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Isolation and characterization of hemagglutinins from *Nereis virens*: A polychaetous annelid

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ISOLATION AND CHARACTERIZATION OF HEMAGGLUTININS FROM *NEREIS VIRENS*: A POLYCHAETOUS ANNELID

BY

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Chair of Examining Committee

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Abstract

ISOLATION AND CHARACTERIZATION OF HEMAGGLUTININS FROM NEREIS VIRENS : A POLYCHAETOUS ANNELID

BY

PI-SHIANG LAI

Advisor : Professor Charlotte S. Russell

Coleomic fluid from Nereis virens was fractionated into low molecular weight (LMW) and high molecular weight (HMW) agglutinins from the supernatant and a hemagglutinin lipid extract (CM) of the particulate matter (mainly coelomocytes).

LMW was shown to consist of four (I-IV) heat-sensitive and Ca^{++}/Mg^{++}-independent glycoproteins with specific erythrocyte (RBC) and inhibition profiles and MW 20, 20, 19, 24 kd respectively. All four are inhibited by fetuin (type III) ≥ bovine submaxillary mucin (BSM) > thyroglobulin (TG) > yeast invertase ≥ mannan. Hog gastric mucin (HGM) inhibits only LMW III and LMW IV; ovine submaxillary mucin (OSM) only LMW I and LMW II and desialylated OSM (DsOSM) only

LMW I, LMW II and LMW IV. The only monosaccharide inhibitor for all four is mannose-1-phosphate (cyclohexylammonium salt). Immunoelectrophoresis of LMW I-IV shows precipitin lines which coincide with activity and protein and carbohydrate staining. LMW I was shown to be cationic, LMW II, uncharged and LMW III & IV anionic at pH 8.3. LMW protects mice from proliferation of Ehrlich ascites tumor cells (EATC). LMW causes swelling of EATC within two hours and ruptures them after two hours. This result indicates that the selective permeability of the cell membrane is destroyed by LMW.

HMW was shown to be a lipid-associated sulfated proteoglycan. It is periodate- and protease-resistant and Ca^{++}/Mg^{++}-independent with strong hemagglutination activity against rabbit and rat RBC. Activity against rat RBC is inhibited by fetuin (type III) > BSM > DsOSM > OSM, but not by a wide variety monosaccharides including sialic acid. In immunoelectrophoresis (at pH 8.3), (a) HMW gives two precipitin lines which coincide with regions of activity, which also stain for lipid, sugar and protein. (b) Heated HMW (HHMW) does not give any precipitin line but the region of activity is the same as that of HMW

and stains for lipid, sugar and protein. (c) Delipidated HHMW (DL-HHMW) also does not give any precipitin line but the region of activity is the same as that of HMW and stains for sugar and protein. HMW gives two precipitin lines with antiserum to coelomic fluid but HHMW does not. DL-HHMW contains protein (12%), hexose (30%), hexuronate (9%), hexosamine (8%), glycosaminoglycan (GAG, at least 22%) and sulfate (7%). Sialic acid, N-sulfate, phosphorus and pentose are absent. Hexuronic acid and hexosamine are present in equal amounts. With dimethylmethylene blue in guanidinium chloride, DL-HHMW gives a metachromatic reaction. The infrared (IR and FTIR) spectrum of DL-HHMW shows a hexose-6-sulfate ester [1243 cm^{-1} (sulfate), 813 cm^{-1}(equatorial-6-sulfate)] and amide [1661 cm^{-1} (amide I) and 1536 cm^{-1} (amide II), protein moiety as well as N-acylhexosamine]. These results suggest that DL-HHMW is a glycosaminoglycan. The depolymerization enzymes, chondroitinase ABC, keratanase and heparinase II, do not change the activity and GAG content of DL-HHMW. Either the protein is protecting GAG or this is a new class of GAG. HMW delays the growth of EATC in mice and causes

agglutination of EATC in vitro.

CM is heat-insensitive, Ca^{++}/Mg^{++}-dependent and agglutinated rat > rabbit > green monkey > rhesus monkey = human (O) = guinea pig >> chicken RBC. Rat RBC agglutination was inhibited by BSM > HGM ≥ fetuin (type III). In immunoelectrophoresis (at pH 8.3), CM does not give any precipitin line and the region of activity most of which stained with Sudan Black B extends along the length of the gel. CM was further fractionated into acetone-insoluble (AI) and acetone-soluble (AS) fractions by acetone-10% $MgCl_2$. Mannan inhibits AS but not AI. The lipid mixtures were resolved by 2D TLC on silica and studied with group specific reagents and IR. One of the two most active spots from AI contains phosphate, $-NH_2$, fatty acid ester and vicinal-OH. It migrates like DOPE. The vicinal-OH may be on a fatty acid side chain. The other spot from AI migrates like DOPI, contains phosphate, vicinal-OH and long chain fatty acid ester and gives a positive choline test (possibly due to double bonds). The most active spot from AS appears to be a long chain amide. CM retards proliferation of EATC in mice. In vitro CM causes swelling of EATC

within 2 hrs and rupturing after 2 hrs.

Contradictory reports in the literature about the hemagglutinating activity of DOPE are partially-explained. While DOPE itself is not an agglutinin, a dry-heated sample of DOPE is powerful one.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

AI : acetone-insoluble faction

AS : acetone-soluble fraction

BSA : bovine serum albumin

BSM : bovine submaxillary mucin

$Ca(OAc)_2$: calcium acetate

CF : coelomic fluid

CM : chloroform-methanol extract

EATC : Ehrlich ascites tumor cells

d : dalton

DEAE : diethylaminoethyl cellulose

DEPE : dielaidoylphosphatidylethanolamine

DL-HHMW : delipidated & heated high molecular weight sample

dH_2O : deionized water

DLNPE : dilinolenoylphosphatidylethanolamine

DLPE : dilinoleoylphosphatidylethanolamine

DMB : 1,9-dimethylmethylene blue

DMSO : dimethylsulfoxide

DOPA : dioleoylphosphatidic acid

DOPC : dioleoylphosphatidycholine

DOPE : dioleoylphosphatidylethanolamine

DOPS : dioleoylphosphatidylserine

DsOSM : desialylated ovine submaxillary mucin

DSPE : distearoylphosphatidylethanolamine

2D TLC : two dimensional thin layer chromatography

EGTA : ethylene-bis-(beta-aminoethylether)-NN' tetraacetic acid

FTIR : Fourier transform infrared spectrum

GAG : glycosaminoglycan

HEPES : N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid

HGM : hog gastric mucin

HHMW : heated high molecular weight sample

HMW : high molecular weight fraction

IR : infrared spectrum

kd : kilodaltons

LMW : low molecular weight fraction

LPC : lysophosphatidylcholine

LPE : lysophosphatidylethanolamine

LPI : lysophosphatidylinositol

MOPE : monooleoylphosphatidylethanolamine

MW : molecular weight

NaOAc : sodium acetate

OSM : ovine submaxillary mucin

PBS : phosphate buffered saline

PBS-N_3 : phosphate buffered saline -N_3

PBS-N_3-T : phosphate buffered saline-N_3-0.001% Triton X-100

PBS-T : phosphate buffered saline-0.001% Triton X-100

PE : phosphatidylethanolamine

PS : phosphatidylserine

PVPP : polyvinylpolypyrrolidone

RBC : erythrocytes

Sp : sphingomyelin

TCA : trichloroacetic acid

TG : thyroglobulin

TLC : thin-layer chromatography

TPCK : L-1-Tosylamide-2-phenylethylchloromethyl ketone

Tris : tris(hydroxymethyl)aminomethane

YI : yeast invertase

INTRODUCTION

Lectins

There are numerous activities mediated primarily at the surface taking place in the living cell. For example, intercellular communication, the regulation of cell growth and differentiation, the immune response, and perhaps transformation. These activities are mediated in particular by the carbohydrate moieties that stud the cell surface. The recognition that this is so, and increasing understanding of the structure and function of the cell surface are due to a class of proteins called lectins (1).

The term ' lectin ' (Latin, legere , to select or pick out) was first applied by Boyd and Shapleigh (1954) (2) to seed extracts of plants, which could agglutinate and distinguish among human blood groups. In 1888, Stillmark (3) discovered that extracts of castor bean seeds could agglutinate animal erythrocytes. Materials which could clump red blood cells, were then called hemagglutinins. Because they were first isolated from plants, they came to be known as phytohemagglutinins. Lectin is a better name because these

materials are found in sources other than plants. Hemagglutinins had been found predominantly in the seeds of plants, in particular those of the legumes, but also in other parts of plants , such as roots, leaves and bark (3). In addition, agglutinins have been found in bacteria, fungi, lichens, fish roe, snails, fish, insects, invertebrates and mammals (4). Hemagglutinins may be membrane bound or free in solution. Some lectins are known to agglutinate not only red blood cells but also other kinds of cells such as lymphocytes, fibroblasts (connective-tissue precursors), spermatozoa, bacteria and fungi (1).

