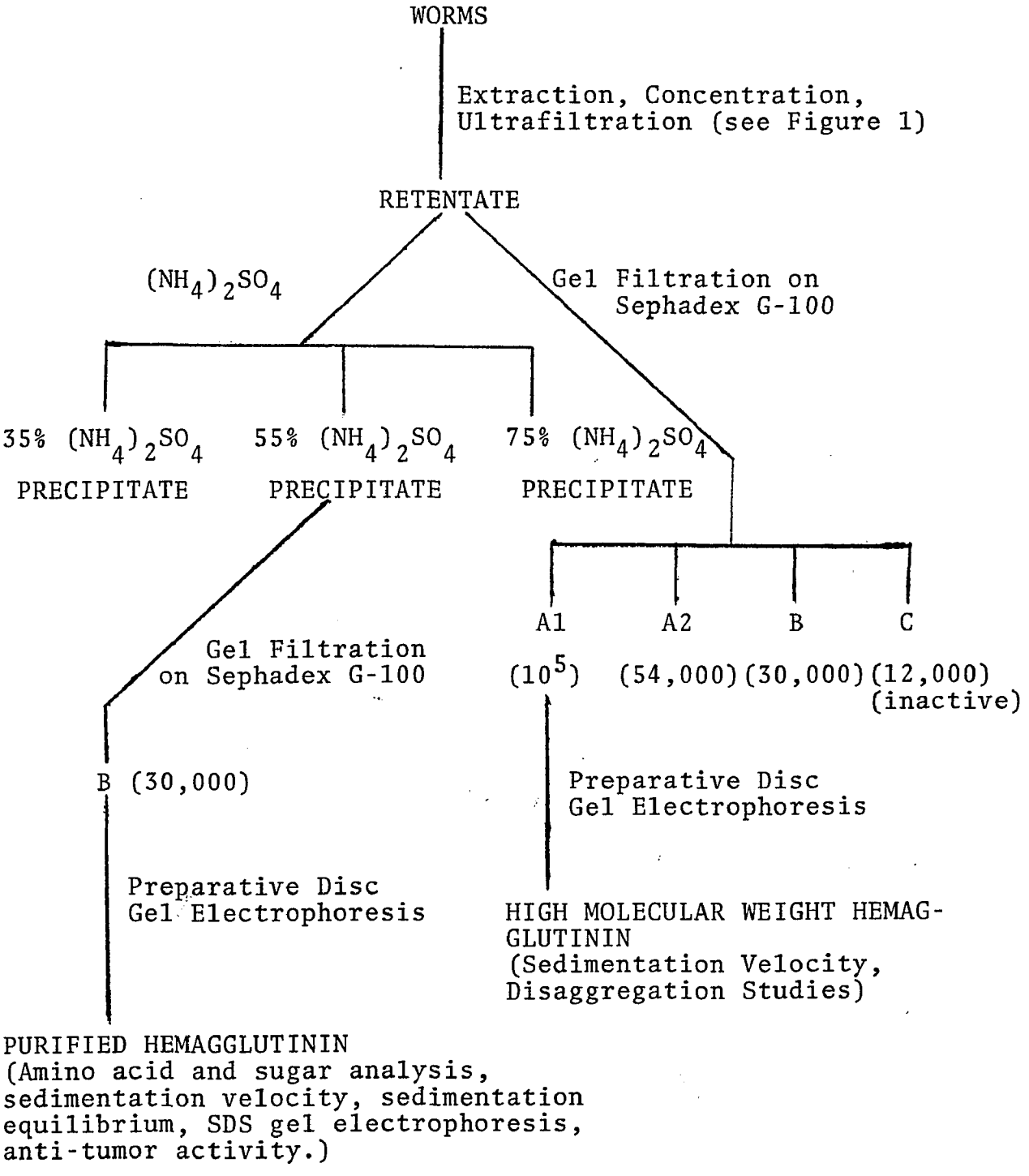
44

FIGURE 2

Experimental Purification Procedures

Fraction B obtained from gel filtration of the 55% ammonium sulfate precipitate was equivalent to fraction B from gel filtration of retentate, as determined by co-chromatography on Sephadex G-100, and by their identical patterns on disc gel electrophoresis. In some experiments fractions A1 and A2 were combined and labelled fraction A. Values for molecular weights of fractions from gel filtration were determined from their elution volumes on calibrated columns of Sephadex G-100. Amino acid analysis, carbohydrate composition, and sedimentation equilibrium, were performed on the hemagglutinin obtained from 55% ammonium sulfate precipitate, and purified by preparative disc gel electrophoresis.

FIGURE 2


WORMS
Extraction, Concentration, Ultrafiltration (see Figure 1)
RETENTATE
$(NH_4)_2SO_4$
Gel Filtration on Sephadex G-100
35% $(NH_4)_2SO_4$ PRECIPITATE
55% $(NH_4)_2SO_4$ PRECIPITATE
75% $(NH_4)_2SO_4$ PRECIPITATE
A1
A2
B
C
(10^5)
(54,000)
(30,000)
(12,000) (inactive)
Gel Filtration on Sephadex G-100
B (30,000)
Preparative Disc Gel Electrophoresis
Preparative Disc Gel Electrophoresis
HIGH MOLECULAR WEIGHT HEMAGGLUTININ (Sedimentation Velocity, Disaggregation Studies)
PURIFIED HEMAGGLUTININ (Amino acid and sugar analysis, sedimentation velocity, sedimentation equilibrium, SDS gel electrophoresis, anti-tumor activity.)

8, p. 109).

Sephadex G-100 gel (24 g.) was swollen by gentle stirring in 2 liters cold PBS and allowed to equilibrate at 5°C for 3-4 days. A Pharmacia K-26 glass column, 100 x 2.6 cm., was packed with the swollen gel by gravity flow. A flow adaptor was used for sample application. The packed column, 77 x 2.6 cm., was operated in a descending direction, using gravity flow from a self-regulating reservoir, with a pressure head of 20 cm. The elution buffer was PBS, the flow rate was 24 ml./hr., and fractions of 6 ml. were collected.

The column was calibrated by measuring the elution volumes of a series of commercially available pure proteins with known molecular weights (Pharmacia, Piscataway, N. J.). These proteins were dissolved in PBS (20 mg./ml.) and applied to the column in separate runs. Sample size was generally 5-7 ml. Proteins used for this purpose were: aldolase - 158,000; bovine serum albumin - 64,000; ovalbumin - 45,000; chymotrypsinogen - 25,000; and ribonuclease A - 13,700 daltons. The void volume was determined by the elution volume of Blue Dextran 2000. For each protein standard, a value of K_{av} was calculated from the formula:

EQ. 4

$$K_{av} = V_e - V_o/V_t - V_o$$

where V_e = elution volume of the standard, V_o = void volume, and V_t = total bed volume of the column. A plot of K_{av} vs. log molecular weight gave a straight line, from which the

approximate molecular weight of the sample could be deduced (56).

Fractions from gel filtration were assayed for hemagglutinin activity with rat RBC by the capillary method. For assay with chicken cells, fractions were pooled (see Fig. 3) and concentrated by ultrafiltration through a PM-10 membrane.

Physical and Chemical Properties of Amphitrite Hemagglutinin

Several physical and chemical properties of the Amphitrite hemagglutinin were studied using pooled active fractions A1, A2, and B from gel filtration (see Results).

Heat Treatment. Capillary tubes containing 20 ul. of an active hemagglutinin fraction (initial titer 2-4 vs. chicken RBC) were sealed with plasticine and placed in one of five water baths maintained at 60°C, 70°C, 80°C, 85°C, or 90°C for 30 minutes. The tubes were then removed from the heat, cooled, opened, and assayed with chicken RBC in the usual manner.

Dialysis. In order to determine the effect of cations on the hemagglutinin activity, samples of active hemagglutinin fractions were dialysed against 4 liters of each of the following: PBS, distilled water, and PBS made 0.05M $CaCl_2$, + 0.05M $MgCl_2$. The dialysed hemagglutinin was assayed by the capillary method with chicken RBC.

EDTA Treatment. Samples of lyophilized hemagglutinin fractions were dissolved in 2 ml. 0.15M EDTA (10 mg./ml.), and dialysed

against 4 liters 0.15M EDTA. After dialysis, the hemagglutinin was assayed by the capillary assay. Controls with 0.15M EDTA showed that this solution had no effect on RBC.

Enzymatic Digestion. A 3 ml. sample of hemagglutinin in PBS pH 8 (5 mg./ml.) was mixed with 150 mg. insolubilized trypsin (Sigma) at 37°C for 18 hours. After the incubation period, the enzyme was removed by centrifugation in an International table-top centrifuge, the supernatant was filtered, and dialysed against 4 liters PBS.

A 5 ml. sample of hemagglutinin solution in PBS (5 mg./ml.) was added to a pronase "Enzite" (Miles) column and incubated for 30 min. at 37°C. Material was eluted from the column with PBS until the eluate had no absorbance at 280 nm. The eluate was dialysed against 4 liters PBS.

Three ml. of fraction A1 (5 mg./ml.) was mixed with 100 mg. of deoxyribonuclease (Sigma) for 18 hours at 24°C. After incubation the digested material was dialysed against 4 liters PBS.

Phenol Extraction. The phenol extraction procedure was adapted from Lampson et al. (57). A dilute sample (0.5 mg./ml.) of active hemagglutinin was mixed with an equal volume (35 ml.) of phenol, and stirred in a water bath at 37°C for 40 minutes. The mixture was resolved into two phases by centrifugation in an International table-top centrifuge. The upper, aqueous phase was removed with a Pasteur pipette, and re-extracted with an equal volume of phenol at 25°C for 20 minutes. The

extraction procedure was repeated once more at 25°C for 20 minutes. The aqueous layer after the third extraction was pipetted off, filtered, and dialysed against two changes of 6 liters PBS for 48 hours. The dialysate was transferred to a 50 ml. ultrafiltration cell equipped with a PM - 10 membrane. Ultrafiltration with periodic addition of PBS to the cell (total volume PBS added was approximately 200 ml.) was continued until the ultrafiltrate had no absorbance at 280 nm., indicating the complete removal of phenol.

Streptomycin Sulfate Precipitation. Samples of hemagglutinin fractions A1 and A2 (5 ml., 15 mg./ml.) were mixed with 1 ml. 0.2M streptomycin sulfate (aq.) at 25°C with stirring. The precipitate was collected after 1 hour by centrifugation in an International table-top centrifuge. The supernatant was dialysed against 4 liters PBS, and assayed for hemagglutinin activity against chicken RBC by the capillary assay.

Erythrocyte Specificity of Amphitrite Hemagglutinin

The agglutinating specificity of fractions A and B from gel filtration was determined by the microtiter well assay. Rat, chicken, rabbit, guinea pig, sheep and human blood group A, B, and O (H) erythrocytes were titered with hemagglutinin fractions. Titrations were performed as described in Methods. Titrations were run in duplicate, with controls of 25 µl. of each 5% RBC suspension + 100 µl. PBS.

Agglutination Inhibition by Saccharides

In order to determine the binding specificity of Amphitrite hemagglutinin, the inhibition of hemagglutinin activity by saccharides was studied as follows: Fraction B was titered by the microtiter well assay with rat RBC as described (p.31), except that 25 µl. of a 0.2M saccharide solution in PBS was added to each well, and allowed to incubate with the hemagglutinin 1 hour before the addition of RBC. The sugars tested are shown in Table 3. Two kinds of controls were run on the same plates as the test titrations: Instead of inhibitor, 25 µl. PBS was added to a titration of the hemagglutinin. Also, 100 µl. PBS + 25 µl. of each sugar solution without hemagglutinin was assayed for non-specific affect on the RBC by the sugars. D-galactose, D-glucosamine, and N-acetyl-D-galactosamine without hemagglutinin caused agglutination of RBC, probably due to high molecular weight impurities in the commercial preparations. This result was also reported by Marquardt and Gordon (58). These solutions were ultrafiltered through a UM-2 membrane (with a molecular weight cut-off of 2000) and the ultrafiltrates, which had no affect on RBC were used in the inhibition tests.

All saccharide solutions were used at pH 7; the solutions of N-acetyl neuraminic acid, D-glucosamine-HCl, D-mannosamine-HCl, and D-galactosamine-HCl were adjusted to pH 7 with dilute NaOH before use. Inhibition was indicated by a reduction in the titer (compared to controls) after addition of 25 ul. of a

saccharide solution.

pH Activity Profile

The effect of pH on Amphitrite hemagglutinin activity was determined with rat RBC by the microtiter well assay. Phosphate buffered saline was adjusted to pH values from 2-12 with HCl or NaOH. To 100 µl. of various dilutions of fraction B, 25 µl. of PBS of a particular pH was added to each well. Controls without hemagglutinin showed no effect on RBC by solutions of pH 4-9. However, cell destruction was observed below pH 4, and above pH 10. Some non-specific agglutination of cells was seen at pH 9.5 and 10. Only points between pH 4-9 were used (see Fig. 5).

Ammonium Sulfate Precipitation of Amphitrite Retentate

Ammonium sulfate precipitates of Amphitrite hemagglutinin were prepared according to the method of Brewer et al. (59). To a solution of retentate in PBS (200 ml., 10 mg./ml.) 42 g. solid ammonium sulfate was added slowly with stirring at 5°C. The resulting solution was 35% saturated in $(NH_4)_2SO_4$. After 18 hours with stirring at 5°C, the suspension was centrifuged at 20,000 RPM for 20 minutes at 5°C in the Beckman JA-20 rotor. Solid ammonium sulfate (25.8 g.) was added to the supernatant resulting in a 55% saturated solution. After 1 hour incubation with stirring at 5°C, the suspension was centrifuged as above. One more fraction was prepared by the addition of

28.2 g. $(NH_4)_2SO_4$ to the supernatant to give a 75% saturated solution. All three precipitates were re-dissolved in 5 ml. PBS and dialysed against 4 liters PBS overnight.

A variation of this experiment was performed by incubating the 35% solution for 1 hour rather than 18 hours at 5°C before further addition of ammonium sulfate (see Results, p.89). All other steps were the same.

Fraction A from gel filtration of retentate was treated in the same way except that the volume of solution was 40 ml., and the amounts of solid $(NH_4)_2SO_4$ added were 8.35 g., 5.15 g., and 5.75 g., for the 35%, 55%, and 75% cuts respectively. For this experiment, the 35% saturated solution was incubated for 18 hours.

Gel Filtration of High Molecular Weight Hemagglutinin Fraction in 6M Urea

A sample of fraction A1 from gel filtration of Amphitrite retentate (see Results, figure 3) was subjected to gel filtration in 6M urea on Sephadex G-100. The sample was concentrated to 2 ml. (approx. 10 mg./ml. in PBS). Solid urea (0.72 g.) was added to give a sample solution of 6M urea in PBS. The sample solution was incubated for 2 hours at 37°C and then for 18 hours at 5°C. Sephadex G-100 was swollen, packed and equilibrated in 6M urea PBS. The gel was packed into a Pharmacia K-15 column (40 x 1.5 cm.) using gravity flow. The elution buffer was 6M urea PBS. The flow rate was 10 ml./hr. and fractions of 6 ml. were collected.

The column was calibrated as described above for gel filtration of Amphitrite retentate.

Fractions from gel filtration of the high molecular weight fraction in 6M urea were dialysed for 72 hours against two changes of 6 liters PBS, and assayed for hemagglutinin activity by the microtiter well assay with rat RBC.

Purification of the Low Molecular Weight Hemagglutinin

The low molecular weight form of the Amphitrite hemagglutinin (molecular weight = 30-32,000) was purified to homogeneity in four steps. The 55% saturated ammonium sulfate precipitate, redissolved in PBS (20 mg./ml.), was subjected to gel filtration on the Sephadex G-100 column (77 x 2.6 cm.) described above (p.43). Sample size was 7-10 ml. The elution buffer was PBS, the flow rate was 20 ml./hour, and fractions of 6 ml. were collected. The eluted fractions were assayed with chicken RBC by capillary assay. Active fractions (see Fig. 12) were pooled, concentrated to 2 ml. (approx. 15 fold) by ultrafiltration through a PM-10 membrane, and subjected to preparative polyacrylamide disc gel electrophoresis. Eluates from gel slices (see Methods) were assayed for activity with chicken RBC by capillary assay. The contiguous active fractions (see Fig. 14) were pooled, dialysed against 4 liters distilled water, and lyophilized.

Analytical Ultracentrifugation

Sedimentation velocity and sedimentation equilibrium experiments were performed according to the methods described by Schachman (60). All analytical ultracentrifuge experiments were performed on a Beckman Model E Analytical Ultracentrifuge, with the titanium AN-D rotor. A single sector cell with plane quartz windows was used with a Schlieren counterweight cell. The Schlieren optical system and light source was used with a phase plate angle of 30°. Kodak Metallographic plates, used for photographs, were developed with Kodak D-19 developer. Measurements on developed film were made with a Gaertner Microcomparator.

Sedimentation Equilibrium. Conditions for the run were as follows: sample - 1.85 mg. purified hemagglutinin in 1.0 ml. water; temperature - 26°C; vacuum - 0.4 microns; rotor speed - 10,000 RPM; duration of run - 4 hours. Calculation of the molecular weight by the approach to equilibrium method was done according to the equation (60):

EQ. 5

$$M = \frac{RT}{(1 - Vp)w^2} \cdot \frac{(dc/dx)_m}{X_m C_m}$$

where M is the molecular weight, R is the gas constant, T is the absolute temperature, V is the partial specific volume of the solute, p is the density of the solvent, w is the rotor velocity in radians, dc/dx is the concentration gradient at the meniscus (top of the cell), X_m is the distance from the center of rotation to the meniscus, and C_m is the solute con-

centration at the meniscus.

C_m is calculated by the equation (60):

EQ. 6

$$C_m = C_o - 1/X_m^2 \sum X_n^2 (dc/dx)_n$$

where C_o is the initial solute concentration, X_n and $(dc/dx)_n$ are the distances from the center of rotation and the concentration gradients respectively at various intervals (n) near the meniscus. The values for $(dc/dx)_m$, $\sum X_n^2 (dc/dx)_n$, and X_m were determined from the photograph taken 1 hour after the start of the run, as described by Schachman (60). The partial specific volume was calculated from the amino acid and sugar composition to be 0.710.

Sedimentation Velocity. Sedimentation velocity experiments were done on purified hemagglutinin to determine homogeneity, and on fraction A1 to determine molecular size of the high molecular weight material. For the purified hemagglutinin the run conditions were: sample - 3.8 mg. purified hemagglutinin in 1.0 ml. water; temperature - 20°C; vacuum - 0.4 microns; rotor speed - 55,560 RPM; duration of run - 6.5 hours. For fraction A1 the run conditions were: sample - 2.7 mg. fraction A1 in 1.0 ml. water; temperature - 20°C; vacuum - 0.4 microns; rotor speed - 57,800 RPM; duration of run - 3 hours.

Photographs were taken during both runs at 30 minute intervals. The sedimentation coefficient S was calculated from the equation:

EQ. 7

$$S = 1/w^2X \quad (dx/dt)$$

were w is the angular velocity in radians, X is the position of the boundary peak, and dx/dt is the rate of sedimentation. The value for 1/X dx/dt was obtained by a plot of log X (as determined from a series of photographs) vs. time (of each exposure).

Ehrlich Ascites Agglutination (in vitro)

In an attempt to elucidate the mechanism of the anti-tumor activity of Amphitrite hemagglutinin, Ehrlich ascites tumor cells were tested for agglutination by the hemagglutinin in vitro. Ehrlich ascites cells were grown in female Swiss Webster mice for 7 days. At that time the animals were killed, and the ascites fluid was diluted 4:1 with saline, the cells were washed three times with saline. The diluted cell suspension contained approximately 2 x 10^6 cells/ml. Equal volumes (1 ml.) of cell suspension and purified hemagglutinin (1 mg./ml.) or fraction A1 (2.7 mg./ml.) were incubated at 25°C without shaking for various times. A 1:1 dilution of the ascites suspension with saline was used as a negative control, and a solution of Concanavalin A (6.5 mg./ml.) was used as a positive control.

The method of Kaneko et al. (61) was used to assay for ascites cell agglutination. The incubated mixture of cells and agglutinin was stirred by gently pipetting three times

into a one ml. cuvette. The absorbance at 600 nm. was immediately determined in a Beckman DU Spectrophotometer. The absorbance at 600 nm. is proportional to the turbidity of the solution. Since agglutination produces a smaller number of particles than were originally present in solution, the turbidity is inversely proportional to the extent of cell agglutination. Positive agglutination is therefore detected by a plot of absorbance at 600 nm. vs. time with a negative slope.

Test suspensions and controls were also subjected to microscopic examination for agglutination.

Results

Hemagglutinin Activity in Annelid Extracts

Hemagglutination activity was discovered in ultrafiltration retentates from Amphitrite ornata by the sedimentation velocity assay with rat RBC. The capillary and microtiter well methods confirmed the presence of a hemagglutinin activity toward rat and chicken erythrocytes. Titers for the Amphitrite retentate were low (2-4 with chicken RBC, 4-8 with rat RBC), even at concentrations of 40 mg. solid material per ml. PBS.

Hemagglutinin activity was also found in Nereis retentates (see below).

Gel Filtration of Amphitrite Retentate on Sephadex G-100

Gel filtration of Amphitrite retentate on Sephadex G-100 gave the elution profile shown in Figure 3. The protein elution pattern (ABS at 280 nm.) was independent of column conditions, and was consistent for several batches of retentate. The hemagglutinin activity profile seen in Figure 3 was not completely consistent for different retentates. The relative activities of the three peaks were not constant. This kind of heterogeneity might result from aggregation-disaggregation phenomena (see below), with the final composition dependent on the particular batch work-up.

The calibration curve for this column with standard pro-

FIGURE 3

Gel Filtration of Amphitrite Retentate on Sephadex G-100

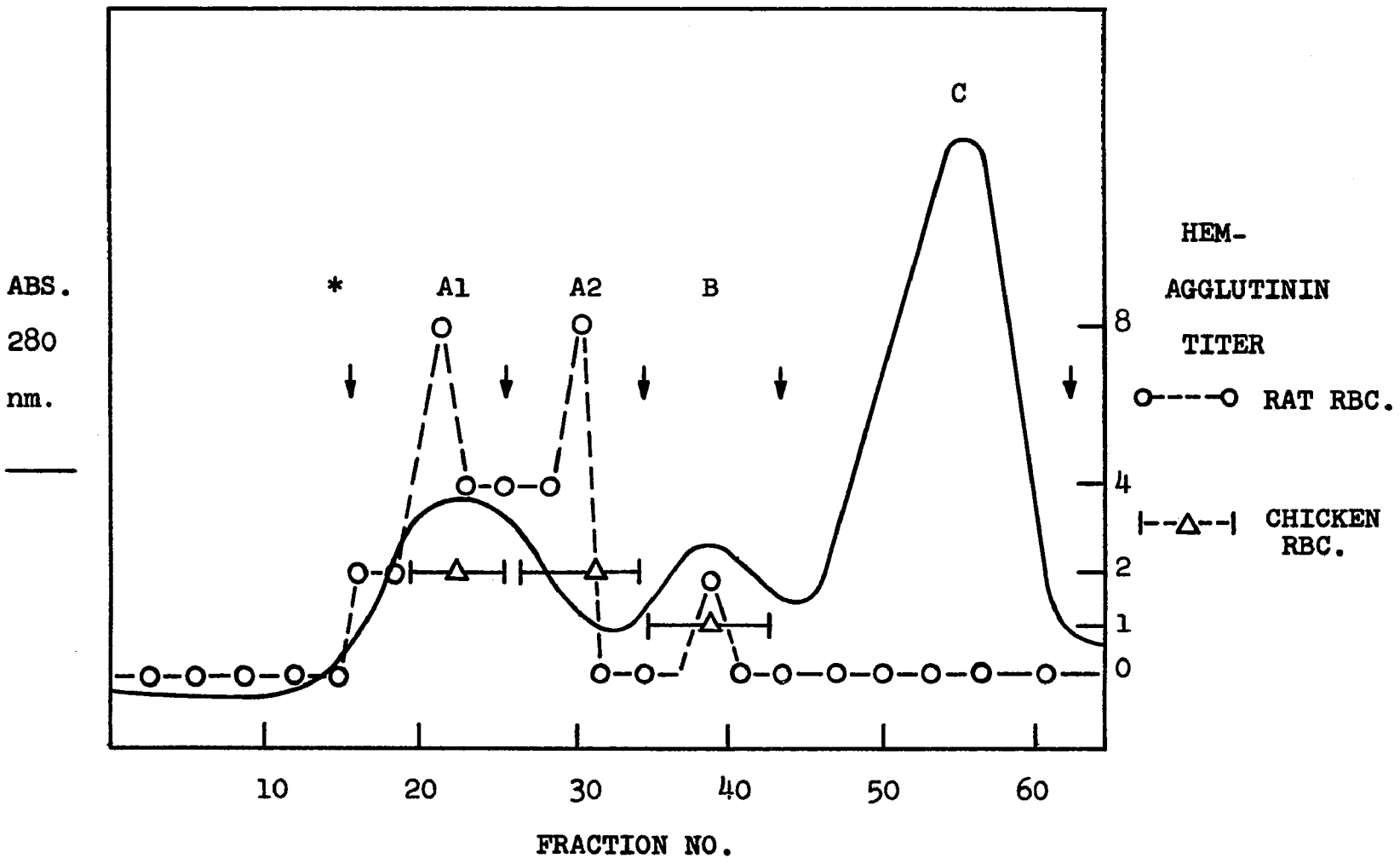
Column dimensions were 77 X 2.6 cm. The equilibration and elution buffer was PBS. Sample size was 15 ml. of retentate (40 mg./ml.) in PBS. The sample was clarified by centrifugation in the JA-20 rotor at 5°C 20,000 RPM for 20 minutes prior to column application. Fractions of 6 ml. were collected.

_____ Absorbance at 280 nm.

o--------o Hemagglutinin titer vs. rat RBC by the capillary assay on individual fractions.

 Hemagglutinin titer vs. chicken RBC by the capillary assay on pooled fractions.

Fractions were pooled as indicated by arrows. Void volume is indicated by the asterisk.


ABS.
280
nm.
*
A1
A2
B
C
HEM-
AGGLUTININ
TITER
8
4
2
1
0
RAT RBC.
CHICKEN
RBC.
10
20
30
40
50
60
FRACTION NO.

teins is shown in Figure 4. The three peaks of hemagglutinin activity represent material of molecular weights $>$100,000 (void volume), 54,000, and 30,000. The pooled active fractions were labelled A1, A2, and B, as indicated in Figure 3. In some subsequent experiments fractions A1 and A2 were pooled as fraction A. The large 280 nm. absorbing peak of low molecular weight ($<$12,000) labelled fraction C had no activity.

No hemagglutinating activity was found when individual fractions were assayed with chicken RBC. However, the pooled and concentrated fractions A1, A2 and B all showed titers of 1-2 against chicken RBC. The three different molecular weight fractions all exhibited the same pattern of specificity for rat and chicken RBC.

Erythrocyte Specificity of Amphitrite Hemagglutinin

The erythrocyte agglutinating specificity of Amphitrite hemagglutinin (fractions A and B) is shown in Table 2. The specificities of high and low molecular weight fractions (A and B, respectively) were parallel for rat, chicken, rabbit, and guinea pig RBC. This is unlike the results found by Hall and Rowlands (10) in the lobster, where different molecular weight fractions exhibited different erythrocyte specificities. The Amphitrite hemagglutinin was most active toward rat and rabbit RBC and was moderately active toward chicken RBC. Only weak activity was seen with sheep and guinea pig cells. The Amphitrite hemagglutinin agglutinated all four human blood

FIGURE 4

Calibration Curve for Sephadex G-100 Column

The column used in Figure 3 (77 X 2.6 cm.) was calibrated. The elution buffer was PBS. Sample size of standard proteins was 5-7 ml., 20 mg./ml. in PBS. Fractions of 6 ml. were collected. Standard proteins used were: bovine serum albumin - 64,000; ovalbumin - 45,000; chymotrypsinogen - 25,000; and ribonuclease A - 13,700 daltons. Values for K_{av} were calculated from elution volumes (EQ. 4, p.46). Hemagglutinin fractions are indicated on the calibration curve by □ . The molecular weights for these fractions were A1 - >100,000; A2 - 54,000; B - 30,000.

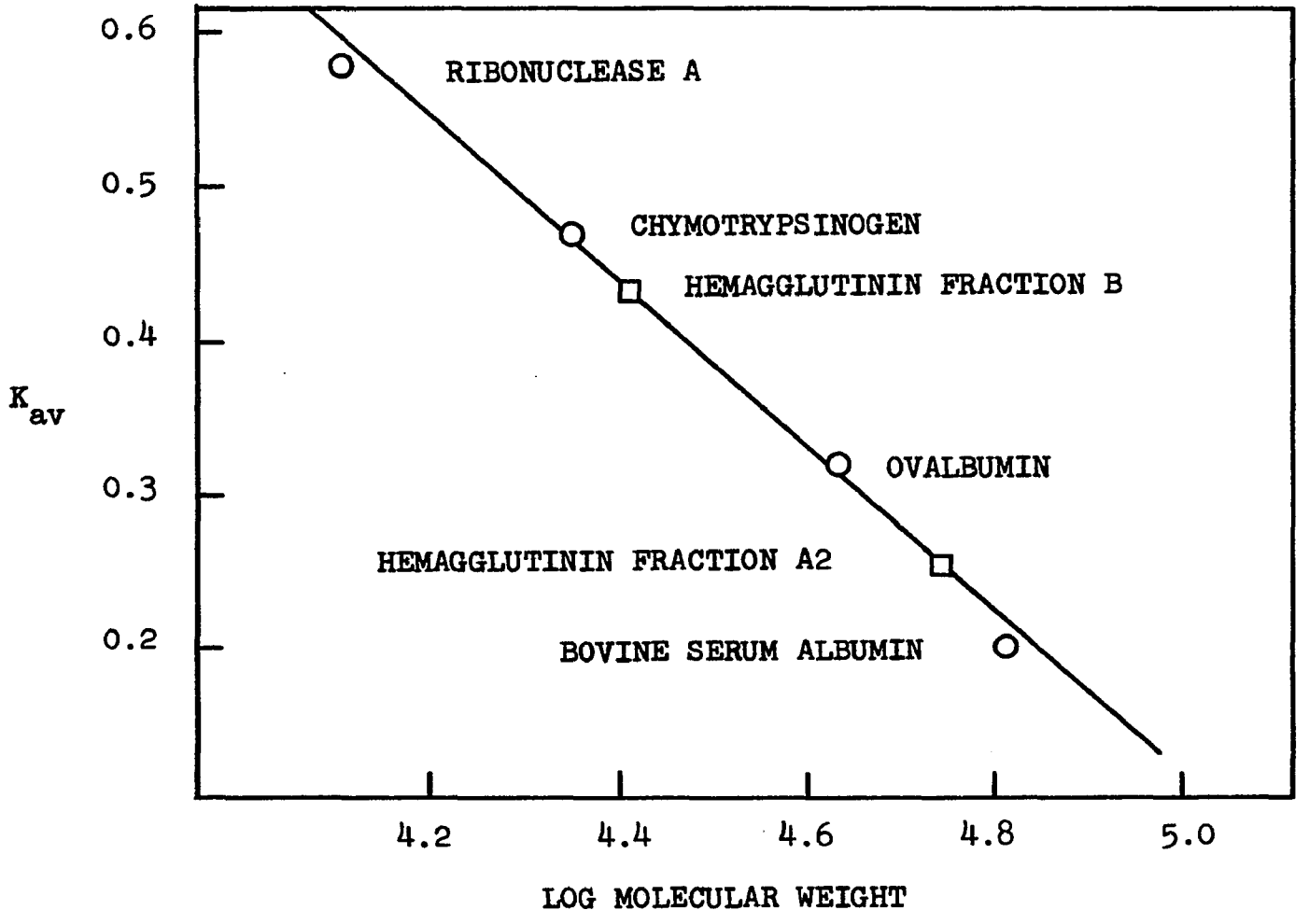

0.6
0.5
0.4
0.3
0.2
K_{av}
RIBONUCLEASE A
CHYMOTRYPSINOGEN
HEMAGGLUTININ FRACTION B
OVALBUMIN
HEMAGGLUTININ FRACTION A2
BOVINE SERUM ALBUMIN
4.2
4.4
4.6
4.8
5.0
LOG MOLECULAR WEIGHT

TABLE 2

RELATIVE ERYTHROCYTE SPECIFICITY
OF AMPHITRITE HEMAGGLUTININ FRACTIONS

	Titer	
Erythrocyte[1]	Fraction B[2]	Fraction A[3]
Rat	32	32
Rabbit	16	32
Chicken	4	4
Sheep	1	
Guinea Pig	1	0
Human Type A	4	
Human Type B	1-2	
Human Type O (H)	1	

[1] 5% suspension in saline

[2] From gel filtration of 55% ammonium sulfate precipitate of retentate.

[3] From gel filtration of retentate (pooled A1 + A2).

groups with low titers, although activity was four times greater toward group A than the others.

Saccharide Inhibition of Amphitrite Hemagglutinin

The data for saccharide inhibition of Amphitrite hemagglutinin activity (fraction B) is presented in Table 3. The only effective inhibitor when tested with rat RBC was N-acetyl-D-galactosamine, the terminal sugar residue of the human blood group A immunodeterminant. None of the saccharides tested inhibited agglutination of chicken RBC. This may be due to different binding sites for the two erythrocyte species on the hemagglutinin.

The finding of some human blood group A specificity for Amphitrite hemagglutinin coincides with the inhibition of activity by N-acetyl-D-galactosamine. However, the hemagglutinin is not totally specific for this saccharide. Human groups B and O were also agglutinated. Moreover, N-acetyl-D-galactosamine never completely inhibited activity, even at high concentrations.

Physical and Chemical Properties of Amphitrite Hemagglutinin

Table 4 summarizes the effects of several physical and chemical treatments on Amphitrite hemagglutinin activity toward chicken RBC. All three fractions gave the same results.

No loss or increase in activity (initial titer 2-4 with chicken RBC) was seen after lyophilization, dialysis against

TABLE 3

SACCHARIDE INHIBITION OF AMPHITRITE HEMAGGLUTININ FRACTION B[1]

Saccharide[2]	Titer
None	32
D-Glucose	32
D-Galactose	32
D-Fucose	32
L-Fucose	32
D-Mannose	32
D-Glucosamine	32
D-Galactosamine	32
D-Mannosamine	32
N-Acetyl-D-Glucosamine	32
N-Acetyl-D-Galactosamine	8
N-Acetyl-D-Mannosamine	32
N-Acetylneuraminic Acid	32
Methyl- -D-Mannopyranoside	32
Methyl- -D-Galactopyranoside	32
Phenyl- -D-Galactopyranoside	32
Chondroitin sulfate	32
Sialic acid-rich glycoprotein	32

[1] 1 mg./ml.

[2] 25 ul. of 0.2M (in PBS) of each saccharide added to hemagglutinin microtiter titrations.