Stein and Cooper (5) classified agglutinins into three categories -- antibodies, lectins and other receptor-specific substances. (a) Antibodies are of immune origin, are produced by lymphocytes or plasma cells, and may be elicited as a response to antigen. They are glycoproteins with highly specific molecular structure composed of multiples of a basic subunit consisting of one heavy and one light polypeptide chain. They have a wide range of binding specificities, with two or more binding sites per molecule. They have been found

only in vertebrates. (b) Lectins are glycoproteins or proteins, are probably of nonimmune origin, and are usually present as constitutent molecules, i.e., are not generally increased by presence of inducing substances. Their binding specificities are confined to carbohydrates and, as a general rule, have two or more binding sites per molecule. They occur ubiquitously in viruses, bacteria, plants and animals. (c) Other receptor-specific substances are those agglutinins excluded from the other two categories by their relatively strict definition. These include the inducible, non-immunoglobulin heteroagglutinins of vertebrates and invertebrates and other lectin-like but possibly nonprotein (carbohydrate or lipid), agglutinins in invertebrates.

Rogers and Loveless (6) found that "hemagglutinins" from extracts of six selected species of brown algae were removed by treatment of the extract with insoluble polyvinylpolypyrrolidone (PVPP) and could not be removed from the PVPP by elution with medium containing Tween 80. Protein-rich extract did not contain "agglutinins" for human erythrocytes but purified polyphenols from

these algae produced powerful "agglutination". They thought that the substances, which caused the hemagglutination of human erythrocytes, were polyphenols and quite different from the known lectin-type hemagglutinins.

Invertebrate lectins

Invertebrates do not possess the adaptive immune systems of the vertebrates. Therefore, the protective mechanisms of invertebrates are the less immune-like phenomena of lysis and agglutination by components in coelomic fluid and phagocytosis by coelomocytes. The recognition of "self" and "non self" by the latter is poorly understood although this discriminatory property of coelomocytes is well documented (7). It has been proposed that agglutinins participitate in the defense mechanism of invertebrates by aiding phagocytosis (8,9).

Yeaton (10,11) has reviewed the immunological significance of invertebrate lectins. Invertebrate lectins may play a role in immune systems, perhaps as (a) a sugar configuration-specific antibody-like

molecule which can agglutinate bacteria, viruses, fungi, sperm and other parasites; (b) an opsonin which enhances hemocyte phagocytosis of foreign particles; (c) a perivitelline protector to maintain sterility inside eggs; (d) a histocompatibility system to recognize allogeneic and xenogeneic differences. Investigation of annelid coelomocytes point to their participation in some form of specific "self" recognition and cellular immunity (7,12). Cooper and coworkers (13) found that when Lumbricus coelomocytes were incubated in vitro with vertebrate erythrocytes, rosettes of both the secretory (a large number erythrocytes adhered to the surface of the coelomocyte surface, giving it the appearance of a grape-like cluster) and E-type (only a single row of erythrocytes surrounded the coelomocytes) were formed around the coelomocytes. The highest percentages of secretory rosettes were formed using rabbit erythrocytes. Coelomocytes, collected from Lumbricus 24 hrs after rabbit erythrocyte-injection, exhibited significantly decreased numbers of secretory rosettes when cultured with erythrocytes from the original donor rabbit. However, culturing the same

coelomocytes with erythrocytes from a different rabbit showed nearly normal numbers of rosettes. It suggests that coelomocytes in Lumbricus are involved in some form of specific immune response.

Among the invertebrate lectins which have been characterized are those from the American lobster (Homarus americanus) (8,9), the horseshoe crab (Limulus polyphemus)(12,14), the starfish (Asterias forbesi) (14), the American oyster (Crassostrea virginica) (15,16,17), the elongated clam (Tridacna maxima) (18,19), the spiny lobster (Panulirus argus) (14,20), the sea hare (Aplysia californica) (21), Amphitrite ornata (22), the garden snail (Helix pomatia) (23,24), the sponge (Axinella polypoides) (25,26) and the ascidian (Styela plicata) (27). The agglutinins isolated from these invertebrates vary greatly in their physical and chemical properties (see Table 2 in ref 5). However, we can still see some common patterns. Most of the invertebrate agglutinins have large molecular weights (up to 10^6 daltons in the oyster Crassostrea virginica), and the agglutinins are usually composed of subunits (from 2 to 18) held

together by noncovalent binding. Calcium is usually required for activity, and occasionally magnesium is required. Most of the invertebrate agglutinins are proteins or glycoproteins, with a binding specificity directed toward carbohydrates. Some agglutinins vary from these patterns. The agglutinins of the sponge Axinella polypoides have relatively low molecular weights (I- 21,000 daltons, II- 15,000 daltons), the subunits of agglutinin I (2 subunits, 15,000 daltons/ subunit) are bound covalently, and there are no requirements for divalent cations. The agglutinins from Amphitrite ornata, Aplysia californica, and Styela plicata have no calcium and magnesium requirements.

One of the more thoroughly characterized agglutinins from annelids are those of Amphitrite ornata. The two higher molecular-weight fractions (100,000 and 54,000 daltons) is thought to be aggregates of a 30,000-dalton fraction name "amphitritin". Amphitritin is a glycoprotein, resistant to digestion by trypsin and pronase, but inactivated by heating at 85^{0}C (22).

Agglutinins in most invertebrates occur naturally and cannot be

induced by exposure, in contrast to vertebrate immunoglobulin. However, three exceptions to this generalization have been noted. Fleshfly larvae (Sarcophaga peregrida) hemagglutinins can be induced by injury to the body wall. In the Pacific oyster (Crassostrea gigas), exposure to bacteria results in the production of higher levels of hemagglutinins. In the earthworm (Lumbricus terrestris) increased titers of hemagglutinins may also be induced by immunization with specific erythrocytes (5,13).

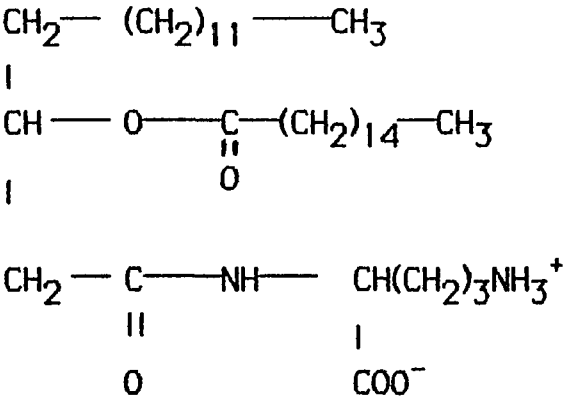
Cooper and his coworkers (13, 28) found that naturally occurring agglutinins from Lumbricus terrestris were inhibited most strongly by bovine submaxillary mucin and fetuin, whereas induced agglutinins were inhibited by thyroglobulin, bovine submaxillary mucin, hyaluronic acid and fetuin. The only monosaccharide inhibitor was 2-keto-3-deoxyoctanoate (KDO) which is a component of lipopolysaccharide found in many gram negative bacteria. This observation suggested an antibacterial role for coelomic fluid. Lumbricus coelomic fluid (normal or induced) was significantly mitogenic against mouse lymphocytes (T cells rather than B cells).

As mentioned on page 5, Lumbricus coelomocytes, cultured in defined media with rabbit RBC, showed secretory and E-type rosettes with RBC, suggesting that coelomocytes secrete some agglutinins as well as contain them on the cell surface (13).

Lipid hemagglutinins

Stone (29) observed that agglutination of avian erythrocytes by some pure lipids (cephalin, lecithin, sphingomyelin, cardiolipin, oleic acid, stearic acid and glycol distearate) and mixtures of these lipids have properties similar to those of organic extracts from a wide variety of tissues. Tsivion and Sharon (30) reported that oleic acid, dioleoylphosphatidic acid, a polar fraction from calf thymocytes and certain bovine lipids has some lectin-like activity, i.e. agglutination-specificity for certain RBC and specific inhibition of activity by certain complex carbohydrates. Kawai and his coworkers (31, 32) found that an ornithine-containing lipid from Bordetella pertussis strongly agglutinated human (type A and B), rabbit, green monkey and BALB/C mouse erythrocytes (RBC), but

weakly agglutinated horse, sheep, chicken and guinea pig RBC. The proposed structure of this amphipathic aminolipid (31, 32) was 3-hydroxyhexadecanoic acid, amide-linked to ornithine and esterified to the second hexadecanoic acid.