TABLE 4

EFFECTS OF PHYSICO-CHEMICAL TREATMENTS ON HEMAGGLUTININ ACTIVITY

Treatment	Hemagglutinin Activity
None	+
Dialysis against H_2O	+
Dialysis against EDTA (.15M)	+
Heat to 70°C, 30 min.	+
Heat to 85°C, 30 min.	-
Trypsin	+
Protease	+
Phenol	-
Streptomycin Sulfate	+

distilled water, EDTA treatment, or the addition of Ca^{++} and Mg^{++}. These results suggest that the Amphitrite hemagglutinin is independent of divalent cation concentration, unlike many plant and invertebrate agglutinins (see Table 1). The hemagglutinin is relatively heat stable, with no loss in activity up to 85°C for 30 minutes. Neither of the proteolytic enzymes trypsin or pronase had any effect on the hemagglutinin activity. Although this result might tend to rule out protein in the active material, the data from analytical disc gel electrophoresis (see below) showed the presence of protein bands resistant to these enzymes in the active fractions. Pauley et al. (11) and Miller et al. (12) report similar findings for the hemagglutinins from crayfish and sea hare. Deoxyribonuclease and streptomycin sulfate had no effect. Phenolic extraction, which removes all proteins, abolished the activity.

pH Activity Profile of Amphitrite Hemagglutinin

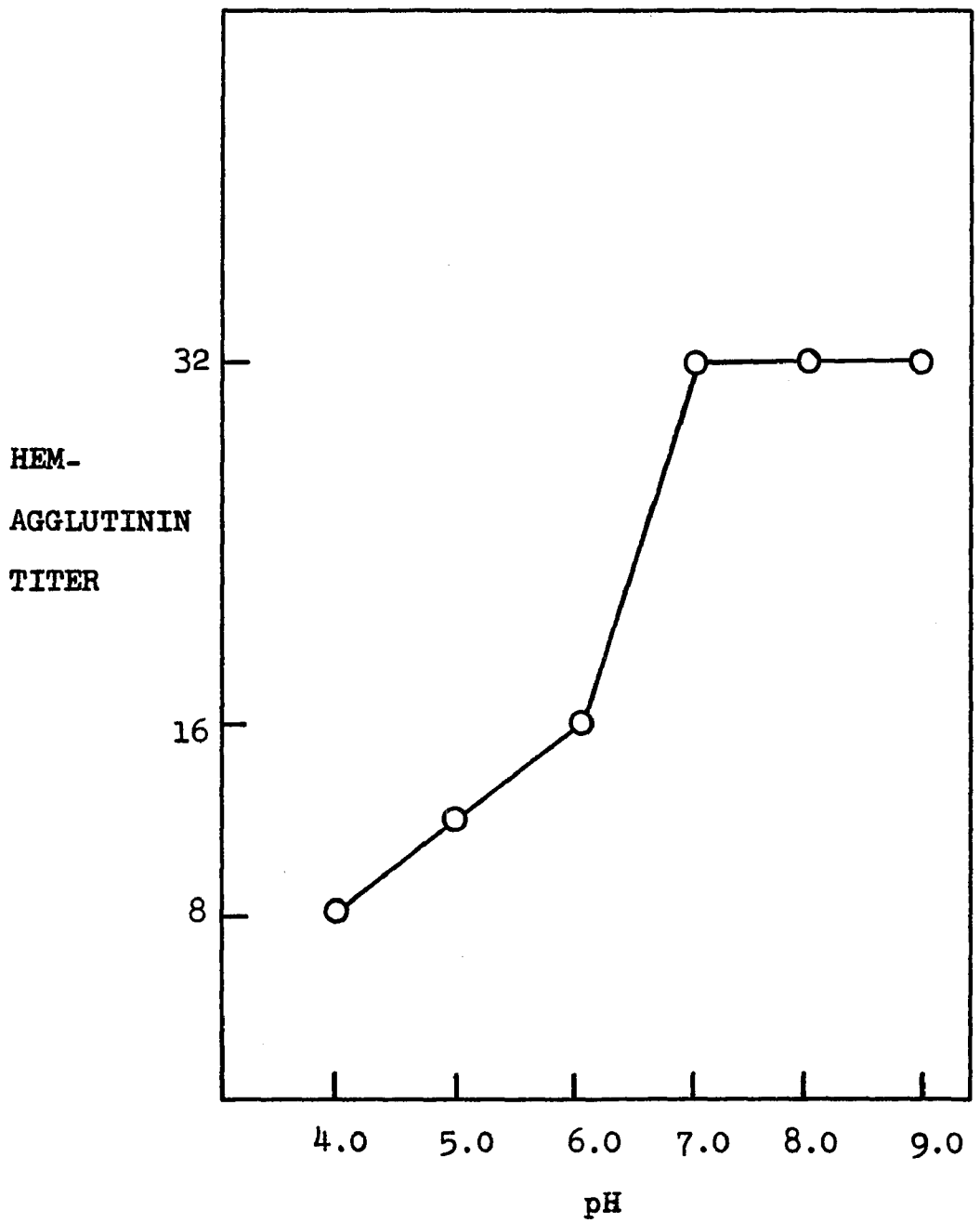
The effects of pH on the activity of Amphitrite hemagglutinin (fraction B) is shown in Figure 5. Acidic pH had an inhibiting effect on the activity, whereas no effect was seen in the alkaline range up to pH 9.

The inhibiting effect at pH 4 was irreversible. When hemagglutinin was pre-incubated at pH 4 for 1 hour, then adjusted to pH 7, the titer remained low. However, the effect at pH 5 and above was reversible. It is likely that at pH 4

FIGURE 5

pH Activity Profile of Amphitrite Hemagglutinin

Hemagglutinin titers with rat RBC were determined by micro-titer well assay. Controls showed no effect on RBC in the pH range 4-9, but cell destruction and non-specific agglutination occurred below pH 4, and above pH 9.


HEM-
AGGLUTININ
TITER
32
16
8
4.0
5.0
6.0
7.0
8.0
9.0
pH

some irreversible destruction of the hemagglutinin molecule occurred, reducing activity. On the other hand, the inhibiting effects at pH 5 and 6 were probably due to reversible inactivation of the hemagglutinin, possibly by protonation of some of the many carboxyl groups (see below) on the molecule. It is also possible that low pH affects the erythrocyte surface, reducing the agglutinability of the cells.

The graph of Figure 5 was not extended beyond pH 4 or 9 because of RBC destruction at these ranges.

Polyacrylamide Disc Gel Electrophoresis

Analytical Disc Gel Electrophoresis. Analytical polyacrylamide disc gel electrophoresis was performed on Amphitrite hemagglutinin fractions in order to elucidate their chemical compositions. Figure 6 shows the results for disc gel electrophoresis of fraction A1 stained for protein (Figure 6A), nucleic acid (Figure 6B), and for carbohydrate (Figure 6C). Six to seven protein bands were observed in all runs. These included an intense fast-moving (anodal) band, a broad diffuse band, and a broad intense band near the origin. The "Stains-all" gels showed several intense blue bands, one of them co-migrating with the fast-moving protein band. Only two pink carbohydrate bands were seen on periodic acid-Schiff reagent stained gels, one co-migrating with the intense fast-moving protein band, and the other near the origin.

Analytical polyacrylamide disc gel electrophoresis of

FIGURE 6

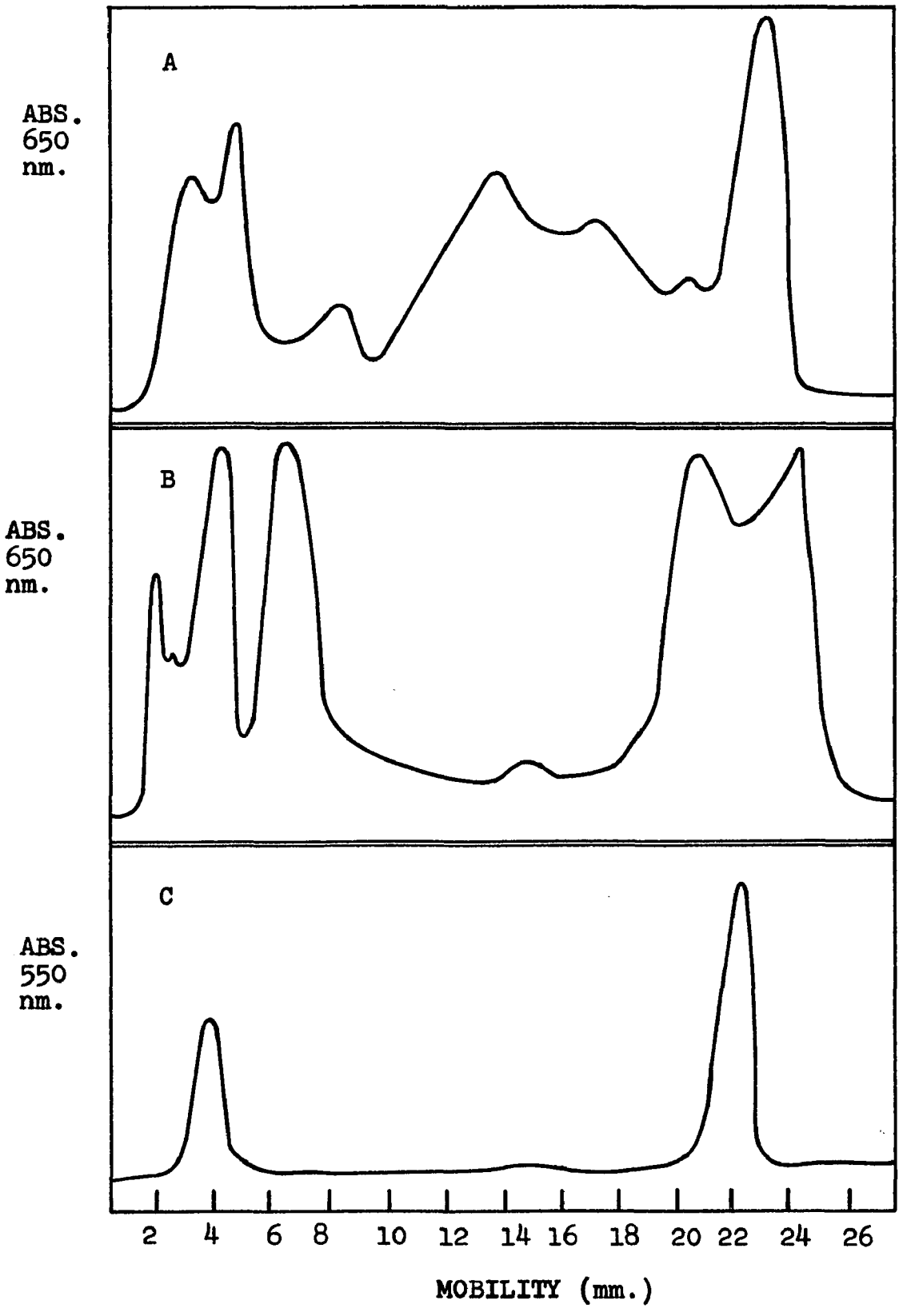
Gel Scan of Analytical Polyacrylamide Disc Gel Electrophoresis of Hemagglutinin Fraction A1

Electrophoresis was carried out for 40 minutes at 5 mA/gel. Gels were 12% acrylamide, pH 8.9. The top of the gel is at left of the scan.

A) Gel stained with Coomassie Brilliant Blue for protein, scanned at 650 nm.

B) Gel stained with "Stains-all" for nucleic acid, scanned at 650 nm.

C) Gel stained with periodic acid-Schiff reagent for carbohydrate, scanned at 550 nm.


A
ABS.
650
nm.
B
ABS.
650
nm.
C
ABS.
550
nm.
2 4 6 8 10 12 14 16 18 20 22 24 26
MOBILITY (mm.)

fraction A2 was identical to A1 for Coomassie Blue and periodic acid-Schiff reagent stained gels, but contained only two "Stains-all" bands, co-migrating with the periodic acid-Schiff bands.

Analytical polyacrylamide disc gel electrophoresis of fraction B gave results shown in Figure 7. Three to four proteins were present, including an intense fast-moving band. Both "Stains-all" and periodic acid-Schiff reagent stained gels showed one band co-migrating with the fast protein band.

Analytical Disc Gel Electrophoresis of Trypsin and Phenol Treated Hemagglutinin. When samples of fraction A1 treated with trypsin and phenol were applied to analytical polyacrylamide disc gel electrophoresis, the results illustrated in Figures 8 through 10 were found. Figure 8 shows the protein staining bands for fraction A1 (Figure 8A), fraction A1 treated with trypsin (Figure 8B) and fraction A1 treated with phenol (Figure 8C). Enzyme treatment removed all but two proteins. The band near the origin was reduced in intensity. After prolonged treatment with trypsin (48 hours) this band also disappeared. No effect was seen by trypsin on the fast-moving band. Pronase treatment gave identical results. Phenolic extraction destroyed all protein material, including the fast-moving band.

Proteolytic enzymes did not affect the "Stains-all" staining pattern of fraction A1 (Figure 9A-B). However,

FIGURE 7

Gel Scan of Analytical Polyacrylamide Disc Gel Electrophoresis of Hemagglutinin Fraction B

Electrophoresis was carried out for 40 minutes at 5mA/gel. Gels were 12% acrylamide, pH 8.9. The top of the gel is at the left of the scan.

A) Gel stained with Coomassie Brilliant Blue for protein, scanned at 650 nm.

B) Gel stained with "Stains-all" for nucleic acid, scanned at 650 nm.

C) Gel stained with periodic acid-Schiff reagent for carbohydrate, scanned at 550 nm.

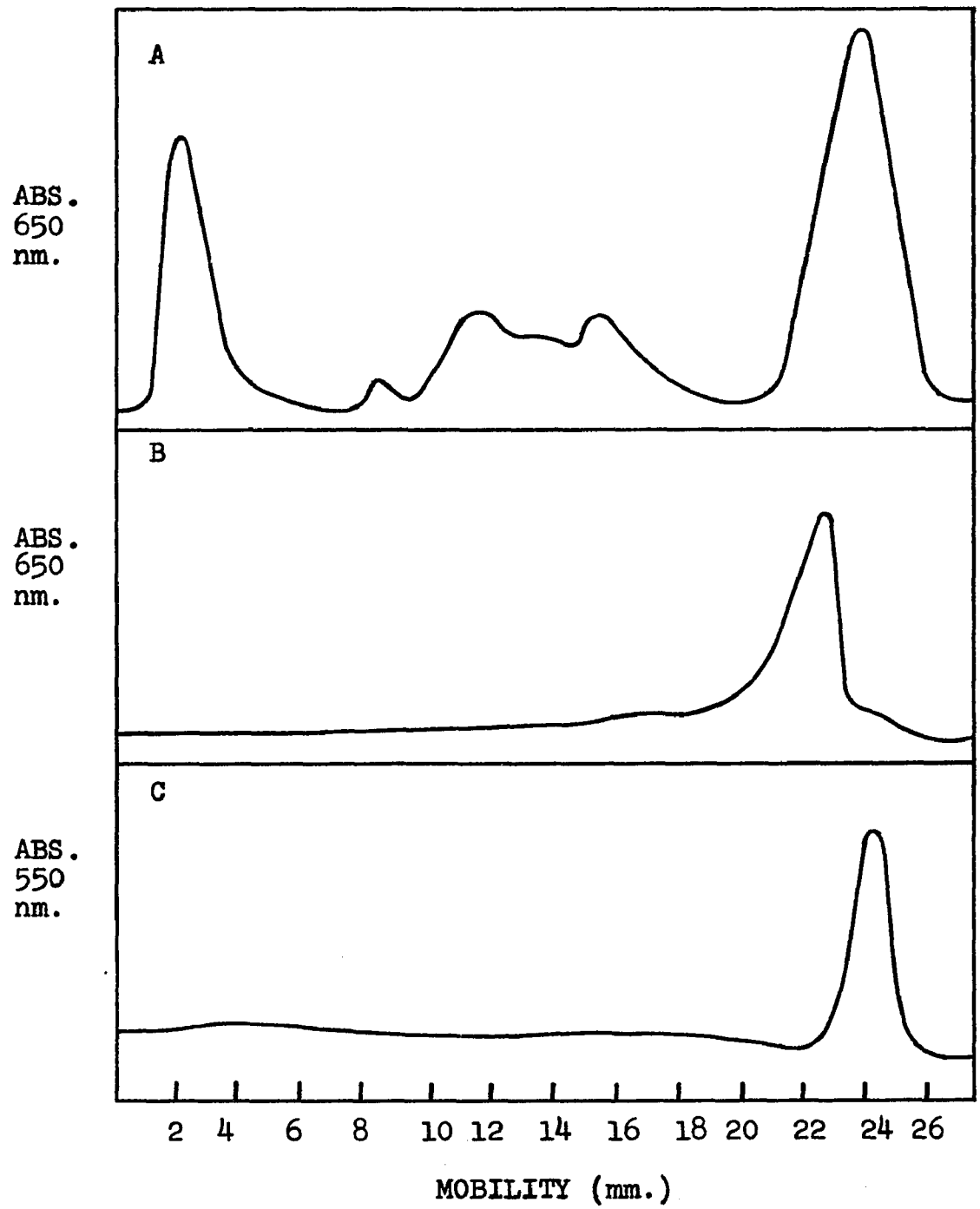

A
ABS.
650
nm.
B
ABS.
650
nm.
C
ABS.
550
nm.
2 4 6 8 10 12 14 16 18 20 22 24 26
MOBILITY (mm.)

FIGURE 8

Gel Scan of Analytical Polyacrylamide
Disc Gel Electrophoresis of Hemagglutinin Fraction A1
After Trypsin and Phenol Treatments
(Coomassie Blue Staining Pattern)

Electrophoresis was carried out for 40 minutes at 5mA/gel. Gels were 12% acrylamide, pH 8.9. The top of the gel is at the left of the scan.

A) Fraction A1 without treatment. Gel stained with Coomassie Blue, scanned at 650 nm.

B) Fraction A1 after trypsin treatment. Gel stained with Coomassie Blue, scanned at 650 nm.

C) Fraction A1 after phenol treatment. Gel stained with Coomassie Blue, scanned at 650 nm.