Kawai (33) reported that bovine brain phosphatidylserine which had a structure similar to the proposed structure of the ornithine-containing lipid of Bordetella pertussis strongly agglutinates human, rabbit, green monkey, chicken and BALB/C mouse RBC. We also found that dioleoylphosphatidylserine agglutinates rat RBC (at a concentration of 100 µg/ml PBS-N_3, specific titer 80/mg/ml). Forbes and his coworkers (34) reported that egg phosphatidylethanolamine (PE) and dioleoylphosphatidyl

ethanolamine (DOPE) agglutinates mouse RBC. Kawai reported that PE (kind unspecified) does not agglutinate human (type O, A and B), rabbit, green monkey, sheep, horse, guinea-pig, chicken and mouse (BALB/C) RBC. Our work will partially-resolve this contradiction.

Proteoglycan hemagglutinins

Fuke and Sugai (27) found that hemagglutinins in the coelomic fluid of the ascidians, Styela plicata and Halocynthia hilgendorfi, strongly agglutinate rabbit, rat and mouse but not sheep or guinea pig RBC. These agglutinins are high molecular weight substances which are heat-stable (no change of activity was observed when the sample was heated at 140^{0}C for 30 minutes in an autoclave), resistant to trypsin digestion, but sensitive to periodate. They thought that the hemagglutinin isolated from the ascidians is polysaccharide or mucopolysaccharide. [The preferred name for the polysaccharide component of proteoglycan is 'glycosaminoglycan' when the polysaccharide component contains derivatives of either glucosamine or galactosamine (35)]. Coombe et al. (36) isolated a

sulfated polysaccharide from the sponge, Ophlitaspongia tenuis, which was implicated as a cell aggregation enhancing factor. They proposed, based on a literature survey [see also Hunt (37)] replete with examples of specific receptors for sulfated polysaccharides on a variety of cells, ranging from mammalian to invertebrate, and cell-adhesion events from macrophage-lymphocyte to egg-sperm, that "sulfated polysaccharide-mediated cell adhesion" is "a basic cell adhesion mechanism."

The effects of some invertebrate hemagglutinins on Ehrlich ascites tumors

Tabrah, Kashiwagi and Norton (38) reported that a Hawaiian folk remedy for cancer patients was a cooked extract of a polychaetous annelid, kaunaoa (Lanice conchilega). They observed that extracts of two Hawaiian polychaetous annelids, Lanice conchilega and Reteterebella queenslandia, retard the growth of Ehrlich ascites tumor cells in mice but they did not test for hemagglutination activity.

Extracts of two other polychaetous annelids, Amphitrite ornata and Nereis virens have strong inhibitory effects on the growth of Ehrlich ascites tumor cells in mice but do not have toxicity in mice (39). The inhibition activity of the extracts parallel the hemagglutination titer against rat RBC.

Purposes of this work

(1) To isolate the agglutinins from Nereis coelomocytes and coelomic fluid supernatant.

(2) To determine the biological properties of each agglutinin.

A. Characterize the hemagglutination function

(a) Erythrocyte agglutination specificity.

(b) Inhibition profile.

(c) Cation requirements.

(d) Light microscopy.

B. Interaction with EATC

(a) In vitro (light microscopy and scanning electron microscopy).

(b) In vivo.

(3) To characterize the chemical nature of each agglutinin.

A. MW.

B. Sensitivity to various chemical, heat and enzymatic treatments

C. Response to antibodies

D. Spectra

E. Response to group-specific reagents, qualitatively and quantitatively

(4) To study hemagglutination of phosphatidylethanolamines.

MATERIALS AND METHODS

Instrumentation

UV-visible spectra were measured on a Cary 15 or a Perkin-Elmer Model Lambda 3B recording spectrophotometer. For RBC and EATC centrifugations an International Tabletop Clinical centrifuge was used. All other centrifugations were done at 4^0C in a Beckman refrigerated J-21 centrifuge with rotors JA-20 and JA-14, or a Sorvall RC-5B refrigerated superspeed centrifuge with rotors ss-34 and GSA. Lyophilizing was done in a Virtis Model 10-020 lyophilizer. Tumor cells were counted in a Coulter counter (Coulter Electronics, Hialeah, FL). Microscopic studies were done with an Olympus light microscope (Model CHA, light source tungsten bulb CH-6V, 100W-TP). Light micrographs were taken with 1 second exposures, with an Olympus OM-2 camera with a microscope adaptor using Kodak Panatomic ASA 32 black and white film. Infrared spectra were measured in Perkin-Elmer Model 247 and Digi-Lab Fourier-Transform Model FT S-40 infrared spectrometers.

Chemicals and Biologicals

Chemicals

Enzymes, glycoproteins, mucopolysaccharides and complex polysaccharides were purchased from Sigma (St. Louis, MO). Monosaccharides were purchased from Sigma and other common suppliers. Sephadex gels, Blue Dextran 2000, and gel filtration protein standards were purchased from Pharmacia (Piscataway, NJ). Bio-Rad reagent for protein determinations, Bio-gel P-200 and Cellex D were purchased from Bio-Rad (Richmond, CA). Lipid standards were purchased from Avanti Polar Lipids Inc. (Birmingham, AL). Solvents for lipid extraction and thin layer chromatography were reagent grade and distilled before use. All other chemicals were reagent grade or better. Dialysis tubing Spectrapor #1 (cut-off, MW 6000-8000), Spectrapor Semi-Micro tubing (MW 12-14,000 Da cut-off) and Microtiter plates (round well) were from Fisher (Springfield, NJ). Polygram Sil G for thin layer chromatography was purchased from Brinkmann Instruments, Inc. (Westbury, NY).

Animals

Live Nereis virens were purchased from Bait Wholesalers (New Rochelle, NY). Male CF_1 mice bred from 12 females and 12 males, given by the Department of Biology of City College, were purchased from Charles River Laboratories (Kingston, NY). All mice were maintained on a rat chow diet.

Erythrocytes

Rat erythrocytes (RBC) were obtained from etherized live Sprague-Dawley rats by cardiac puncture using 3.8% Na-citrate as anticoagulant. Sheep, steer, guinea pig, rabbit, chicken, African green monkey, rhesus monkey and human (O) RBC were purchased from M. A. Bioproducts (Rockville, MD), Rockland (Gilbertsille, PA) and Flow Laboratories (McLean, VA). Erythrocytes were washed with PBS-N_3 (0.9 % NaCl-1mM phosphate, pH 7.2, 0.01% NaN_3) three times at room temperature, spun down each time for 3 minutes in a Table-top International Clinical centrifuge, and suspended in PBS-

N_3 to make a 5% suspension. Erythrocytes which were stored in citrate solution could be used within a week. However, erythrocytes which were washed and suspended in PBS-N_3 had to be used the same day.

Analytical methods

(1) Microtiter assay for hemagglutination

RBC were washed with PBS-N_3 (0.9% NaCl-1 mM phosphate pH 7.2, 0.01% NaN_3) three times at room temperature in a Table-top International Clinical centrifuge and suspended in PBS-N_3 to make a 5% suspension. 50 µl of PBS-N_3 or PBS-N_3-0.001% Triton X-100 (PBS-N_3-T) or test inhibitor solutions consisting of glycoproteins, mucins, lipopolysaccharides, amino acids, phospholipids, monosaccharides, mannan or PEG (MW 8,000 d) dissolved in PBS-N_3, was added to each well. 50 µl of the sample to be assayed was

diluted serially with the same medium starting with the second well of a row. The first well containing PBS-N_3 or PBS-N_3-T served as control. A second 50 µl of PBS-N_3 or PBS-N_3-T or test solution was added to each well, making a total of 100 µl. When a test solution was used, it was allowed to preincubate for 30 minutes at room temperature. Then 25 µl of 5% RBC suspension was added to each well. The plates were sealed and agitated on a Tektator Plate Shaker (Scientific Products) for 3 minutes, observed after 30 minutes (for inhibition studies, every 10 minutes), and 1 hour, and up to 2 hours, at room temperature. They were stored in the refrigerator at 4^0C overnight and observed again.

The plates were read by tilting. In a positive result, the RBC's remained clumped or formed a film at the bottom of the well and did not flow. In a negative result, the RBC's flowed at the bottom of the well. The titer is defined as the reciprocal of the highest dilution which still has agglutination activity. Titers were performed in duplicate. If the results differed by one well, the titer is presented

as the average value (fractional titer). If titers differed by more than one well, the determination was repeated. The titers are reported as exponentials of two for simplicity. The specific titer for LMW is defined as titer/ mg protein/ml and for HMW and CM was defined as titer/ mg dry wt/ml.

(2) Protein determination

Protein was determined by the method of Bradford (40), using Bio-Rad dye reagent and bovine gamma-globulin as a standard.