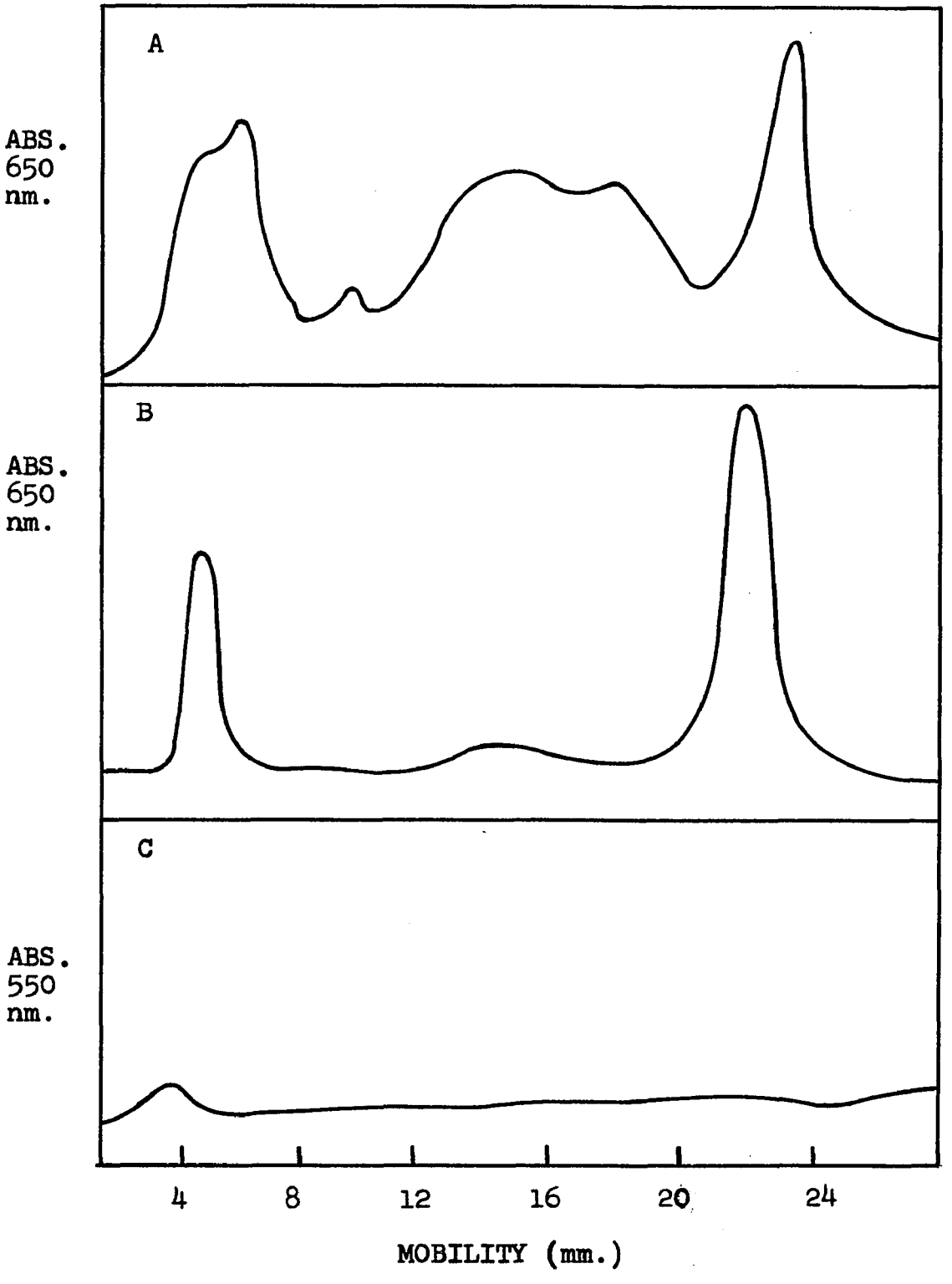

A
ABS.
650
nm.
B
ABS.
650
nm.
C
ABS.
550
nm.
4
8
12
16
20
24
MOBILITY (mm.)

FIGURE 9

Gel Scan of Analytical Polyacrylamide
Disc Gel Electrophoresis of Hemagglutinin Fraction A1
After Trypsin and Phenol Treatments
("Stains-all" Staining Pattern)

Electrophoresis carried out for 40 minutes at 5 mA/gel. Gels were 12% acrylamide, pH 8.9. The top of the gel is at the left of the scan.

A) Fraction A1 without treatment. Gel stained with "Stains-all," scanned at 650 nm.

B) Fraction A1 after trypsin treatment. Gel stained with "Stains-all," scanned at 650 nm.

C) Fraction A1 after phenol treatment. Gel stained with "Stains-all," scanned at 650 nm.

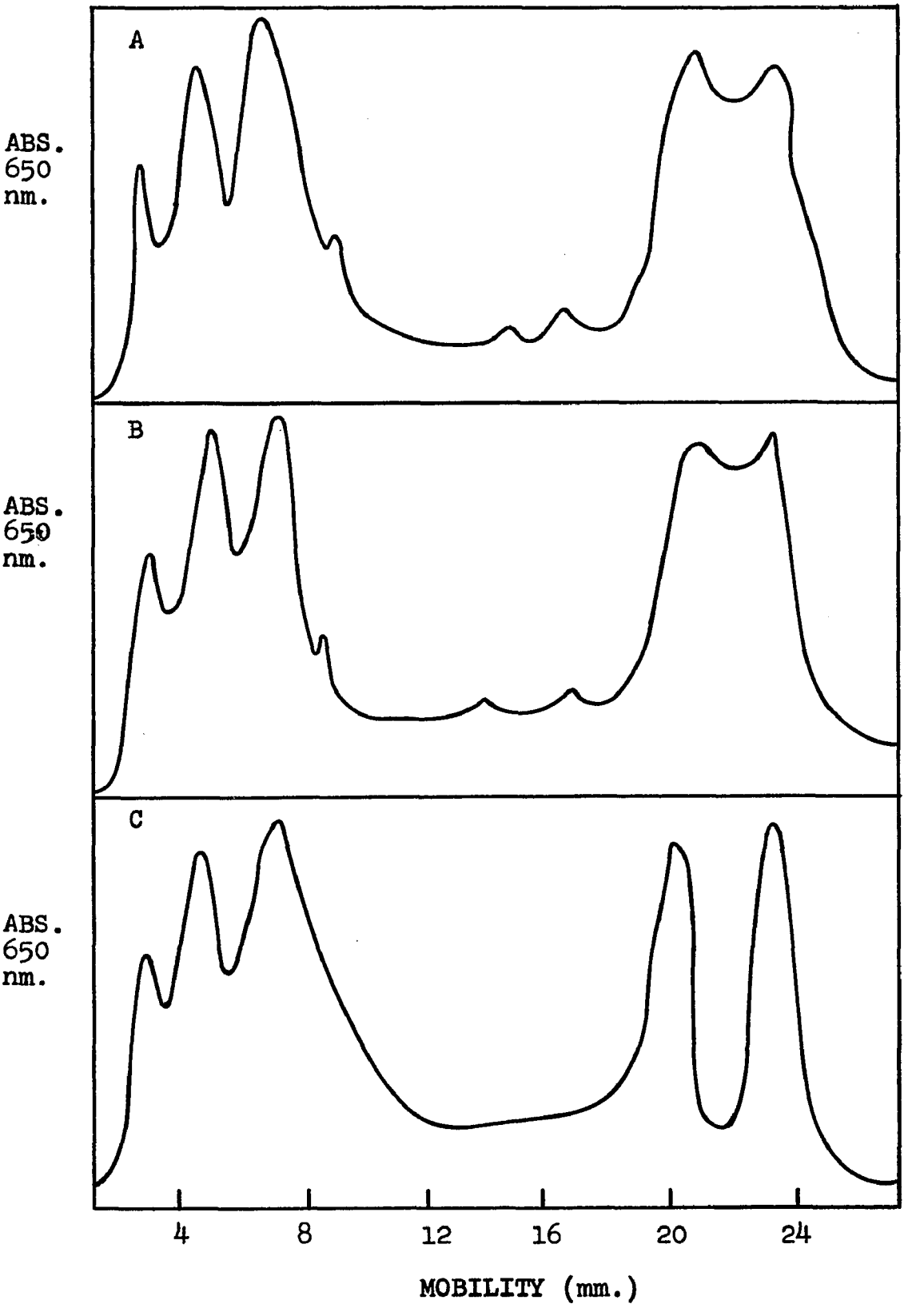
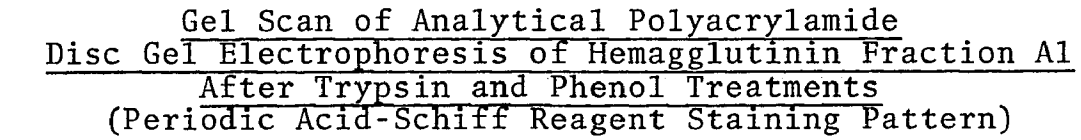

A
ABS.
650
nm.
B
ABS.
650
nm.
C
ABS.
650
nm.
4
8
12
16
20
24
MOBILITY (mm.)

FIGURE 10

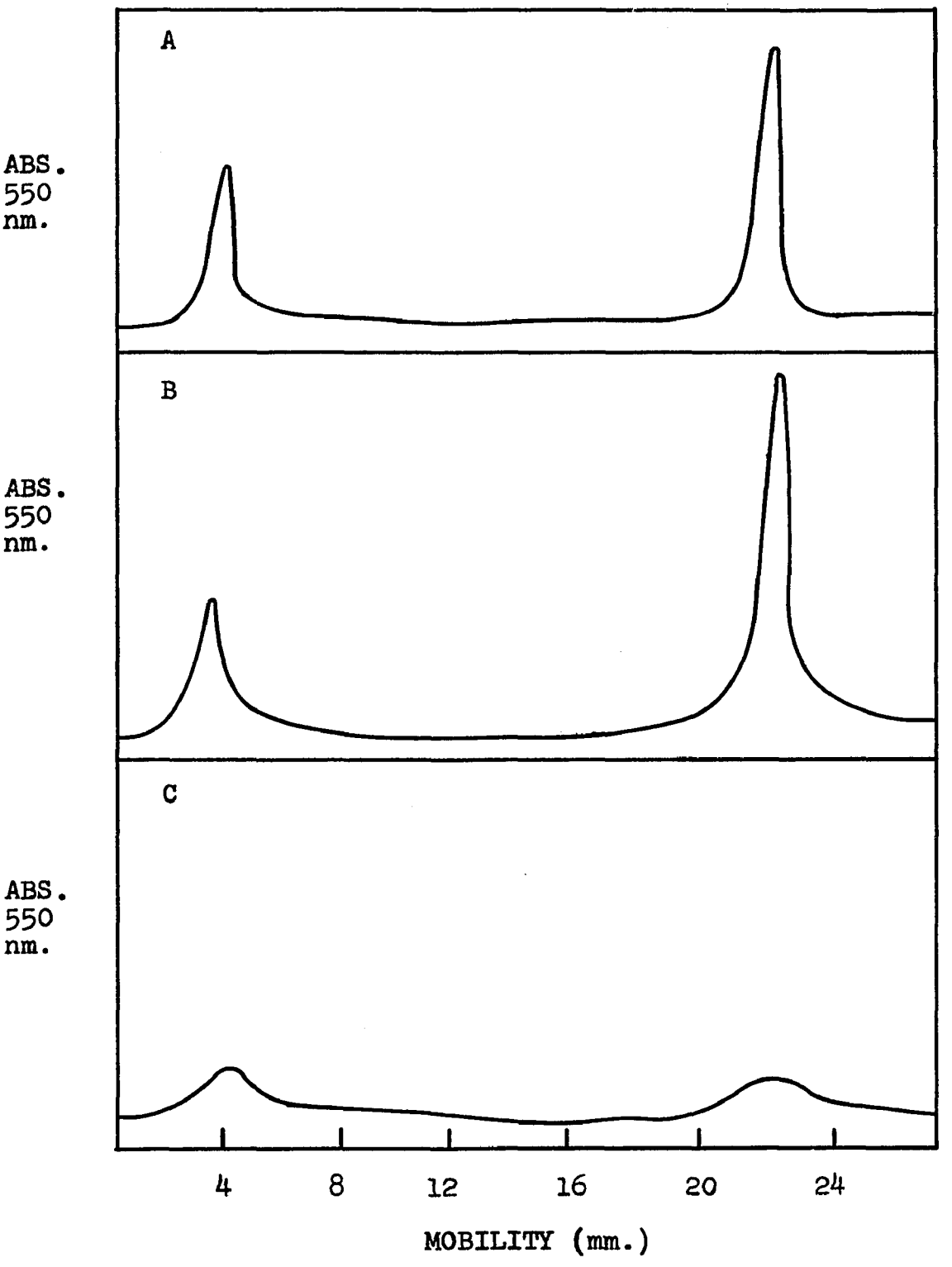


Electrophoresis was carried out for 40 minutes at 5mA/gel. Gels were 12% acrylamide, pH 8.9. The top of the gel is at the left of the scan.

A) Fraction A1 without treatment. Gel stained with periodic acid-Schiff reagent, scanned at 550 nm.

B) Fraction A1 after trypsin treatment. Gel stained with periodic acid-Schiff reagent, scanned at 550 nm.

C) Fraction A1 after phenol treatment. Gel stained with periodic acid-Schiff reagent, scanned at 550 nm.


A
B
C
ABS.
550
nm.
ABS.
550
nm.
ABS.
550
nm.
4
8
12
16
20
24
MOBILITY (mm.)

phenolic extraction removed a portion of the fast-moving broad band (Figure 9C). The mobility of the region affected corresponded exactly to the mobility of the fast-moving protein band. A similar result was found in gels stained for carbohydrate. Neither band was affected by enzymes, but both were removed by phenol (Figure 10).

These data showed that one protein component of an active hemagglutinin fraction was resistant to proteolytic enzyme digestion. This component was strongly anionic, and was destroyed by phenolic extraction. In addition, it appeared that this protein was associated with nucleic acid and/or carbohydrate.

Preparative Polyacrylamide Disc Gel Electrophoresis. Considering the data on the effects of trypsin and phenol on hemagglutinin activity presented above, the trypsin-resistant protein bands were considered most likely to be responsible for the activity. This was confirmed by preparative disc gel electrophoresis of fraction A1 as illustrated in Figure 11. All the hemagglutinin activity was found in the fast-moving trypsin resistant band. Preparative disc gel electrophoresis on fraction A2 was unsuccessful due to low yields of active material. Preparative disc gel electrophoresis of fraction B is discussed on p. 92. The hemagglutinin fraction prepared from preparative disc gel electrophoresis of fraction A1 was not a pure preparation due to contamination with DNA which co-migrated with the protein, as determined by a posi-

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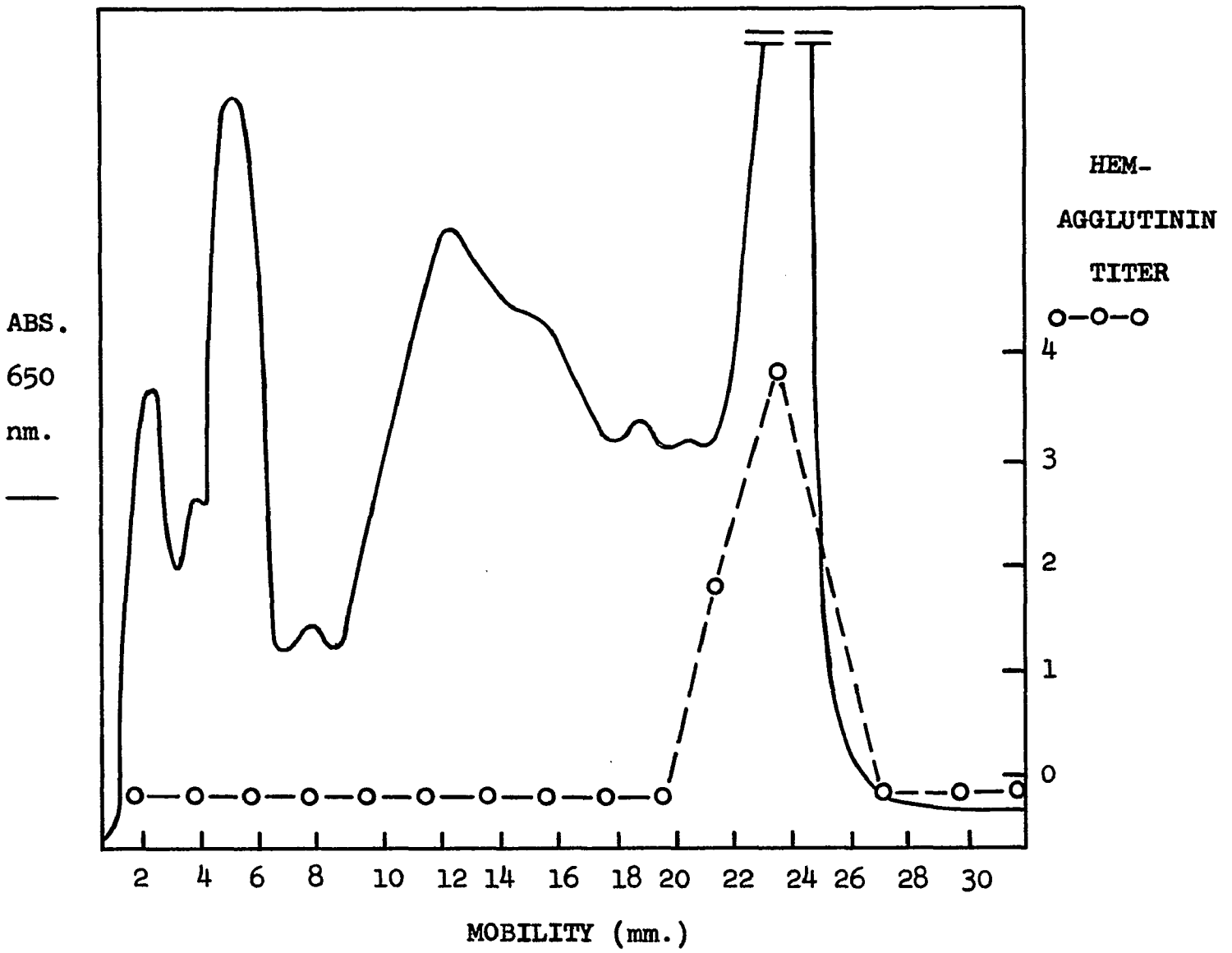
FIGURE 11

Preparative Polyacrylamide Disc Gel Electrophoresis of Hemagglutinin Activity from Fraction A1

Electrophoresis was carried out for 60 minutes at 8 mA/gel. Gels were 12% acrylamide, pH 8.9. Six gels (8 mm. i.d. x 85 mm.) were run simultaneously; one was stained with Coomassie Blue, scanned at 650 nm. The others were sliced, eluted, and assayed with rat RBC by the capillary assay. The top of the gel is at the left of the scan.

_____ Absorbance at 650 nm. for Coomassie Blue stained gel.

o--------o Hemagglutinin titer vs. rat RBC for preparative gels.