(3) Hexose determination

Hexose concentration was determined by the micro-assay phenol-sulfuric method of Dubois et al (41) using glucose as a standard.

(4) Hexosamine determination

Hexosamine concentration was determined by the Morgan-Elson method (42) using glucosamine hydrochloride as a standard.

(5)Pentose determination

Pentose concentration was determined by the orcinol method (43) using ribose as a standard.

(6) Deoxysugar determination

Deoxysugar concentration was determined by the method of Webb (44) using deoxyribose as a standard.

(7) Sialic acid determination

Sialic acid was determined by the periodate-resorcinol method of Jourdian et al (45) and the thiobarbituric acid method of Warren (46) using N-acetylneuraminic acid as a standard.

(8)Glycosaminoglycan determination

Glycosaminoglycan content was determined by the binding assay of Chandrasekhar et al (47) using DMB [1,9-dimethylmethylene blue, Serva Feinbiochemica (Heidelberg/New York)]-0.24 M guanidinium chloride and chondroitin sulfate A or B or C as standards. An aliquot

(10-50 µl) of each test sample was mixed with 12 µl of 4M guanidinium chloride and 138-178 µl of 0.05 M sodium acetate, pH 6.8 was added to make a final volume of 200 µl and then 800 µl of DMB solution (16 mg of DMB was dissolved in 5 ml of ethanol followed by the addition of 2.0 ml of formic acid and 2.0 g sodium formate and made up to 1 liter with deionized water) was added. GAG content was measured by the ratio of absorbance at 550/610 nm.

(9) Hexuronic acid determination

Hexuronic acid content was determined by the sulfuric-carbazole method of Dische (48) using glucuronic acid as a standard.

(10) Sulfate determination

Sulfate content was determined by the trichloroacetic acid-gelatin/$BaCl_2$ method of Carney (49) using K_2SO_4 as a standard.

(11) N-sulfate determination

N-sulfate content was determined by the method of Dische et al (50) using heparin as a standard.

(12) Elemental sulfur and phosphorus determinations

Elemental sulfur and phosphorus analyses were performed by Schwarzkopf Microanalytical Laboratories (Woodside, NY).

(13) Group-specific reagents used on Sil G plates for TLC

(13-a) Zinzade's reagent for phosphate

The procedure of Beiss (51) was followed using dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidyl serine (DOPS), dioleoylphosphatidylcholine (DOPC) or dioleoylphosphatidylinositol (DOPI) as standards. 0.685 g of $Na_2MoO_4 \cdot 2H_2O$ and 40 mg of hydrazine sulfate were dissolved in 10 ml of water, 25 ml of concentrated H_2SO_4 was added , the mixture was cooled and diluted to 60 ml with dH_2O. The silica TLC plates

were immersed for 5 minutes at room temperature. The phosphatides appeared as blue spots on a white background.

(13-b) Ninhydrin reagent for free amino group ($-NH_2$)

The procedure of Marinetti (52) was followed using DOPE or DOPS as standards. 0.25 g of ninhydrin was dissolved in 100 ml of acetone-lutidine (9 : 1, v/v) to make a 0.25 % ninhydrin solution. The silica TLC plates were immersed for 2-3 minutes at room temperature. The chromatograms were hung in the hood for 1-2 hours at room temperature. Materials which had free amino groups appeared as mauve spots on a white background.

(13-c) Phosphomolybdic acid reagent for choline

The procedure of Levine and Chargaff (53) was followed using DOPC or sphingomyelin (SP) as standards. The ninhydrin-stained chromatogram was well-washed with dH_2O for 10 minutes and then immersed in a phosphomolybdic acid solution (1 g of phospho-

molybdic acid in 100 ml of dH_2O) for 5-10 minutes at room temperature. The chromatogram was washed with three 50 ml portions of butanol (5-10 minutes each), put under running tap water for 1 hour, washed briefly with dH_2O to remove traces of reagent and finally immersed in a dilute stannous chloride solution [1 ml of $SnCl_2$ stock solution (40 g of $SnCl_2$ in 100 ml of concentrated HCl) was diluted to 100 ml with dH_2O] in a few minutes. Choline-positive spots gave a blue color on a white background.

(13-d) <u>-NH group stain</u>

The procedures of Schwartz and Pallansch/ Mazur et al (54) were followed using SP, DOPE or DOPS as standards. The reagents for -NH group staining consisted of solution (i) 1% tert-butylhypochlorite solution in cyclohexane and solution (ii)1% starch-iodide solution (1 g of KI and 1 g of soluble starch were dissolved in 100 ml of dH_2O,

the mixture brought to a boil and cooled to room temperature). The chromatogram was immersed in solution (i) for 2-3 minutes, hung in the hood at room temperature for 1 hour and immersed in solution (ii) for 2-3 minutes. Materials which had NH groups (e.g. SP) as well as NH_2 groups (e.g. DOPE or DOPS) gave blue spots on a very light-blue background.

(13-e) Periodate-Schiff reagent for vicinal-OH (- CH- CH-)
OH OH

The procedure devised by Baddiley et al (55) was followed using DOPI as a standard. The periodate-Schiff reagent consisted of (i) 0.25% sodium periodate in dH_2O, (ii) 1% $Na_2S_2O_5$ solution and (iii) Schiff reagent (Sigma) diluted 1 : 2 (v/v) with 1% $Na_2S_2O_5$ solution. The chromatogram was passed through the sodium periodate solution, hung for 15-20 minutes at room temperature, and the paper was passed repeatedly through a 1% $Na_2S_2O_5$ solution until

iodine was completely decolorized. The chromatogram was passed through the diluted solution of Schiff reagent. Materials which had vicinal-OH gave pink-mauve spots.

(13-f) Alpha-naphthol reagent for sugar

The procedure of Siakotos and Rouser (56) was followed using glucose as a standard. The sugar reagents consisted of (i) 0.5% alpha-naphthol in methanol-water (1 : 1) and (ii) 95% sulfuric acid/H_2O. The chromatogram was sprayed with alpha-naphthol solution until damp, dried in air, sprayed with sulfuric acid solution and then heated in the oven at 120^{0}C until maximum color was developed. Glucose or glycolipids gave blue-purple spots and other polar lipids gave yellow spots.

(13-g) $AgNO_3$ reagent for reducing sugar

The procedure of Trevelyan et al (57) was used. The chromatogram was sprayed first with 1% $AgNO_3$ in acetone, dried in the hood, followed by spraying with 2% NaOH in ethanol and hung at

room temperature for 2 hours using glucose, galactose,maltose, arabinose and lactose as standards. Reducing sugars gave a brown color.

(13-h) Resorcinol stain for gangliosides

The procedure of Svennerholm/ Miettinen and Takki-Luukainen (58) was followed using N-acetylneuraminic acid from sheep submaxillary mucin as a standard. The chromatogram was sprayed with the resorcinol reagent (10 ml of 2% resorcinol in water was mixed with 80 ml of concentrated HCl containing 0.5 ml of 0.1 M $CuSO_4 \cdot 5\ H_2O$ and the mixture was diluted to 100 ml with water), covered with a glass plate and heated in oven at 120^0C until maximum color was developed. The gangliosides or sialic acid derivatives appeared as blue-violet spots on a white background.

(13-i) Ferric chloride reagent for sterols and sterol esters

The procedure of Lowry (59) was followed using cholesterol as

a standard. 50 mg of $FeCl_3 \cdot 6\,H_2O$ was dissolved in 90 ml of water and mixed with 5 ml of glacial acetic acid and 5 ml of concentrated H_2SO_4 to make a ferric chloride reagent. The chromatogram was sprayed with the reagent and heated at 100^{0}C. Cholesterol appeared as a red to violet spot on a yellow background within 2-3 minutes.

(13-j) Ester stain

The procedure of Skidmore and Entenman (60) was followed using ethyl acetoacetate as a standard. The ester stain consisted of (i) alkaline hydroxylamine solution (100 ml 10% ethanolic hydroxylamine hydrochloride was mixed with 200 ml 12% ethanolic sodium hydrochloride solution, the mixture was centrifuged and the clear supernatant was used and (ii) ferric chloride reagent (10 g of $FeCl_3 \cdot 6\,H_2O$ and 20 ml of concentrated HCl were ground in a mortar and the solution was shaken with 300 ml of ethyl ether to make an ethereal ferric chloride solution). The chromatogram was first sprayed with alkaline hydroxylamine solution, dried briefly and

sprayed with ethereal ferric chloride solution. Esters appeared as purple spots on a yellow background.

(14) Immunoelectrophoresis

Immunoelectrophoresis was carried out according to instructions from the supplier of the apparatus, Helena Laboratories (Beaumont, TX).

24 µl of LMW DEAE peak I sample were applied to each well [see Figures 11-13, #2-7 (from the left side) except LMW DEAE peak III-#1-7]. Bromphenol blue (marker) was applied to the first well (#1) and the last well (#8) of the 8 wells (except LMW DEAE peak III-only applied to the last well (#8)). The samples were subjected to electrophoresis at constant voltage (100 volts) for 25 minutes. The same procedure was followed for LMW DEAE peaks II-IV, HMW, heated HMW, heated & delipidated HMW and CM extract of coelomocytes.

Each gel was cut into 5 parts (Figures 11-13). The first part was cut into 15 slices/sample and each slice was soaked in 200 µl

of PBS-N_3 overnight, centrifuged and titered. The second part was for Coomassie Blue (0.1% Coomassie Blue in 7% acetic acid) protein staining. The third part was stained for carbohydrate staining with the periodate-Schiff reagent (61) [(i) soaked in solution C (0.54 ml acetic acid, 0.89 g anhydrous sodium acetate and 10.0 g hydroxylamine hydrochloride were dissolved in dH_2O to a final volume of 100 ml) for 15 min, (ii) washed under running water for 15 min, (iii) soaked in solution B (1.0 g periodic acid and 0.82 g anhydrous sodium acetate were dissolved in dH_2O to a final volume of 100 ml) for 10 min, (iv) washed under running water for 10 min, (v) soaked in solution A [Schiff reagent (Sigma) which had been diluted with an equal volume of water] for 30 min, (vi) washed three times, each for 2 min duration, in solution D (5 ml 10% sodium metabisulphite in water, 2 ml 2 N HCl and 90 ml dH_2O) and (vii) followed with three washes, each of 60 minutes, in solution E (Solution D + 20% glycerol by volume) and dried at room temperature]. The fourth part was for lipid staining with Sudan

Black B (62) (200 mg of Sudan Black B in 100 ml 60% ethanol, was soaked for 60 minutes at room temperature, and allowed to stand overnight to allow the undissolved dye to settle out. The working solution was prepared by diluting the stock with an equal volume of 60% ethanol). The fifth part was used for immunodiffusion : 75 µl rabbit antiserum to crude CF was applied to the trough. The plate was placed in a humid chamber. If the material was antigenic, a precipitin arc appeared within 24 hours. The plate was soaked in 0.85% NaCl, 0.01% NaN_3 for 48 hours with 3 changes of saline-NaN_3 daily and the precipitin arc was visualized by staining with 0.1% Amido Black in 5% acetic acid for 30 minutes and destaining with 5% acetic acid until the background was colorless (63).

(15) Infrared analysis

13 mm KBr pellets using 100-150 mg KBr and 0.7-1.50 mg of sample were prepared. Spectra were recorded in a spectrophotometer Perkin-Elmer Model 247 and a Digi-Lab Fourier transform infrared spectrophotometer Model FTS-40.

Antiserum (to crude CF) production

0.5 ml coelomic fluid (CF) from Nereis virens, emulsified with 0.5 ml of Freund's adjuvant was injected into the thigh of a New Zealand rabbit. Four weeks later a booster containing 1 ml of CF in Freund's adjuvant (v/v = 1 : 1) was administered and 3 weeks later blood was drawn from an ear vein. After standing at room temperature to allow clotting, the blood sample was centrifuged in the table-top centrifuge for 3 minutes, and serum containing antibody against crude CF was collected. Aliquots were stored at $-15^{0}C$.

8 ml of rabbit antiserum to crude CF was centrifuged in the table-top centrifuge for 5 minutes and the top clear supernatant was divided into two equal parts. One part of this centrifuged rabbit antiserum was heated on the steam bath for 1 hour. The heated antiserum solution was centrifuged in the table-top centrifuge for 5 minutes to remove precipitate and transferred to a 15 ml sterile centrifuge tube. 100 µl of each sample (CM extract from coelomocytes, HMW fraction and LMW DEAE peak I-IV samples)

was treated with 100 µl of rabbit antiserum to crude CF, or 100 µl of heated rabbit antiserum, or 100 µl of PBS-N_3. After mixing, the solutions were incubated at 37^{0}C for 1 hour, and centrifuged in a Beckman Microfuge for 2 minutes. The supernatants were tested for hemagglutination activities.

Tumor cells

(1) Tumor transfer

The Ehrlich ascites tumor stock, in 2 live BDF_1 mice was a generous gift from Sloan-Kettering Institute for Cancer Research (New York, NY). A tumor stock mouse was killed by etherization. The ascites fluid was removed using a 20 gauge needle and a 5 ml syringe. It was centrifuged for 3 minutes in a tabletop centrifuge, and the supernatant was discarded. The residue was washed with sterile PBS to remove RBC's, which were heavier and settled to the bottom. The RBC's were aspirated out from underneath the tumor cells. The tumor cells were washed and the RBC's removed until as

many RBC's as possible had been aspirated, centrifuging for 2-3 minutes between washings. 1 ml of sterile PBS was added to the EATC's. The concentration of the cells was determined by using a Coulter counter. 20 µl of ascites fluid was diluted to 10 ml [1: 500 dilution in isotonic saline, using Unopette (Becton, Dickinson)]. The diluted cells were counted three times and an average taken and properly diluted with sterile PBS to get 10^6 cells to inject into each CF_1 mouse to grow tumor cells.

(2) Long-term storage of tumor cells with glycerol

A slight modification of the Cassel (64) procedure was used for storing Ehrlich ascites tumor cells in the deep freezer. Tumor cells were aspirated from CF_1 mice using a 20 gauge needle with 5 ml syringe. One ml portions were added to sterile 15 ml polystyrene centrifuge tubes (with screw caps) each containing 1 ml of 40% glycerol (sterilized using a 45 µm Millipore filter [Swinex-Millipore Corp., Bedford, MA)].

The tubes were capped, vortexed and placed at 4^0C for 30 minutes. They were again mixed and left at 4^0C for another hour. The cells were then resuspended, and placed directly in a -76^0C freezer. According to Cassel's report, they could be stored for one year without affecting viability.

(3) The determination of the optimal number of Ehrlich ascites tumor cells needed for tumor growth in mice

Two ml of the frozen EATC was defrosted and washed three times with 10 ml of sterile PBS each time to remove the glycerol, centrifuged in a table-top clinical centrifuge and packed cells were then resuspended in 1 ml of sterile PBS. The concentration of EATC was determined as described above to be 4×10^7 cells/ml. Five groups, containing 3 mice each, were injected intraperitoneally with 5×10^5, 10^6, 2×10^6, 5×10^6, 10^7 cells/mouse (using a 1 ml sterile disposable syringe and a 25 gauge disposable needle). Tumor cell growth became evident in about two weeks. It was found that the optimal number of Ehrlich ascites tumor cells needed for tumor

growth in mice was 10^6 cells/mouse (Figure 20).

Modification of bovine submaxillary mucin (BSM)

(a) BSM (Sigma, type I, 1 mg/ml in PBS-N_3) was heated on the steam bath for 2 hours and centrifuged at maximum speed in a table-top centrifuge for 3 minutes. The clear solution was dialyzed against PBS-N_3. (b) BSM (type I, 1 mg/ml in PBS-N_3) solution was treated with 0.12 N HCl (10 µl of concentrated HCl/1ml solution), incubated at 80°C in a water bath for 1 hour and centrifuged at maximum speed in a table-top centrifuge for 3 minutes. The clear solution was dialyzed against PBS-N_3. (c) BSM (type I, 8 mg/ml in PBS-N_3) was dialyzed against 0.1 M Tris pH 8.0 containing 0.2 M ß-mercaptoethanol for 48 hours at 4°C, then dialyzed against PBS-N_3 for 48 hours at 4°C.

Preparations

(1) Collection and processing of coelomic fluid

Nereis virens worms were washed in artificial sea water (NaCl 24.72 g/l, KCl 0.67 g/l, $CaCl_2 \cdot 2H_2O$ 1.36 g/l, $MgCl_2 \cdot 6H_2O$ 4.66 g/l, $MgSO_4 \cdot 7 H_2O$ 6.29 g/l and $NaHCO_3$ 0.18 g/l, pH adjusted to 7.2-7.4) and placed in a glass container in batches of 10-15 worms. The container was put into a closed bell jar system saturated with ether vapor. The coelomic fluid (~ 1 ml/ worm) released by the worms which relaxed in ether vapor, was collected with a Pasteur pipette. The fluid was centrifuged at 2-3 Krpm (ss-34 rotor in a Sorvall RC-5B refrigerated superspeed centrifuge) for 15 minutes. The particulate fraction was frozen as a pellet or taken up in the minimum volume of artificial sea water (in Scheme 3, 6 ml of packed cells was taken up in 24 ml of artificial sea water) and stored at $-15^{0}C$. The supernatant was made 1 mM in TPCK to avoid protease activity, ammonium sulfate was added to 75% saturation and the mixture was allowed to stir overnight at $4^{0}C$. The mixture

was centrifuged at 12 Krpm (GSA rotor) for 30-60 minutes. The brown oily layer sample which had separated was pipetted and dissolved in the minimum volume of dH_2O and dialyzed against PBS or PBS-N_3 overnight (using Spectrapor #1 dialysis tubing, cut off MW 6-8,000 d).