ABS.
650
nm.
HEM-
AGGLUTININ
TITER
o—o—o
4
3
2
1
0
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
MOBILITY (mm.)

tive diphenylamine test and an absorbance ratio at 280/260 of 1.0-1.1.

Chemical Nature of Hemagglutinin from Polyacrylamide Gel Electrophoresis. The results of phenolic extraction of fraction A1 on "Stains-all" stained gels seemed to imply the presence of a nucleoprotein in the active fast-moving band. The results with periodic acid-Schiff reagent stained gels implied a glycoprotein. Since sialic acid has been shown to give a blue stain with "Stains-all" (62), one explanation for the data presented is that the active hemagglutinin is a glycoprotein containing sialic acid. Subsequent analysis of the carbohydrate composition of purified hemagglutinin confirmed the presence of sialic acid in the molecule (see p. 100)

Ammonium Sulfate Precipitation

The relative hemagglutinin activities of the three ammonium sulfate fractions were determined by titration with rat RBC. Titers for the 35%, 55% and 75% saturated precipitates were 1, 32 and 4, respectively.

Gel Filtration of 55% Ammonium Sulfate Precipitate

The 55% saturated ammonium sulfate precipitate of Amphitrite retentate was applied to a Sephadex G-100 column (77 x 2.6 cm.) described on page 43. The elution profile shown in Figure 12 was obtained. The elution volumes of fractions A and B corresponded to those from gel filtration of retentate.

FIGURE 12

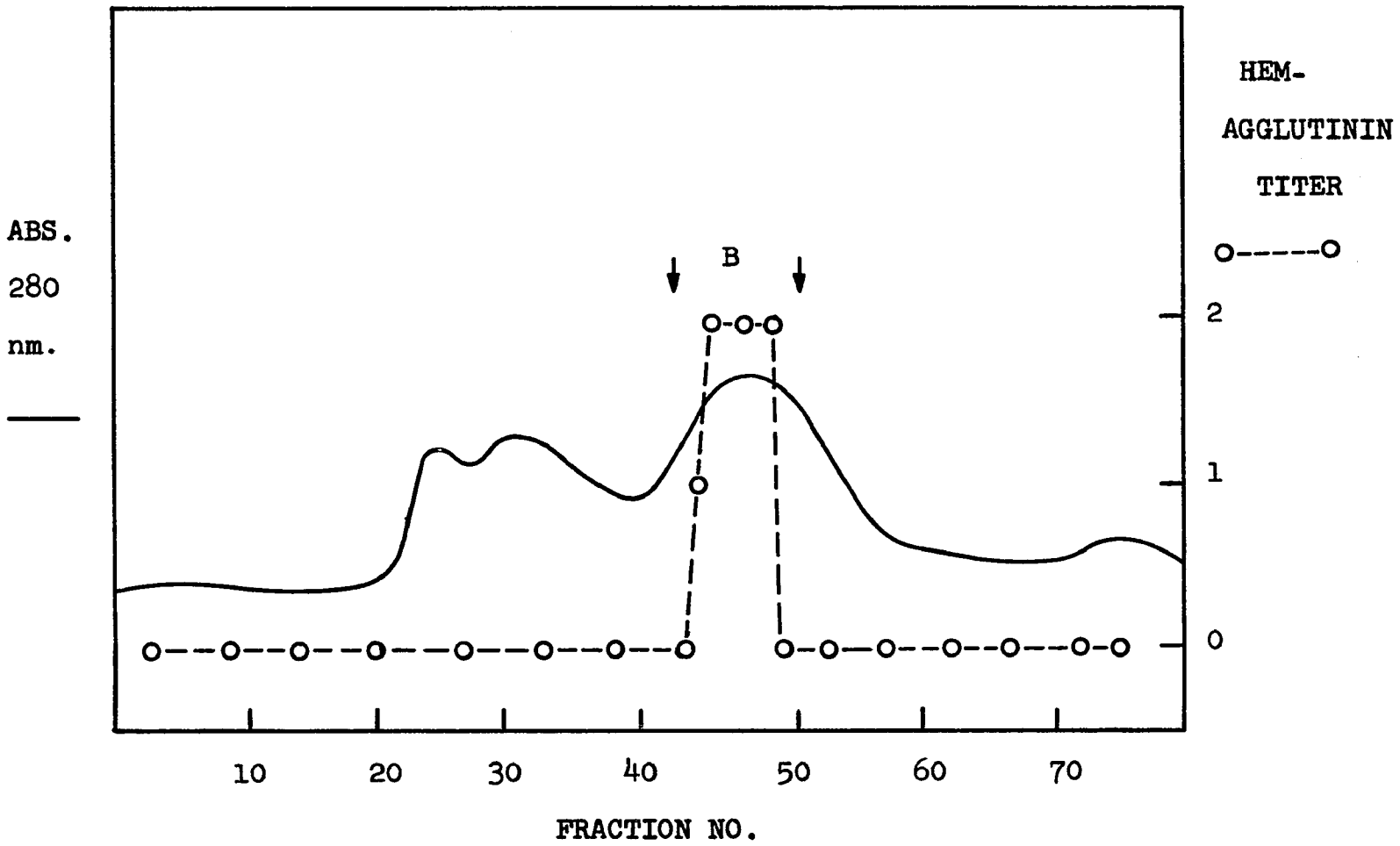
Gel Filtration of 55% Saturated Ammonium Sulfate Precipitate of Amphitrite Retentate on Sephadex G-100

Column dimensions were 77 x 2.6 cm. Elution buffer was PBS. Sample size was 8 ml., 20 mg./ml. Fractions of 6 ml. were collected.

_____ Absorbance at 280 nm.

o--------o Hemagglutinin titer vs. rat RBC by capillary assay.

The active fractions were pooled as indicated by arrows.


ABS.
280
nm.
B
HEM-
AGGLUTININ
TITER
O-----O
2
1
0
10
20
30
40
50
60
70
FRACTION NO.

However, the activity profile had shifted to the lower molecular weight (~30,000) fraction B. This result was reproducible for different retentates, but was contingent on 18-hour incubation of the retentate in 35% saturated ammonium sulfate. When the 55% cut was prepared only 1 hour after the 35% saturated solution had been prepared, the activity was found distributed between all three fractions as was the case for retentate. It seemed possible, therefore, that incubation in the high ionic strength 35% (1.6M) ammonium sulfate for a prolonged period was responsible for the shift in the molecular weight of the active material.

Gel Filtration of High Molecular Weight Hemagglutinin Fraction in 6M Urea

In order to test the possibility of disaggregation of the high molecular weight hemagglutinin by high ionic strength (such as 35% ammonium sulfate) the high molecular weight hemagglutinin fraction (A1) from gel filtration of Amphitrite retentate was incubated in 6M urea. When this was subjected to gel filtration on Sephadex G-100 the elution profile shown in Figure 13 was obtained. Most of the hemagglutinin activity was eluted in a low molecular weight range. The activity peak at fraction 6 corresponded to a molecular weight of 27-29,000. Fraction B was eluted at an identical volume when applied to this column. This result confirms the aggregate nature of the high molecular weight hemagglutinin fraction. The aggregate is maintained by non-covalent bonds, and

FIGURE 13

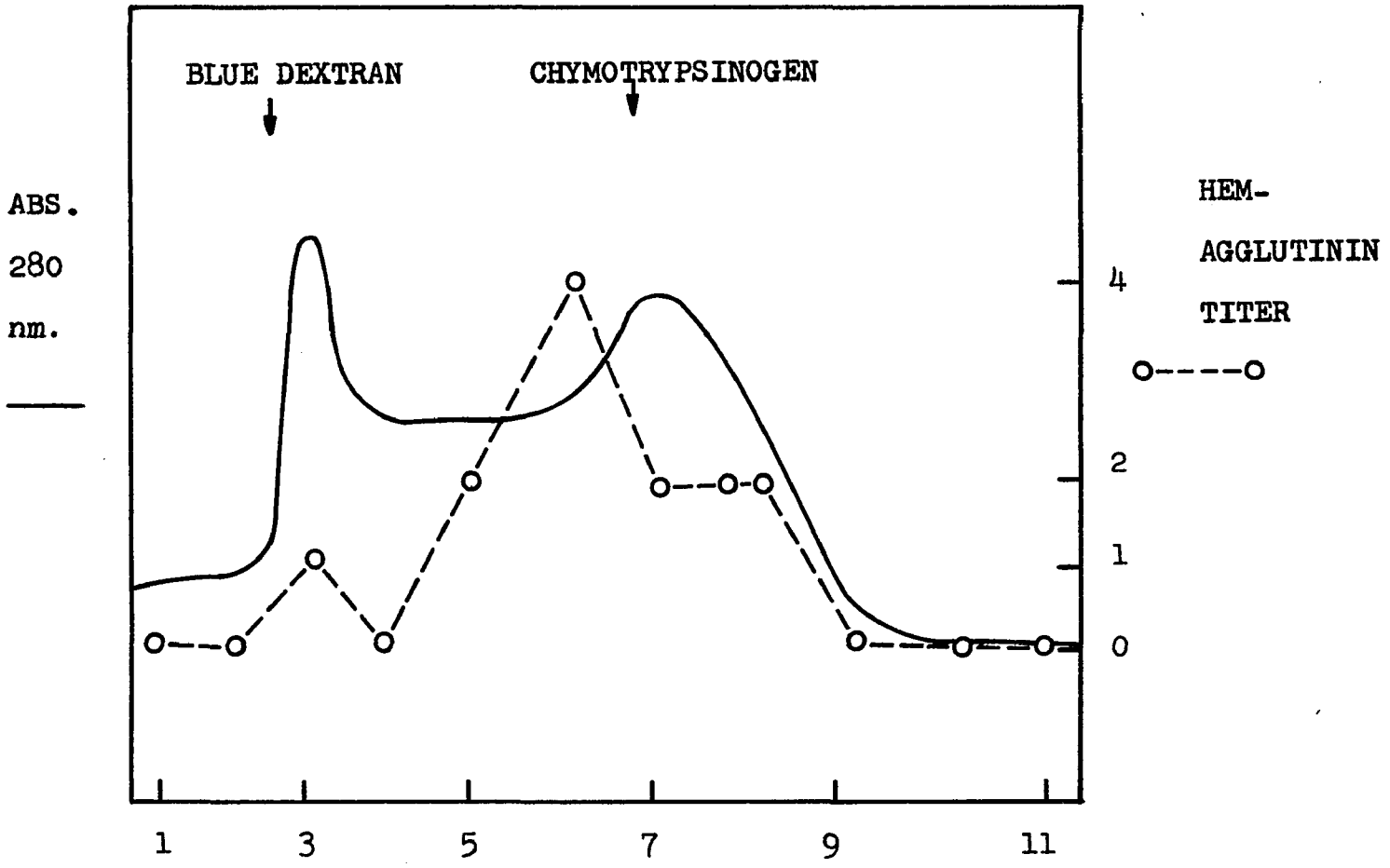
Gel Filtration of High Molecular Weight Hemagglutinin Fraction in 6M Urea

Column dimensions were 1.5 x 40 cm. Elution buffer was 6M urea - PBS. Sample size was 2 ml., 10 mg./ml. Fractions of 6 ml. were collected.

_____ Absorbance at 280 nm.

o--------o Hemagglutinin titer vs. rat RBC by the microtiter well assay.

Elution volumes of Blue Dextran, and chymotrypsinogen (molecular weight = 25,000) are indicated by arrows.


BLUE DEXTRAN
CHYMOTRYPSINOGEN
ABS.
280
nm.
HEM-
AGGLUTININ
TITER
4
2
1
0
1
3
5
7
9
11
FRACTION NO.

can be disaggregated by high ionic strength media, such as 35% (1.6M) ammonium sulfate or 6M urea.

Re-aggregation of the low molecular weight fraction was not observed (by gel filtration) after the removal of urea by dialysis. The addition of Ca^{++} and Mg^{++} ions (these ions have been reported to stabilize the high molecular weight forms of some hemagglutinins) did not promote re-aggregation.

Preparative Polyacrylamide Disc Gel Electrophoresis of Low Molecular Weight (30,000) Hemagglutinin

Fraction B from gel filtration of the 55% ammonium sulfate precipitate (see Figure 12) was subjected to preparative polyacrylamide disc gel electrophoresis. The result is shown in Figure 14. All the hemagglutinin activity was found in the fast-moving protein band. This region was also stained with periodic acid-Schiff reagent, and "Stains-all." The eluted material gave a negative diphenylamine test, indicating the absence of DNA. The 280/260 absorbance ratio of the material was 1.86, another indication of the lack of nucleic acid.

Purification Summary of Amphitrite Hemagglutinin

The purified hemagglutinin was prepared in four steps as described in the Experiments section on page 53. Table 5 gives a summary of the purification data. The hemagglutinin activity was purified 72 fold from crude retentate. Total yields were low, probably due to the loss of some material by

FIGURE 14

Preparative Polyacrylamide Disc Gel Electrophoresis of Hemagglutinin Activity from Fraction B

Electrophoresis was carried out for 60 minutes at 8mA/gel. Gels were 12% acrylamide, pH 8.9. Six gels (8 x 85 mm.) were run simultaneously; one was stained with Coomassie Blue, scanned at 650 nm. The others were sliced, eluted, and assayed. The top of the gel is at the left of the scan.

_____ Absorbance at 650 nm. for Coomassie Blue stained gel.

o--------o Hemagglutinin titer vs. rat RBC by the capillary assay for preparative gels.

FIGURE 14

Preparative Polyacrylamide Disc Gel Electrophoresis of Hemagglutinin Activity from Fraction B

Electrophoresis was carried out for 60 minutes at 8mA/gel. Gels were 12% acrylamide, pH 8.9. Six gels (8 x 85 mm.) were run simultaneously; one was stained with Coomassie Blue, scanned at 650 nm. The others were sliced, eluted, and assayed. The top of the gel is at the left of the scan.

_____ Absorbance at 650 nm. for Coomassie Blue stained gel.

o--------o Hemagglutinin titer vs. rat RBC by the capillary assay for preparative gels.

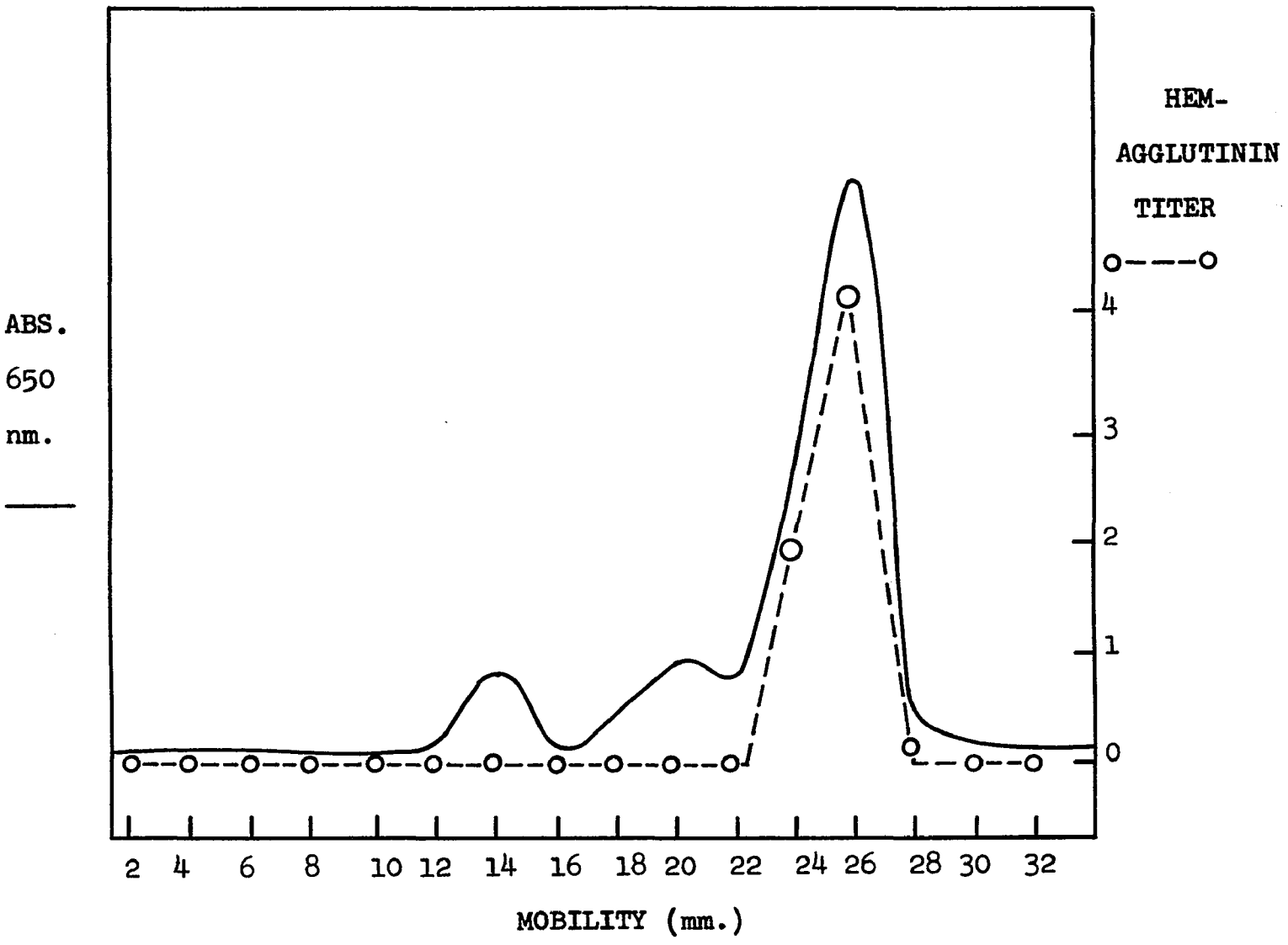

ABS.
650
nm.
HEM-
AGGLUTININ
TITER
o---o
4
3
2
1
0
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
MOBILITY (mm.)

TABLE 5

PURIFICATION SUMMARY OF AMPHITRITE HEMAGGLUTININ

Fraction	Protein (mg./ml.)	Hemagglutinin Titer (Units Activity)	Specific Activity (Units/mg.)	Purification (fold)
Retentate	2.28	16	7.02	1
Ammonium Sulfate Precipitate	1.16	32	27.5	4
Gel Filtration Fraction B	0.465	32	68.8	10
Disc Gel Electrophoresis Eluate	0.016	8	500	72

binding to the dextran of Sephadex gels, to incomplete recovery of material from gels, and to the fact that only one form of the hemagglutinin was purified.

Homogeneity and Chemical Composition of the Purified Hemagglutinin

The low molecular weight hemagglutinin prepared by preparative polyacrylamide disc gel electrophoresis was tested for homogeneity by analytical disc gel electrophoresis at pH 8.5, 8.0, 7.0, and 6.5. At each pH value only one sharp band was seen which stained for both protein and carbohydrate. Figure 15 shows the gel scan for a run at pH 7.0. On the basis of this evidence and the lack of nucleic acid contaminants, the hemagglutinin was considered pure, and is referred to subsequently as the purified hemagglutinin.

The purified hemagglutinin was found to be a glycoprotein containing 12.8% $\pm$ 0.4% carbohydrate as determined by the anthrone reagent method of Scott (53).

Amino Acid Composition. The results of amino acid analysis of the purified hemagglutinin are shown in Table 6. The values for serine, threonine and tyrosine were obtained by extrapolation to zero time of hydrolysis. Values for isoleucine and valine were from the 48-hour hydrolysis. Tryptophan was determined spectrophotometrically (see Methods). Assuming 3 histidines per molecule, and normalizing the values for the other amino acids appropriately, the total molecular weight for the hemagglutinin including 12.8% carbohydrate = 32,087. The last

97

FIGURE 15

Analytical Polyacrylamide Disc Gel Electrophoresis of Purified Hemagglutinin from Fraction B at pH 7

Electrophoresis was carried out for 60 minutes at 8 mA/gel. Gels were 12% acrylamide pH 7.0. The top of the gel is at the left of the scan.

A) Gels stained with Coomassie Blue for protein scanned at 650 nm.

B) Gel stained with periodic acid-Schiff reagent, scanned at 550 nm.

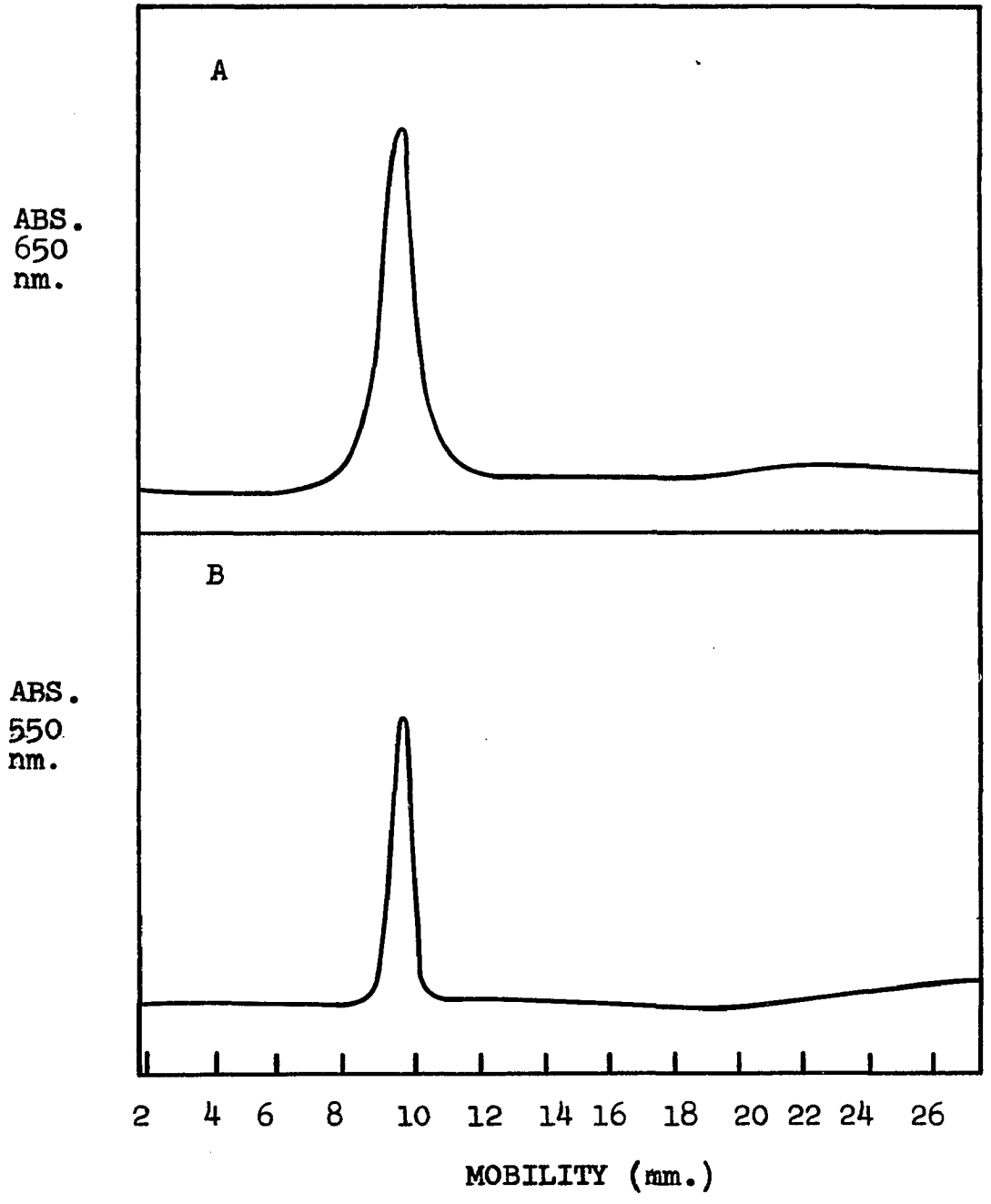

A
ABS.
650
nm.
B
ABS.
550
nm.
2 4 6 8 10 12 14 16 18 20 22 24 26
MOBILITY (mm.)

TABLE 6

AMINO ACID COMPOSITION
OF PURIFIED AMPHITRITE HEMAGGLUTININ

Amino Acid	Peak Area (Sample) / Peak Area (STD.)	No. Residues/ Molecule (HIS = 3)	No. Residues (Integer)
Lysine	0.290	3.72	4
Histidine	0.233	3.00	3
Arginine	0.471	6.06	6
Aspartic Acid	2.43	31.29	31
Threonine	1.02	13.14	13
Serine	0.879	11.31	11
Glutamic Acid	1.87	24.09	24
Proline	1.40	18.03	18
Glycine	1.72	22.11	22
Alanine	1.39	17.88	18
Cysteine	1.09	14.01	14
Valine	1.17	15.10	15
Methionine	0.247	3.18	3
Isoleucine	0.530	6.84	7
Leucine	0.927	11.94	12
Tyrosine	0.633	8.16	8
Phenylalanine	0.670	8.64	9
Tryptophan		3.8	4

Total Amino Acids = 222

Total Molecular Weight = 27,980

Total Molecular Weight Assuming 12.8% Carbohydrate = 32,087.

column in Table 6 gives the integer values of amino acid residues/molecule.

The amino acid composition of the Amphitrite hemagglutinin is marked by a large number of acidic (aspartic and glutamic) and neutral amino acids, and an unusually small number of basic residues. This result is consistent with the strong negative charge exhibited by the molecule on disc gel electrophoresis at pH 7 and above. The similarities of the amino acid composition of Amphitrite hemagglutinin to those of other invertebrate hemagglutinins is discussed below (see Discussion).

Two interesting features of the amino acid composition are the large number of proline and cysteine residues.

Carbohydrate Composition. The purified hemagglutinin was found to contain mannose (5.7%), galactose (3.7%), sialic acid (1.7%), and glucosamine (1.1%). Gas liquid chromatography of the purified hemagglutinin (hydrolysate) gave two peaks with retention times equal to those of the galactose and mannose standards. This identification was confirmed by co-injection of the TMS derivatives of hemagglutinin hydrolyzate plus galactose, and hemagglutinin hydrolyzate plus mannose. In each experiment the peak corresponding to the appropriate co-injected sugar was augmented. Analysis of amino sugars by the Beckman Model 118 amino acid analyser showed no trace of galactosamine or mannosamine.

Molecular Weight of Amphitrite Hemagglutinin by SDS Disc Gel Electrophoresis

The molecular weight of the purified hemagglutinin was determined by disc gel electrophoresis in the presence of sodium dodecyl sulfate. Figure 16 shows the plot of mobility vs. log molecular weight for five standard proteins and for the purified hemagglutinin. The molecular weight of the purified hemagglutinin by this method was 32,000. Two minor bands were seen, corresponding to molecular weights of 22,000 and approximately 5,000.

When SDS disc gel electrophoresis of the purified hemagglutinin was repeated after sample incubation in 1% mercaptoethanol, bands corresponding to molecular weights of 5,000 and 10,000 were seen (Figure 16). Both SDS disc gel electrophoresis experiments gave reproducible results within 3% deviation.

Analytical Ultracentrifugation

Sedimentation Equilibrium of Purified Hemagglutinin. The molecular weight of the purified hemagglutinin was obtained by the approach to equilibrium method according to equation 5 as shown:

$$M.W. = \frac{8.314 \times 10^7 \times 299}{(1-0.710) \times [10{,}000 \times (2/60)]^2} \cdot \frac{0.518}{6.243 \times .206} = 31{,}500$$

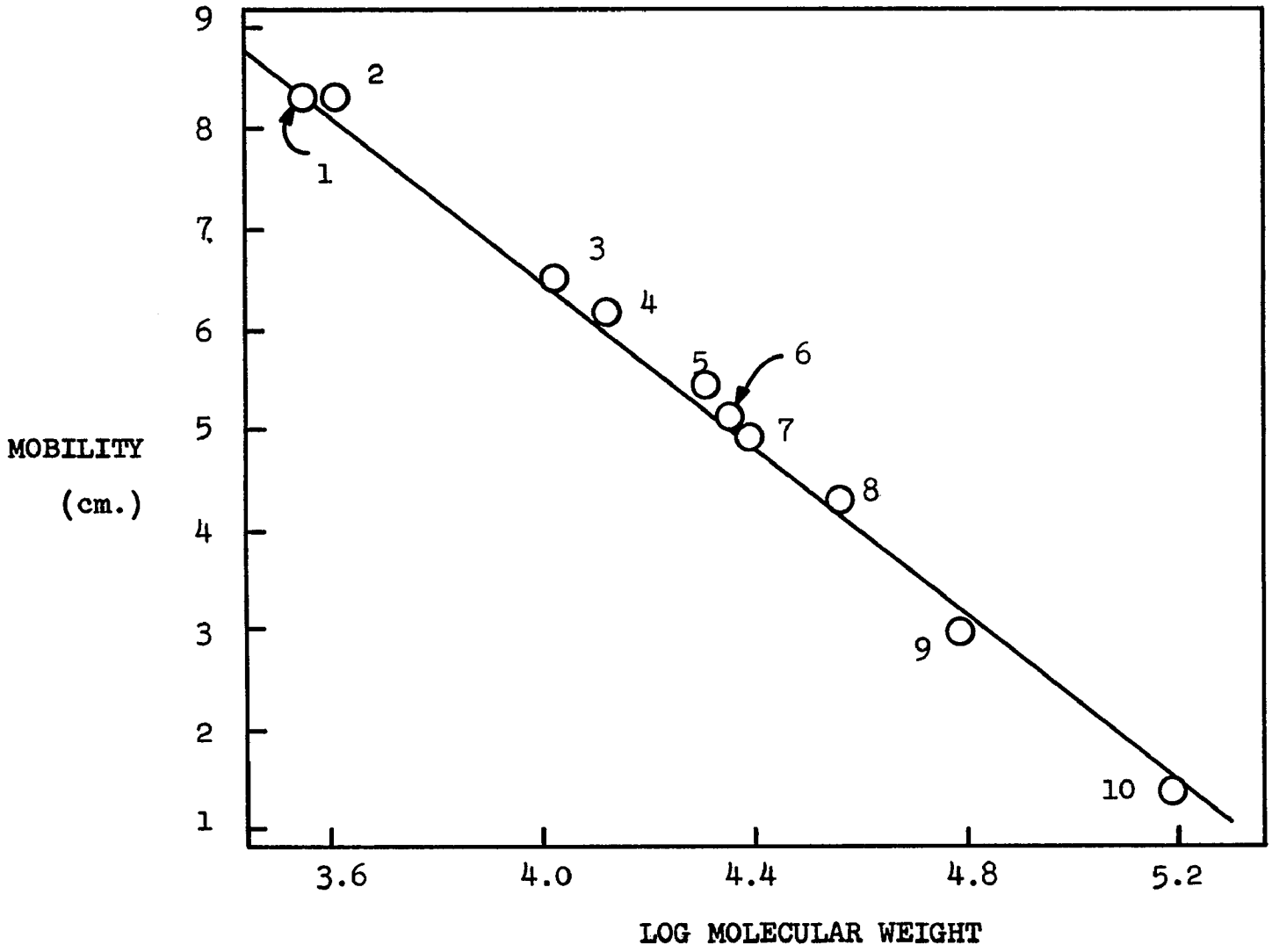
The molecular weight of 31,500 is in good agreement with the values from gel filtration (30,000), SDS gel electrophoresis (32,000) and amino acid analysis (32,087).

FIGURE 16

Sodium Dodecyl Sulfate Disc Gel Electrophoresis of Purified Hemagglutinin

Electrophoresis was carried out for 3 hours at 8 mA/gel. Gels were 5% acrylamide pH 7.0. Three gels were run: one of purified hemagglutinin, one of standard proteins, and one of purified hemagglutinin + standard proteins. Gels were stained with Coomassie Blue. Mobilities were measured in mm. from the top of the gel to the center of bands. The points on the curve and their molecular weights are as follows:

1) Hemagglutinin (minor band, SDS without mercaptoethanol [MCE]) MW = 5000.
2) Hemagglutinin (SDS plus MCE) MW = 5000.
3) Hemagglutinin (SDS plus MCE) MW = 10,000.
4) Ribonuclease MW = 12,700.
5) Hemagglutinin (minor band, SDS without MCE) MW = 22,000.
6) Chymotrypsinogen MW = 25,000.
7) Hemagglutinin (major band, SDS without MCE) MW = 32,000.
8) Ovalbumin MW = 45,000.
9) Bovine Serum Albumin MW = 65,000.
10) Aldolase MW = 158,000.


MOBILITY
(cm.)
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
10
3.6
4.0
4.4
4.8
5.2
LOG MOLECULAR WEIGHT

Sedimentation Velocity of Purified Hemagglutinin. Sedimentation velocity ultracentrifugation confirmed the homogeneity of the purified hemagglutinin. Only one sharp peak migrated across the cell and the plot of log distance vs. time was linear (Figure 17). The sedimentation coefficient calculated from this plot according to equation 7 was 2.6 S.

Sedimentation Velocity of High Molecular Weight Hemagglutinin Fraction A1. Sedimentation velocity ultracentrifugation of fraction A1 produced a broad non-homogeneous peak with sedimentation coefficients ranging from 5-8 (average sedimentation coefficient was 6.5).

Anti-Tumor Activity of Amphitrite and Nereis Extracts

Extracts of the marine polychaetous annelids Amphitrite ornata, and Nereis were found to possess anti-tumor activity against Ehrlich ascites tumor in mice. When extracts were ultrafiltered through UM-10 or PM-10 membranes, the retentates (10,000 daltons) of both organisms retained all anti-tumor activity. Ultrafiltrates were consistently inactive. A summary of the anti-tumor activity data for several preparations is presented in Table 7. Amphitrite retentates from several batches of worms worked up over a period of three years gave fairly consistent anti-tumor relative activities. The organisms were harvested at different seasons and were of varying sizes and ages. The anti-tumor activity was apparently independent of such variables.

FIGURE 17

Sedimentation Velocity of Purified Hemagglutinin

Plot of log distance of Schlieren peak from top of cell vs. time, to give the rate of sedimentation. Values of log X for each of 4 photographs were determined by:

$$\log X = \frac{\log [\text{distance from reference hole to peak}]}{2} + 5.72$$

where 2 = camera lens magnification factor, and 5.72 cm. is the distance from the center of rotation to the reference hole. Time (in seconds) from the start of the run for each photograph was calculated from odometer readings and rotor speed. The slope of the line (dlogX/dt) is 3.82×10^{-6}.

The insert shows a tracing of a photograph of the Schlieren peak after 5 hours from the start of the run. Phase plate angle was 30°. The exposure time was 15 seconds.