(2) Low molecular weight (LMW) and high molecular weight (HMW) fractions (Scheme 1)

25 ml of the resuspended brown layer sample (titer > 2^{12}) was obtained from the 75% $(NH_4)_2SO_4$ precipitation of 128 ml of the coelomic fluid supernatant. Five ml of this brown layer sample was put on a Sephadex G-100 column (height 41.5cm, diameter 2.7 cm, 60 drops/tube, eluted with PBS-N_3) or Bio-gel P-200 column (height 23.4 cm, diameter 2.7 cm, 60 drops/tube, eluted with PBS-N_3) (see Figures 1 & 2). An active peak (fractions #18-23 from Sephadex G-100, or fractions #6-10 from Bio-gel P-200) corresponded to the void volume of the column. It was called high molecular weight

(HMW). Another active peak was in the low MW region (the position of the peak was fraction * 36, MW 19,000 d for Sephadex G-100, fraction * 21, MW 18,000 d for Bio-gel P-200).

(a) LMW fraction

LMW fractions were pooled and concentrated by lyophilization and dialyzed against PBS-N_3, pH 7.2, overnight for titer assay (specific titer ≥ 3413/mg protein/ml) , total volume was 16 ml , protein content was 19.2 mg. This sample was dialyzed against 0.01M Tris buffer, pH 8.2, overnight. The dialyzed LMW sample (15 ml, 18 mg protein) was put on a DEAE column (diameter 1.7 cm, height 15.2 cm) in 0.01 M Tris buffer, pH 8.2. After the baseline was reached, a gradient of 250 ml of 0-0.4 M NaCl in 0.01 M Tris buffer, pH 8.2 was started. Finally, the column was washed with 0.4 M NaCl in 0.01 M Tris buffer, pH 8.2. All DEAE column samples (tubes 1-120) were dialyzed against PBS-N_3, pH 7.2 overnight and titered individually. Four active peaks (I-IV) were obtained (see Figure 3

and Scheme 1).

The four active fractions from DEAE chromatography were concentrated by lyophilization separately and dialyzed against PBS-N_3 overnight. 1.5 ml-2.0 ml of each lyophilized active fraction was put on a Sephadex G-75 column (height 27.4 cm, diameter 1.7 cm, 30 drops /tube, eluted with PBS-N_3) separately to determine its molecular weights (see Scheme 1).

(b) HMW fraction (Scheme 2)

(b-1) HMW

HMW fractions (total volume was 114 ml, obtained from ~ 250 ml of coelomic fluid) were pooled, concentrated by lyophilization and dialyzed against dH_2O overnight. The final concentration was 8.68 mg dry wt/ml (The specific titer was 334/mg dry wt/ml).

(b-2) Heated HMW (HHMW)

The concentrated HMW (8.68 mg dry wt/ml) was heated on a

steam bath for 1 hour and centrifuged at 16.5 Krpm (32,000 g) for 30 minutes at 5^0C (ss-34, Sorvall RC-5B centrifuge). The clear supernatant was heated HMW (HHMW) (The specific titer was 287/mg dry wt/ml).

(b-3) Heated delipidated HMW (DL-HHMW)

5 ml of HHMW (7.12 mg dry wt/ml) in a Teflon centrifuge tube was treated with a total of 125 ml of chloroform-methanol (v : v = 2 : 1). The mixture was shaken vigorously by hand for 10 minutes and allowed to stand for 2 hours at room temperature. The sample was centrifuged at 10 Krpm for 30 minutes. The particulate fraction was separated from the organic layer. The particulate fraction was dried by placing the tube in a desiccator containing beakers of paraffin oil to remove organic vaporand desiccant . The desiccator was stored in the cold room at 4^0C. The dried sample (7.90 mg) was dissolved in 2 ml dH_2O , mixed by vortexing and centrifuged in the table-top centrifuge at maximum speed for 3 minutes. The clear supernatant was DL-HHMW. Its protein content was determined and

after dialysis against PBS-N_3, its titer was determined (The specific titer was 132/mg/ml, see Scheme 2). The $CHCl_3$-CH_3OH solution was treated with 2.25 ml dH_2O/ml $CHCl_3$-CH_3OH, mixed well, kept in the refrigerator overnight and centrifuged at 10 Krpm at 5^0C for 30 minutes (ss-34 rotor, Sorvall RC-5B centrifuge). The aqueous phase was aspirated, dialyzed against PBS-N_3 and tested for its hemagglutination activity. It showed no activity. The chloroform phase was dried by sweeping with inert gas and stored in a desiccator with a beaker of paraffin oil and desiccant in it to let it dry to constant weight. The dried chloroform phase material (16.27 mg dry wt was obtained from 35.60 mg HHMW, 39.7%) was dissolved in 4 ml of PBS-N_3, mixed by vortexing and centrifuged at 16.5 Krpm, 5^0C for 30 minutes (ss-34 rotor, Sorvall RC-5B centrifuge). The clear supernatant was tested for hemagglutination activity (The specific titer was 32/mg/ml, see Scheme 2).

(b-4) Determination of molecular weights of HMW agglutinins

Four ml of concentrated HMW sample (protein content 332.443 µg/ml, sugar 1196.44 µg/ml, specific titer ≥ 12321/mg protein/ml) was put on a Sepharose 4B column (diameter 2.7 cm, height 34.3 cm, 60 drops/tube, eluted with PBS-N_3). Two active peaks were obtained at tube 18 and tube 31(Figure 4).

Two ml of unheated HMW (17.1 mg/ml), 1.8 ml of heated HMW (15.7 mg/ml) and 1.3 ml of delipidated heated HMW (1.09 mg/ml) were put on the Sepharose 4B column separately (diameter 1.7 cm, height 26.5 cm, 30 drops/tube, eluted with PBS-N_3) (see Figure 5).

The molecular weight standards were aldolase (MW 158 kd), catalase (MW 232 kd), ferritin (MW 440 kd) and throglobulin (MW 669 kd). They were loaded separately (described in p 52).

(3) Extraction of lipids

(a) Folch extraction (Scheme 3)

The particulate fraction (packed cells, 6 ml) was stored in 24 ml

of sea water and stored at -15^{0}C. The Folch (65) procedure was used to extract the lipid components. A mixture of chloroform - methanol (v : v=1 : 2, 30 ml : 60 ml) was added to the particulate fraction in sea water and shaken by hand at room temperature. This was centrifuged at 12 Krpm for 30 minutes (ss-34 rotor in a Sorvall RC-5B). To the supernatant, $CHCl_3$-H_2O (57 ml : 57 ml) was added. This separated into two layers : the organic (CM) extract (78 ml),which had 87.75 mg of lipid, and an aqueous layer (132 ml). A 5 ml aliquot (40 mg) of aqueous layer (pH 6.4) from which the methanol had been blown off with N_2, was centrifuged, dialyzed against PBS-N_3, pH 7.2 and had a specific titer vs rat RBC of 4/mg/ml. A 5 ml aliquot of CM containing 11.25 mg of material, was dried with N_2 gas, taken up in 5 ml PBS-N_3 -0.001 % Triton X-100 (PBS-N_3-T) by vortexing for 30 minutes and centrifuged in a table-top centrifuge for 3 minutes. The clear supernatant (pH 6.5, the specific titer was 57 mg/ml) was dialyzed against PBS-N_3-T,

pH 7.2 and had a specific titer vs rat RBC of 160/mg/ml.

(b) Acetone precipitation

Ten ml of CM extract from coelomocytes (packed cells, 6 ml) was evaporated with N_2 gas at room temperature to dryness to constant weight (22.5 mg). This material was treated with 1.125 ml of acetone and 0.0225 ml of 10% $MgCl_2 \cdot 6H_2O$ (in methanol), vortexed, kept in the ice bath for 1 hour and centrifuged in the table-top centrifuge at medium speed for 3 min. The acetone- insoluble (AI) fraction was washed with 0.225 ml of cold acetone twice, vortexed and centrifuged, evaporated by N_2 gas at room temperature to dryness to constant weight (4 mg) and taken up in 10 ml PBS-N_3 -T to test its hemagglutination activity. For TLC the material was taken up in $CHCl_3$ (see Scheme 4). The acetone-soluble (AS) fraction was combined with the acetone washings of AI, evaporated by N_2 gas at room temperature to dryness to constant weight (17 mg) and

taken up in 10 ml PBS-N_3 -T to test its hemagglutination activity. For TLC the material was taken up in $CHCl_3$ (see Scheme 4).