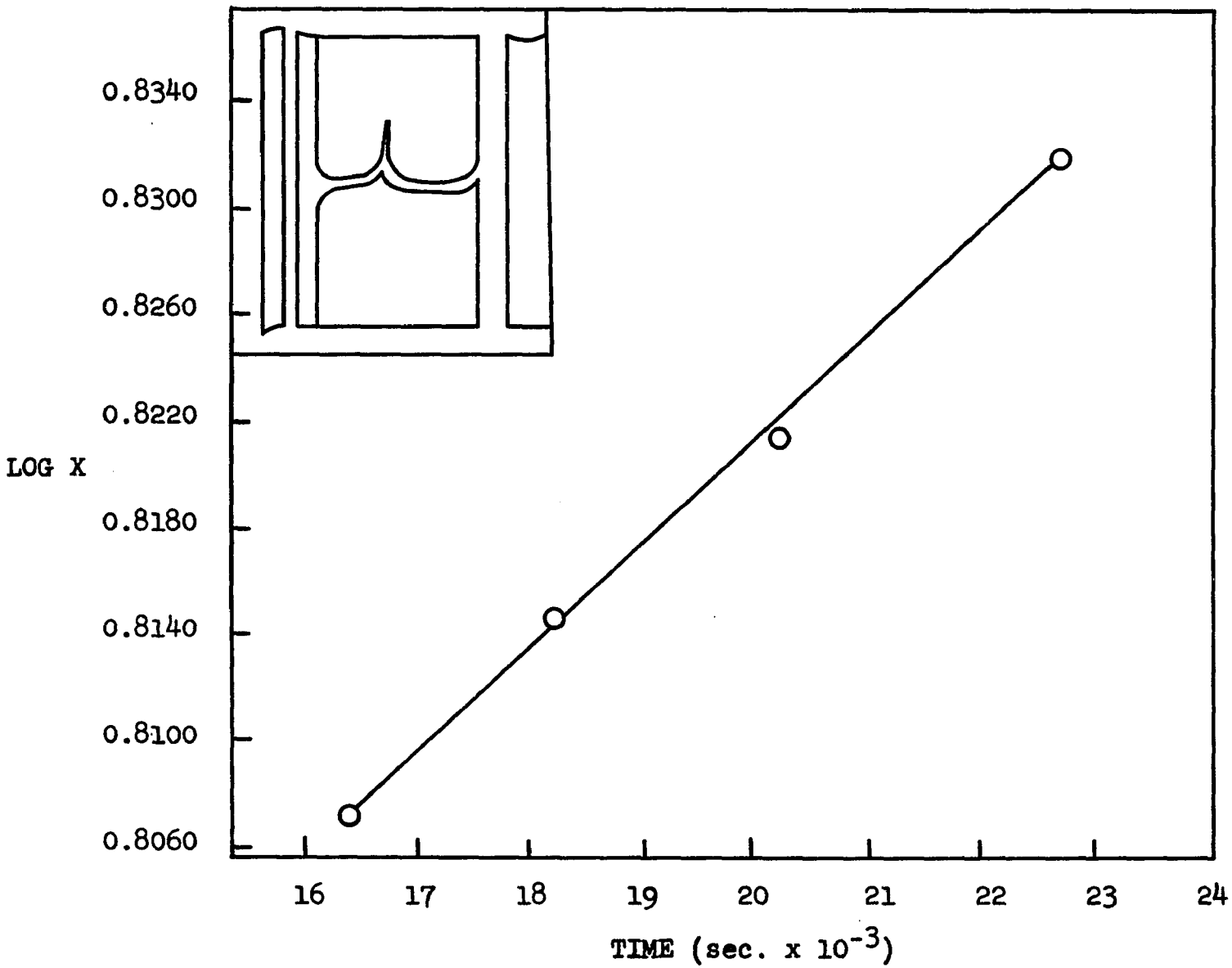

0.8340
0.8300
0.8260
0.8220
0.8180
0.8140
0.8100
0.8060
LOG X
16
17
18
19
20
21
22
23
24
TIME (sec. x 10^{-3})

TABLE 7

ANTI-TUMOR ACTIVITIES OF AMPHITRITE AND NEREIS PREPARATIONS

Sample	Sample Number	Dosage[1]	TPCV T/C[2]	% Act.[3]	Relative Act.[4]
Amphitrite Extract	101471	50	0.09	77.5	1.55
"	110471	50	0.14	65	1.30
"	110471	10	0.31	22.5	2.25
Amphitrite Retentate	020372	25	0.14	65	2.6
"	031472	25	0.13	67.5	2.7
"	061672	25	0.11	72.5	2.9
"	031273	25	0.12	70	2.8
"	091473	25	0.19	52.5	2.1
Nereis Retentate	110971	100	0.18	55	0.55
"	110371	10	0.29	27.5	2.75
"	102971	50	0.28	30	0.60
"	234	100		--TOXIC--	
"	234	50		--TOXIC--	
"	234	20	0.10	75	3.75
"	567	100		--TOXIC--	
"	567	50		--TOXIC--	
"	567	20	0.09	77.5	3.87

[1] Dosage in units of mg. sample per kg. body weight per day.
[2] TPCV = Tumor Packed Cell Volume, T/C = Test/Control.
[3] % Act. = Percent Anti-Tumor Activity = (0.04 - T/c) x 250
[4] Relative Act. = % Act./Dosage

Although Nereis retentates gave higher relative activities at low dosages, at higher dosages the material was toxic toward test animals. Amphitrite retentates tested at these higher dosages (50 and 100 mg./kg.) were not toxic.

The anti-tumor activity from Nereis was not affected by treatment with trypsin, pronase, DNAse or amylase (personal communication from C. S. Russell).

Anti-Tumor Activity of Gel Filtration Fractions

Fractions A, B, and C from gel filtration of Amphitrite retentate on Sephadex G-100 (see Figure 3), were tested for anti-tumor activity. The results for two separate experiments are shown in Table 8. Both fractions A and B were active, but the relative activities of the two fractions differed between the two experiments. This inconsistency in activity vs. molecular weight is similar to that found for the hemagglutinin activity.

Anti-Tumor Activity of Purified Hemagglutinin

In order to test the possibility of a correlation between the hemagglutinin and anti-tumor activities in Amphitrite extracts, the purified, homogeneous hemagglutinin was assayed for anti-tumor activity. The result is shown in Table 9. The purified hemagglutinin exhibited anti-tumor activity 14 times greater than crude extracts.

TABLE 8

ANTI-TUMOR ACTIVITIES OF GEL FILTRATION FRACTIONS OF AMPHITRITE EXTRACTS ON SEPHADEX G-100

Sample	Sample Number	Dosage	T/C	Relative Act.
Retentate	031273	25	.12	2.8
Insoluble Residue	031273	4	.49	0
Fraction A	031273	6	.26	5.8
Fraction B	031273	9	.10	8.3
Fraction C	031273	5	.42	0
Retentate	091473	25	.19	2.1
Fraction A	091473	5	.25	7.5
Fraction B	091473	10	.36	1.0

TABLE 9

ANTI-TUMOR ACTIVITY
OF PURIFIED AMPHITRITE HEMAGGLUTININ

Sample	Dosage	T/C	Relative Act.
Retentate	25	.23	1.7
Purified Hemagglutinin	2.5	.16	24

In Vitro Assay for Agglutination of Ehrlich Ascites Cells by Amphitrite Hemagglutinin

No evidence of agglutination of Ehrlich ascites tumor cells *in vitro* by either fraction of *Amphitrite* hemagglutinin was obtained. Figure 18 shows the plot of absorbance at 600 nm. (turbidity) vs. time of incubation for the purified hemagglutinin, and control suspensions. As is seen in the figure, Concanavalin A did agglutinate the tumor cells. This result was confirmed by microscopic examination, which showed large clumps of cells after 20 minutes incubation. The negative results obtained for purified *Amphitrite* hemagglutinin were also confirmed by microscopic examination which showed no discernible difference from saline controls. Similar negative results were obtained for the high molecular weight fraction A1.

Hemagglutinin Activity from Nereis Extracts

The data presented in this section was collected in collaboration with Wesley Chin. Hemagglutinin activity was found in extracts of the marine polychaetous annelid *Nereis*. When *Nereis* retentate was subjected to gel filtration on Sephadex G-100, the elution profile shown in Figure 19 was obtained. The molecular weights of the activity peaks obtained by calibration of the column were: >100,000 (void volume) for fraction 9; 52,000 for fraction 17; and 30,000 for fraction 20. These results are strikingly similar to the molecular weights of the *Amphitrite* hemagglutinin fractions. Assay

FIGURE 18

Agglutination of Ehrlich Ascites Cells in Vitro

Ehrlich ascites cells (2 x 10^6 cells/ml.) were incubated at 25°C with Amphitrite hemagglutinin and controls, and the absorbance at 600 nm. (turbidity) was measured at various times.

□———□ Saline

o----------o Purified hemagglutinin (1 mg./ml.)

△ — — — △ Concanavalin A (0.5 mg./ml.)

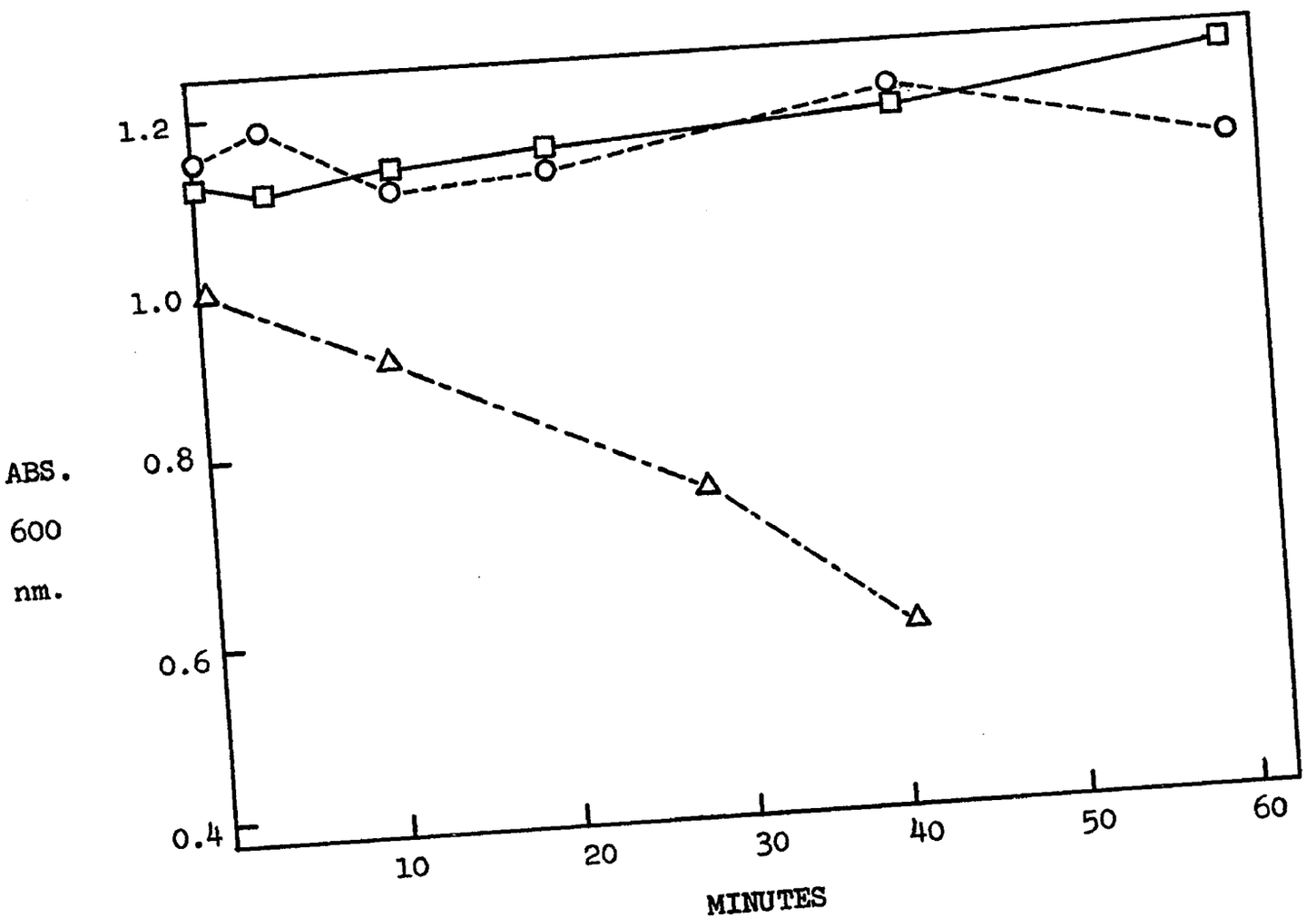

1.2
1.0
0.8
0.6
0.4
ABS.
600
nm.
10
20
30
40
50
60
MINUTES

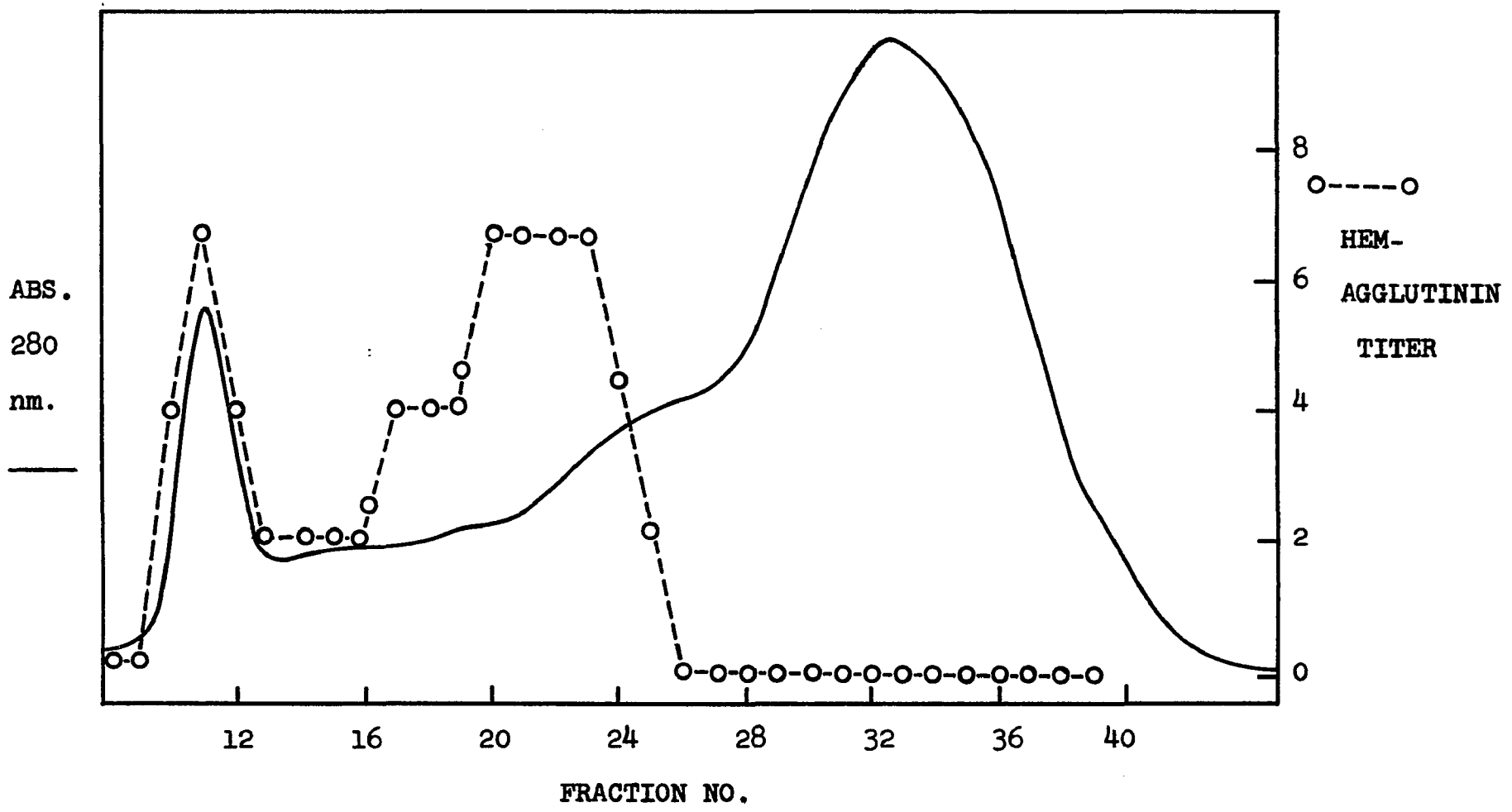
FIGURE 19

Gel Filtration of Nereis Retentate on Sephadex G-100

Column dimensions were 39 x 2.6 cm. The equilibration and elution buffer was saline. Sample size was 200 mg. of lyophilized Nereis retentate in 5 ml. saline (40 mg./ml.). The sample was clarified by centrifugation in the JA-20 rotor at 5°C, 20,000 RPM for 30 minutes prior to column application. Fractions of 6 ml. were collected. Flow rate was approximately 20 ml./hr.

_____ Absorbance at 280 nm.

o--------o Hemagglutinin titer vs. chicken RBC by the micro-titer well assay.


ABS.
280
nm.
8
6
4
2
0
HEM-
AGGLUTININ
TITER
12
16
20
24
28
32
36
40
FRACTION NO.

with chicken and rat RBC gave parallel results for all fractions.

In an attempt to localize the source of the hemagglutinin activity from Nereis, annelids were dissected, and the coelomic fluid was assayed. The Nereis coelomic fluid was considerably more active than extracts of the whole animal.

DISCUSSION

In the past decade, much progress has been made in understanding the molecular and cellular basis of the immune response. The detailed chemical structure of antibodies has been elucidated (63), and the theory of clonal selection (64,65) has been accepted as the cellular mechanism by which adaptive immunity functions. One area of immunology in which many questions have yet to be answered, is the evolution of the immune response. Up to the present time, humoral antigen-induced antibodies have been found in almost all vertebrate species tested, but are uniformly lacking in invertebrates. The consensus of investigators (66-68) is that invertebrates do not possess a complete vertebrate-type immune system. However, what many consider to be immune-like defense systems do exist in some invertebrates. Earthworms exhibit graft rejection (69), a tissue recognition reaction believed to be a primal immune function (70). An excellent review of other lesser known examples of immune-like behavior in invertebrates is presented by Hildemann (68).

One of the most promising developments in the field of immunological evolution has been the demonstration in a growing number of invertebrate species of humoral hemagglutinins, molecules which may represent the evolutionary precursors to antibodies (67). The hemagglutinin from oyster has been found to aid phagocytosis of foreign cells (71). Attempts to in-

crease the titer of hemagglutinin by previous inoculation with an antigen have repeatedly failed (68). There is no doubt that the invertebrate hemagglutinins are not the product of a true immune system, but the question remains as to where they belong in the evolution of immunity.

One approach to this problem taken by several investigators has been to study the physical and chemical properties and structures of purified invertebrate hemagglutinin molecules. This has been accomplished most extensively with the agglutinin from the horseshoe crab (Limulus polyphemus) or limulin (14, 45). To date, many invertebrate hemagglutinins have been discovered, but only a few have been purified to the extent permitting amino acid analysis. The hemagglutinin from Amphitrite ornata is the first hemagglutinin isolated from a polychaetous annelid, and is the only annelid hemagglutinin to be purified.

In order to place the present work in a comparative perspective, the results presented in this dissertation on Amphitrite ornata hemagglutinin will be reviewed and discussed in relation to the reported data for other invertebrate hemagglutinins.

Biological Properties

Table 10 summarizes the erythrocyte binding specificities, the chemical (saccharide) binding specificities (as determined by agglutination inhibition) and other biological acti-

TABLE 10

BIOLOGICAL PROPERTIES OF INVERTEBRATE HEMAGGLUTININS

Species (Phylum)	Erythrocyte Specificity	Saccharide Inhibition	Biological Activities	Ref.
Oyster (Mollusca)	Sheep, trout, rabbit, human		Aids phagocytosis (opsonin)	39, 40, 63
Snail (Mollusca)	Human group A	N-acetyl-D-galactosamine		13
Earthworm (Annelida)	Chicken horse, sheep, rabit		Hemolysin induced?	42
Sandworm, Amphitrite ornata (Annelida)	Rat chicken, rabbit, human	N-acetyl-D-galactosamine	Anti-tumor activity *in vivo*	
Horseshoe Crab (Arthropoda)	Horse rabbit, chicken, sheep	Sialic acid		14
Lobster (Arthropoda)	Mouse, horse, hamster, human	N-acetyl-D-galactosamine, sialic acid, glucosamine, N-acetyl-D-mannosamine		10
Crayfish (Arthropoda)	Chicken, rabbit		Agglutinates pseudomonas bacteria	12

TABLE 10
(continued)

Species (Phylum)	Erythrocyte Specificity	Saccharide Inhibition	Biological Activities	Ref.
Blue Crab (Arthropoda)	Rabbit human, chicken		Aids phagocytosis (opsonin)	79

vities of a number of invertebrate hemagglutinins. Although each species demonstrates a certain specificity for some vertebrate RBC, no clear pattern is seen in erythrocyte specificity between species or phyla. The Amphitrite hemagglutinin shows highest activity toward rat RBC, followed by rabbit, chicken, and sheep cells. This is only roughly parallel to the specificity of the most closely related species, the earthworm (Lumbricus terrestris), which gave highest titers for chicken, followed by rabbit, horse and sheep RBC. Of the invertebrate hemagglutinins which agglutinate human RBC, only the one from the snail (Helix pomatia) shows definite blood group (group A) specificity. This is in contrast to the plant lectins, many of which are group A specific. The Amphitrite hemagglutinin shows higher titers with group A erythrocytes, but is also active toward groups B and O.

Studies of saccharide inhibition of invertebrate hemagglutinins have been done on only a few species. Sialic acid is a potent inhibitor of limulin. Hall and Rowlands (10) found a number of inhibitors of lobster hemagglutinin, including N-acetyl-D-galactosamine and sialic acid. The Amphitrite hemagglutinin is inhibited only by N-acetyl-D-galactosamine when assayed with rat RBC. The snail hemagglutinin is also inhibited by N-acetyl-D-galactosamine; this sugar is the terminal residue of the group A immunodeterminant. The paucity of inhibition data for other species makes any generalization

about the chemical binding specificity of invertebrate hemagglutinins impossible.

Plant lectins possess biological activities other than agglutination of RBC. The search for other biological activities of invertebrate hemagglutinins has concentrated on immunological type behavior (such as defense against invasion). The oyster hemagglutinin has been found to act as an opsonin, aiding phagocytosis and clearance of foreign cells (71). Crayfish and sea hare hemagglutinins will agglutinate pseudomonas bacteria. The hemagglutinin from Amphitrite ornata is the only invertebrate hemagglutinin reported to possess anti-tumor activity in vivo. Although several plant lectins agglutinate tumor cells in vitro, they are too toxic for in vivo tests.

Physical and Chemical Properties

Plant lectins have been found to require Ca^{++} for maximum activity and stability. Calcium ion dependence is also found in most of the invertebrate hemagglutinins (those from Molluscs and Crustaceans), a characteristic distinguishing them from vertebrate antibodies which lack such a requirement. However, the Amphitrite hemagglutinin (as well as the ascidian hemagglutinin) are independent of Ca^{++} ion concentration. These two molecules also exhibit greater heat stability than is seen for the other hemagglutinins. Table 11 summarizes the physical and chemical data for invertebrate hemagglutinins.

TABLE 11

PHYSICAL - CHEMICAL PROPERTIES

Species (Phylum)	Heat Stability[1]	pH Stability	Chemical Stability	Ca^{++} Ion Dependence	Ref.
Oyster (Mollusca)	Stable at 56°, labile at 70°			Ca^{++} dependent, EDTA sensitive	39,40
Sea Hare (Mollusca)	Stable at 60°, labile at 70°		Trypsin and pronase insensitive; phenol and TCA sensitive		11
Sandworm Amphitrite ornata (Annelida)	Stable at 70°, labile at 85°	Stable at pH 5-10, irreversibly inactive below pH 4	Trypsin and pronase insensitive; phenol sensitive	Ca^{++} independent, EDTA insensitive	
Crayfish (Arthropoda)	Stable at 50°, labile at 70°	Active at pH 5-10, inactive at 1.6, 11.	Trypsin and pronase insensitive; phenol and TCA sensitive		12
Lobster (Arthropoda)	Labile at 56°	Irreversibly inactive below pH 5	Trypsin sensitive	Ca^{++} dependent, EDTA sensitive	10
Horseshoe Crab (Arthropoda)	Labile at 65%			Ca^{++} dependent, EDTA sensitive	14

TABLE 11
(continued)

Species (Phylum)	Heat Stability[1]	pH Stability	Chemical Stability	Ca^{++} Ion Dependence	Ref.
Blue Crab (Arthropoda)	Stable at 50°, labile at 60°	Stable at pH 7-9	Trypsin and pronase insensitive; phenol and TCA sensitive	Ca^{++} dependent	79
Starfish (Echinoderm)	Labile at 65°			EDTA sensitive	37
Ascidian (Chordata)	Stable at 100°C		Trypsin insensitive, periodate sensitive	Ca^{++} independent; EDTA insensitive	43

[1] Heated for 30 minutes at each temperature.

The resistance to proteolytic enzyme digestion shown by the Amphitrite hemagglutinin is shared by the hemagglutinins from ascidian, crayfish and sea hare, although lobster hemagglutinin is sensitive to trypsin. Fuke and Sugai (43) concluded from their data on the heat stability and trypsin insensitivity of ascidian hemagglutinin that the molecule was a polysaccharide, but all other invertebrate hemagglutinins, including Amphitrite, are proteinaceous. Protection against digestion may be provided by the carbohydrate moiety, or be due to the small number of basic amino acid residues (see amino acid composition Table 13) which are susceptible to attack by trypsin, or be due to some property of the protein conformation.

The pH activity profile of Amphitrite hemagglutinin resembles that of the lobster hemagglutinin (the only other invertebrate hemagglutinin studied with respect to pH) which also shows irreversible loss of activity at low pH. Both molecules contain an unusually large number of aspartic and glutamic acid residues (see below), and the Amphitrite molecule is anionic at neutral pH (from disc gel electrophoresis). Partial protonation of some of these many carboxyl groups may reduce activity by changes in conformation, or by altering the characteristics of the binding site. At low pH (below 4) further protonation of the carboxyl groups may result in irreversible alteration of the protein structure, destroying agglutinating activity. Therefore, at least some of the carboxyl groups

in the Amphitrite hemagglutinin molecule are probably necessary for the expression of activity and for molecular stability.

Chemical Structure and Composition

Analysis of the molecular weight and size of many of the invertebrate hemagglutinins is at a rudimentary stage at the present time. The only general picture that emerges from the data illustrated in Table 12 is that the invertebrate hemagglutinins tend to form large aggregates of non-covalently linked subunits. The sedimentation coefficients of the aggregates range from 5.6 S to 33.4 S, with reported molecular weights of 100,00 to 460,000. Subunit molecular weights vary from 19,000 to 68,500. No phylogenetic pattern is seen in these species variations in molecular size. Even values for the molecular weight of the same hemagglutinin (Limulus polyphemus) from different laboratories are inconsistent. It is possible that discrepancies may be due to the use of different methods for molecular weight determination. Of the commonly used methods, gel filtration and SDS gel electrophoresis have been shown to be often inaccurate for glycoproteins (72). Sedimentation velocity requires precise measurements of the diffusion constant, and the partial specific volume of the protein, values not always available. Sedimentation equilibrium is the preferred method for glycoprotein molecular weight determination.

For the Amphitrite hemagglutinin, several methods were

TABLE 12

CHEMICAL STRUCTURE AND COMPOSITION

Species (Phylum)	Aggregate Size or Molecular Wt.	Subunit Molecular Wt.	Carbohydrate Composition	Ref.
Oyster (Mollusca)	33.4 S	20,000	8.8% Mannose (3.7%) Glucosamine (2.7%) Galactose (1.5%) Fucose (0.6%) Sialic Acid (0.2%)	39, 40
Snail (Mollusca)	5.28 S 100,000		7% Galactose (4%) Mannose (3%)	13
Sea Hare (Mollusca)	18.5 S 31 S			11
Sandworm Amphitrite ornata (Annelida)	6.5 S	32,000 2.6 S	12.8% Mannose (5.7%) Galactose (3.7%) Glucosamine (1.1%) Sialic Acid (1.7%)	
Lobster (Arthropoda)	19 S 11 S	55,000 55,000		10
Crayfish (Arthropoda)	150,000			12
Horseshoe Crab (Arthropoda)	12.6 S	25,000		37
	13.5 S 390,000 - 408,000	22,500		14

TABLE 12
(continued)

Species (Phylum)	Aggregate Size or Molecular Wt.	Subunit Molecular Wt.	Carbohydrate Composition	Ref.
Horseshoe Crab (Arthropoda) (continued)	13.9 S 335,000	19,000 1.9 S	3.5% Glucosamine (1.8%) Mannose (1.8%)	45
	460,000	22,000	4% Glucosamine	80
Spiny Lobster (Arthropoda)	400,000 10.3 S	68,500	4.6% Mannose (2.9%) Glucosamine (0.8%) Galactose (0.6%) Sialic Acid (0.2%) Fucose (0.1%)	40, 75
Starfish (Echinoderm)	6.5 S	30,000 major 18,000 minor		37

used to establish the molecular weight of the purified hemagglutinin. Gel filtration gave an approximate value of 30,000, while SDS gel electrophoresis gave 32,000. Close agreement was obtained between these values and that from approach to equilibrium sedimentation: 31,500. Furthermore, amino acid and carbohydrate analysis gave the value 32,087 assuming 3 Histidine residues/molecule.

The results of the ammonium sulfate and 6M urea experiments on the interconversion of the high molecular weight hemagglutinin in fraction A1 to the low molecular weight hemagglutinin in fraction B confirm the aggregate nature of the Amphitrite hemagglutinin. The data presented do not, however, prove that the active high molecular weight material is composed exclusively of a number of non-covalently bound subunits (of molecular weight = 32,000), as is the case for the hemagglutinin from Limulus polyphemus. An alternate possibility is that the active hemagglutinin of fraction B (molecular weight = 32,000) is non-covalently linked to some large inactive "carrier" molecule. The lack of any observed re-aggregation of the activity from low molecular weight (B) to high molecular weight A1 supports the latter hypothesis. The Limulus polyphemus hemagglutinin aggregation is readily reversible. Although the existence of large inactive carrier molecules is not discussed in the literature on other invertebrate

hemagglutinins, this possible explanation for the results cannot be excluded.

The starfish hemagglutinin (from an Echinoderm) is the most similar to the Amphitrite hemagglutinin in terms of aggregate and subunit size.

The only correlation of invertebrate hemagglutinin and vertebrate immunoglobulins with respect to molecular size is the similarity in molecular weight of the vertebrate light chain (25,000) and the subunit of Limulus polyphemus (20 - 25,000). However, Fernandez-Moran et al. (73) have shown that Limulus polyphemus hemagglutinin is composed of an octamer arranged in an octagon, as opposed to the more complex quarternary structure of antibodies (two light and two heavy chains).

The carbohydrate composition of vertebrate antibodies varies from 3-12% and consists of the sugars mannose, galactose, glucosamine, fucose and sialic acid. The same sugar residues are found in the invertebrate hemagglutinins. The oyster and lobster hemagglutinins contain all five of these sugars, and the Amphitrite hemagglutinin contains all but fucose. Fucose may in fact be part of the carbohydrate moiety of Amphitrite hemagglutinin, but in an undetectably small concentration. The function of carbohydrate in hemagglutinins and

antibodies is unknown; Lotan et al. (74) have shown that the lectin from soy bean retains activity after destruction of the carbohydrate portion by periodate oxidation.

Amino Acid Composition

The amino acid composition of the purified Amphitrite hemagglutinin is quite similar to those of three other purified invertebrate hemagglutinins. Table 13 shows a comparison of the order of prevalence of amino acid residues in the hemagglutinins from horseshoe crab, oyster, and snail with the Amphitrite hemagglutinin. In all cases aspartic acid or glutamic acid are the most prevalent residues. The neutral residues glycine, alanine, and valine, and the hydroxy acids serine and threonine are prevalent in all four molecules to similar extents. With the exception of histidine in oyster hemagglutinin, the basic amino acids are the least prevalent in all four molecules. The Amphitrite hemagglutinin appears to be most closely related to oyster hemagglutinin with respect to amino acid composition. Sequence analysis of these four invertebrate hemagglutinins would provide more useful information regarding the structural similarities and differences between these molecules.

A theory of immunological evolution recently proposed by Acton and Weinheimer (75), suggests that invertebrate hemagglutinins are primitive receptor molecules, whose function is the recognition of foreign cells. These authors postulate

TABLE 13

ORDER OF PREVALENCE OF AMINO ACIDS
IN FOUR PURIFIED INVERTEBRATE HEMAGGLUTININS

Amino Acid	Oyster (39)	Horse-Shoe Crab (14)	Snail (13)	Amphi-Trite	
Aspartic acid	1	2	1	1	Most Prevalent
Glutamic acid	3	1	3	2	
Glycine	4	4	10	3	
Proline	-	11	5	4	
Alanine	5	10	8	4	
Valine	10	6	4	5	
Cysteine	7	14	12	5	
Threonine	6	9	7	6	
Leucine	5	3	6	7	
Serine	9	5	2	8	
Phenylalanine	11	11	13	9	
Tyrosine	12	12	10	10	
Isoleucine	9	9	7	11	
Arginine	8	13	7	12	
Lysine	14	6	9	13	
Tryptophan	13	13	11	13	
Methionine	13	15	14	14	
Histidine	2	8	15	14	Least Prevalent

that a precursor gene coding for a molecule of approximately 20,000 daltons underwent duplication, translocation and mutation to give genes for the larger size hemagglutinin subunits, and for the lysins, bactericidins, and clotting factors also found in the hemolymph of many invertebrates.

In his most recent discussion of invertebrate precursors to immune responses, Burnet (76) reports that Tyson has proposed that the invertebrate hemagglutinins may provide recognition of foreignness, and that these molecules may be associated with, and/or produced by the circulating hemocytes (coelomocytes), a situation roughly analogous to the vertebrate immune system. Although Baskin (77) has recently observed the presence of phagocytotic coelomocytes in Amphitrite, there is no evidence at present regarding the role of the Amphitrite hemagglutinin as a possible opsonin, aiding phagocytosis in vivo.

The traditional view that adaptive immunity evolved as a system for defense against infection has been challenged in recent years by the concept of immune surveillance. First proposed in 1959 by Lewis Thomas (78), this theory proposes that the primary function of the immune system was to identify and destroy spontaneous neoplastic cells arising within the organism. Evidence for this concept comes from clinical observations of a correlation between immunological deficiency and cancer (70) and the discovery of tumor specific antigens on transformed cell surfaces.

> The thesis is that when aberrant cells with proliferative potential arise in the body they will carry new antigenic determinants on their cell surface. . .a thymus dependent immunological response will be initiated which eventually eliminates the aberrant cells. . . (70).

The data presented in this thesis is relevant to the theory of immunological surveillance in that a hemagglutinin from an invertebrate, a molecule which may represent a precursor to modern antibodies, or belong to a primitive immune-like system, has been shown to possess anti-tumor activity. It is not known whether the Amphitrite hemagglutinin is the functional agent of an evolutionary primitive immunological surveillance system in annelids. However, in light of this theory, the existence of anti-tumor and hemagglutinin activities on the same molecule may be of evolutionary significance. More definite conclusions await the investigation of other invertebrate hemagglutinins for anti-tumor activity, a course of research which this work hopes to stimulate.

The biochemical mechanism of cell agglutination involves the binding of macromolecules to the surface of cells, ultimately forming a lattice. The mechanism of the anti-tumor activity of invertebrate extracts is unknown. With the discovery of homogeneity between the hemagglutinin and anti-tumor activities of Amphitrite ornata extracts, it appears likely that the Amphitrite anti-tumor activity may involve the tumor cell surface. This is reinforced by the finding of increased

agglutination of transformed cells by lectins, a definite cell surface phenomenon. However, the Amphitrite anti-tumor agent apparently does not act by agglutination of tumor cells. No in vitro evidence of ascites cell agglutination by the Amphitrite hemagglutinin was found. Although the anti-tumor activity of Amphitrite is therefore not due to simple agglutination of the Ehrlich ascites cells (as is the case for the lectin Concanavalin A), a cell surface reaction mechanism cannot be ruled out. For example, the well-known mitogenic affect of some lectins on lymphocytes might apply to the Amphitrite hemagglutinin. A stimulation of mouse lymphocyte multiplication might lead to enhanced host defenses against the tumor.

It is hoped that further research on the relationship between the hemagglutinin and anti-tumor activities found in invertebrate extracts will be stimulated by the present work. Of particular interest would be the anti-tumor assay of the purified and well-characterized hemagglutinin from Limulus polyphemus, an organism known to possess anti-tumor activity (32). The crucial work required for further analysis of the evolutionary relationships, if any, between invertebrate hemagglutinins and vertebrate immunoglobulins is sequence analysis of several purified hemagglutinins including that from Amphitrite, which may reveal homologous regions. If constant and variable domain regions are found in the amino acid sequences of these proteins, then Burnet's precursor hypothesis

would be strongly supported. For the Amphitrite hemagglutinin, sequence analysis should be followed by study of the active site chemistry, to determine if the anti-tumor and hemagglutinin active sites are identical or different.

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