(c) Thin layer chromatography (TLC)

All solvents for TLC were distilled before use or HPLC grade. All plastic-backed Sil-G TLC plates (Brinkmann Instruments Inc., Westbury, NY) were prewashed with developing solvents and activated by heating in the oven under vacuum at 80^0C or 120^0C for 30 minutes.

For one dimensional TLC, the acetone-insoluble (AI) and acetone-soluble (AS) fractions from the CM extract of coelomocytes were developed separately in $CHCl_3$-CH_3OH-acetone-acetic acid-H_2O [65 : 10 : 20 : 10 : 3 (v/v)]. For two- dimensional TLC (2D TLC), AI was developed in $CHCl_3$-CH_3OH-acetone-acetic acid-H_2O [65 : 10 : 20 : 10 : 3 (v/v)] for the first dimension and $CHCl_3$-CH_3OH-concentrated ammonia [65 : 25 : 5 (v/v)] for the second

dimension.For AS, hexanes : ethyl ether : acetic acid [80 : 20 :1 (v/v)] was used for the first dimension and hexanes : ethyl ether : acetic acid [90 : 10 : 1 (v/v)] was used for the second dimension. The TLC plates were dried and visualized in I_2 vapor or stained for specific functional groups (described in pp 24-30). Lipid standards were developed and stained under the same conditions.

For preparative work, bands were extracted in the first developing solvent system and rechromatographed in the second developing solvent system. (a) For AI, (i) the TLC bands (except the bands near the origin) were eluted twice with 3.8 ml of $CHCl_3$-CH_3OH-dH_2O [1 : 1 : 0.8 (v/v)] and centrifuged. Two ml each of $CHCl_3$ and dH_2O were added to the supernatant, mixed, centrifuged and the bottom chloroform phase was collected. The chloroform phase was diluted with an equal volume of benzene (4 ml), evaporated by rotary evaporation under reduced pressure at 30^0C (66) and taken up in 2 ml PBS-N_3 -T to determine its hemagglutination titer. (ii) the TLC bands near the origin (highly

polar lipids) were eluted twice with 3.8 ml of $CHCl_3$-CH_3OH-0.2 N HCl [1 : 1 : 0.8 (v/v)] and centrifuged. Two ml each of $CHCl_3$ and dH_2O were added to the supernatant, mixed, centrifuged and the bottom chloroform phase was collected. The chloroform phase was neutralized by adding 1.6 ml of 0.2 N methanolic ammonia, diluted with 5.6 ml of benzene, evaporated by rotary evaporation under reduced pressure at 30^0C (66) and taken up in 2 ml PBS-N_3 -T to determine its hemagglutination titer. (b) For AS, each TLC band was eluted twice with 3.8 ml of $CHCl_3$-CH_3OH-ether [1 : 1 : 0.8 (v/v)] and centrifuged. Two ml each of $CHCl_3$ and dH_2O were added to the supernatant and centrifuged. The bottom chloroform phase was collected, diluted with 4 ml of benzene, evaporated by rotary evaporation under reduced pressure at 30^0C (67) and taken up in 2 ml PBS-N_3 -T to determine its hemagglutination titer.

(4) Preparation of gel filtration and anion exchange columns

(a) Sephadex G-100 and G-75 columns

Sephadex G-100 and G-75 were swollen in PBS-N_3 on a steam bath for 5 hours and 3 hours respectively. The height of the gel bed was 41 cm, diameter 2.7 cm for Sephadex G-100 or 27.4 cm, diameter 1.7 cm for Sephadex G-75. The void volume of the Sephadex gel was determined with 2 ml Blue Dextran 2000 (1.5 mg/ml in PBS-N_3). The molecular weight standards were bovine serum albumin (MW 67 kd), ovalbumin (MW 43 kd), alpha-chymotrypsinogen A (MW 25 kd), myoglobin (MW 17 kd) and ribonuclease A (MW 13.7 kd). They were loaded in 2 ml aliquots of 10 mg of each protein per ml. The buffer was PBS-N_3 and fractions of 60 drops/tube (4 ml) for Sephadex G-100, 30 drops/tube (1.9 ml) for Sephadex G-75, were collected using an LKB Ultrarac fraction collector with elution monitored at 254 nm and recorded by an LKB 8300 Uvicord II monitor.

(b) Sepharose 4 B column

180 ml of Sepharose 4 B gel solution was mixed with 70 ml of PBS-N_3 and poured into a column. The height of the gel bed was 34.3 cm, diameter 2.7 cm. The void volume of the Sepharose gel was determined with 2 ml of Blue Dextran 2000 (2.0 mg/ml in PBS-N_3). The molecular weight standards were aldolase (MW 158 kd), catalase (MW 232 kd), ferritin (MW 440 kd) and thyroglobulin (MW 669 kd). They were loaded in 1 ml aliquots separately from each other (aldolase 22 mg/ml, ferritin 6 mg/ml, thyroglobulin 10 mg/ml and catalase 10 mg/ml). Loading volume was 1 ml. Fractions of 60 drops/tube (~4 ml) were collected. For the smaller column, the height of the gel bed was 26.5 cm, diameter 1.7 cm and fractions of 30 drops/tube (2 ml) were collected.

(c) Bio-gel P-200 column

Bio-gel P-200 was swollen by following the instructions of Bio-Rad. The height of the gel was 23.4 cm, diameter 2.6 cm. The void

volume of the Bio-gel P-200 column was determined with 2 ml ferritin (6 mg/ml in PBS-N_3). The Bio-Rad molecular weight standards kit was used to calibrate the column. It contained a mixture of bovine thyroglobulin (MW 670 kd), bovine gamma-globulin (MW 158 kd), chicken ovalbumin (MW 44 kd), horse myoglobin (MW 17 kd) and vitamin B_{12} (MW 1.35 kd) at a concentration of 36 mg/ml. 0.2 ml of the mixture was loaded on the column. Fractions of 60 drops/tube were collected.

(d) DEAE (Cellex D) anion exchange column

10-20 grams of Cellex D was suspended in 500 ml of 0.25 M NaOH and allowed to swell for 30 minutes. It was filtered and rinsed with dH_2O, suspended in 500 ml of 0.25 M HCl and allowed to stand for 10 minutes. It was then filtered and rinsed with dH_2O. The NaOH treatment was repeated. After rinsing with dH_2O, it was equilibrated in 0.01 M Tris buffer, pH 8.2. The height of the equilibrated column was 15.2 cm, diameter 1.7 cm.

Light micrographic studies on the hemagglutination of rat erythrocytes by Nereis hemagglutinins

Rat erythrocytes were obtained from etherized live Sprague Dawley rats by cardiac puncture using 3.8% Na citrate as an anticoagulant. Before suspending and storing rat erythrocytes in 3.8 % Na-citrate, attempts were made to keep rat erythrocytes in the following different buffer systems-- (i) 1mM phosphate buffer saline (0.9% NaCl, pH 7.2), (ii) 10 mM phosphate buffer saline (0.85% NaCl, pH 7.2), (iii) 1 mM phosphate buffer saline (0.9% NaCl, pH 7.2) : 6% glucose (v/v) = 1 :3, (iv) 10 mM phosphate buffer saline (0.85% NaCl, pH 7.2) : 6% glucose (v/v) = 1 : 3 and (v) 1 X balanced salt solution [10 ml of stock solution #1 (dextrose 10.0 g, KH_2PO_4 0.6 g and $Na_2HPO_4 \cdot 7H_2O$ 3.58 g, dissolved and brought up to 1 liter with dH_2O) and 10 ml of stock solution #2 ($CaCl_2.2H_2O$ 1.86 g, KCl 4.0 g, NaCl 80 g, $MgCl_2$ anhydrous 1.04 g and $MgSO_4.7H_2O$ 2.0 g, dissolved and brought up to 1 liter with dH_2O) and brought to 100 ml with dH_2O (pH 7.25)]. It was found that rat erythrocytes were "happy" in

3.8% Na citrate (all cells were in the normal cell condition up to overnight). It was also found that the titers of the Nereis hemagglutinins using 3.8% Na citrate as a dilution buffer were the same as those in 1 mM phosphate buffer saline (0.9% NaCl, pH7.2) as a dilution buffer, up to a 2 hour period. After 2 hours, rat erythrocytes in 3.8% Na citrate started to aggregate. When this experiment was done, fresh rat erythrocytes were used and the micrographs were taken during a 2-hr period.

Rat erythrocytes were centrifuged in the table-top centrifuge at medium speed for 30 seconds. The packed erythrocytes were washed with 3.8% Na citrate once and centrifuged at medium speed in the table-top centrifuge for 30 seconds. The washed erythrocytes were diluted to a 5% suspension in 3.8% Na citrate. Aliquots of 100 µl of 3.8% Na citrate were added to the first well of each row of a microtiter plate. These served as controls. For the serially-diluted agglutinin sample, aliquots of 50 µl of 3.8% Na citrate were added to each well (#2-12) of a row of the microtiter plate. 50 µl LMW, (or HMW or AI or AS) was added to the second

well. From this well, 50 µl was removed and added to the next well. This process was repeated for each succeeding well thus making each well more dilute by a factor of 2. Aliquots of 50 µl of 3.8% Na citrate were added to each well (#2-12). To each of these wells 25 µl of 5% rat erythrocytes in 3.8% Na citrate was added. Samples were removed from each well and examined under the microscope over a 2-hr period. One drop of the sample was placed on a cover slip, inverted, and carefully placed on the slide to avoid air bubbles. A sample from each well was examined with a light microscope at 400 X magnification. Observations were made after 5 min (erythrocytes just added to the sample), 30 min, 1 hour, and up to 2 hrs. Light micrographs were taken as described on page 16. The results are shown in Figures 6 - 10.

The effects of cations on hemagglutination activities of hemagglutinins

The test samples were CM extract from coelomocytes, HMW and LMW fractions from coelomic fluid supernatant. The protocol for

this experiment is as described as below :

(a) 1 ml of each test sample was in PBS-N_3 as control.

(b) 1 ml of each test sample was dialyzed against HEPES buffered saline with 1 mM EGTA (4.76 g/l HEPES, 0.9% NaCl, 1 mM EGTA, pH 7.5) for 3 hours at 4^0C.

(c) 1 ml of each test sample was dialyzed against HEPES buffered saline with 10 mM EGTA for 3 hours at 4^0C.

(d) 1 ml of each test sample was treated as in (c), then dialyzed against HEPES saline buffer with 1 mM $CaCl_2$ for another 3 hours at 4^0C.

(e) 1ml of each test sample was treated as in (c), then dailyzed against HEPES saline buffer with 1 mM $MgCl_2$ for another 3 hours at 4^0C.

The volume of dialyzing buffer was 500 ml in each case. All 15 samples were titered against rat erythrocytes. The control wells in the titer plates were PBS-N_3 in (a), HEPES saline buffer with 1 mM EGTA in (b), HEPES saline buffer with 10 mM EGTA in (c), HEPES

saline buffer with 1 mM $CaCl_2$ in (d), and HEPES saline buffer with 1 mM $MgCl_2$ in (e) respectively. The results are shown in Table 6.

Degradation or denaturation treatments

(1) Heat treatment

Aliquots of each sample (CM extract from coelomocytes, HMW fraction, and LMW DEAE peak I-IV samples) were heated on a steam bath for 1-2 hours, centrifuged at the table-top centrifuge for 3 minutes and the clear supernatants were titered. The results are shown in Table 7.

(2) Phenol treatment

1 ml of each sample (CM extract from coelomocytes, HMW fraction and LMW fraction, or 1 ml of PBS-N_3) was treated with 1 gram of phenol. The samples were mixed well by vortexing, then centrifuged in the table-top centrifuge for 3 minutes and the aqueous layers collected. The aqueous layers were dialyzed against

PBS-N_3 for 4 hours (fresh PBS-N_3 was replaced every hour) and titered. The results are shown in Table 7.

(3) Polyvinylpolypyrrolidone (PVPP)

Five grams of PVPP (insoluble form, Sigma) was purified by boiling for 10 minutes in 100 ml of 10% HCl, washing with dH_2O several times, neutralizing with 10 drops of concentrated KOH and then washing with dH_2O until free of chloride ion (68). The pellet was washed with PBS-N_3 once and then equilibrated with PBS-N_3 until the supernatant pH remained 7.2. Three ml of packed PVPP, pH 7.2 was mixed with 1.5 ml of CM extract from coelomocytes or HMW or LMW, shaken overnight at 4^0C, centrifuged in the table-top centrifuge for 3 minutes and the clear supernatant was titered. The results are shown in Table 7.

(4) Periodate treatment

(4-a) 500 µl of each sample (CM extract from coelomocytes, HMW

fraction and LMW fraction) was treated with 500 µl 0.05 M NaOAc, pH 5.0 (control) or 500 µl 0.05 M $NaIO_4$, in 0.05 M NaOAc, pH 5.0. 500 µl PBS-N_3 and 500 µl of 0.05 M $NaIO_4$ in 0.05 M NaOAc, pH 5.0 served as controls also. The samples were covered with aluminum foil and kept in the cold room overnight. Then one drop of 0.1 M ethylene glycol was added to each sample to remove excess $NaIO_4$. The samples were dialyzed against PBS-N_3 for 4 hours (buffer was changed once after 2 hours) and titered (69).

(4-b) 1 ml of HMW sample was treated with (i) 0.3 ml 0.2 M citrate buffer, pH 5.4 and 0.2 ml 0.004 M $NaIO_4$, pH 5.4 or (ii) 0.3 ml 0.2 M citrate buffer, pH 5.4 and 0.2 ml 0.04 M $NaIO_4$, or (iii) 0.5 ml 0.2 M citrate buffer, pH 5.4 (the last set served as control). The samples were incubated at room temperature for 3 hours in the dark, dialyzed against PBS-N_3 overnight and titered (27).

(4-c) DL-HHMW (prepared as described in p 43) , 8.434 mg in 1 ml

of dH_2O, was divided into two equal parts. The first part (0.5 ml) was mixed with an equal volume (0.5 ml) of 0.1 M sodium metaperiodate and the second part (0.5 ml) was mixed with an equal volume (0.5 ml) of dH_2O. The latter served as DL-HHMW sample control. 0.5 ml of PBS-N_3 were also mixed with 0.5 ml of 0.1 M sodium metaperiodate to serve as another control. The samples were wrapped with aluminum foil and kept in the dark at room temperture. Forty-eight hours and ninety-six hours later, 0.5 ml of the sample (DL-HHMW + 0.1 M $NaIO_4$ or DL-HHMW +dH_2O or PBS-N_3 + $NaIO_4$) was taken out, 0.01 ml of ethylene glycol was added to each sample to remove excess $NaIO_4$ and the sample allowed to stand 1 hr (70). The sample was dialyzed against PBS-N_3 overnight (with one change of fresh PBS-N_3) and titered. The results are shown in Table 7.

(5) Protease treatment

(5-a) 10 mg of insoluble protease from Streptomyces griseus (Sigma) was added to each of ten 1.5 ml microtubes (tubes 1-10), washed with PBS three times (1 ml of PBS each time) and centrifuged at a Beckman microfuge for 2 minutes. To half of the microtubes (tubes 6-10) was added 200 µl of PBS. They were heated on a steam bath for 30 minutes and centrifuged in a Beckman microfuge for 2 minutes to get the heated "heated protease" samples.

The protocol for this experiment is described as followed :

Tube * 1-4 : 200 µl of LMW DEAE peak I-IV + 10 mg of protease

* 5 : 200 µl of PBS + 10 mg of protease (control)

* 6-9 : 200 µl of LMW DEAE peak I-IV + 10 mg of heated protease

* 10 : 200 µl of PBS + 10 mg of heated protease (control)

The samples were shaken at 37^{0}C overnight and centrifuged at a Beckman microfuge for 2-3 minutes and the supernatants titered.

The results are shown in Table 7.

(5-b) DL-HHMW (6.0 mg in 150 µl of 1 mM phosphate buffer-2mM $CaCl_2$, pH 7.5) was divided into three equal portions. The first (50 µl) was mixed with 20 µl of 1 mM phosphate buffer-2 mM $CaCl_2$, pH 7.5 (DL-HHMW control), the second part (50 µl) was mixed with 20 µl of pronase (Streptomyces griseus, Boehringer Mannheim, 1% by wt) and the third part (50 µl) was mixed with 20 µl of heated pronase (1% by wt, heated on a boiling water bath for 30 minutes). 50 µl of 1 mM phosphate- 2 mM $CaCl_2$, pH 7.5 was also mixed with 20 µl of pronase (1% by wt) to serve as control. The samples were incubated at 37^0C for 20 hours, heated in boiling water bath for 10 minutes, diluted to 500 µl with buffer, centrifuged in the Beckman microfuge for 3 minutes and the clear supernatants titered. The results are shown in Table 10.

(6) TCA (Trichloroacetic acid) treatment

1 gram of trichloroacetic acid was added to 10 ml of

concentrated HMW in a 40 ml Teflon centrifuge tube. The solution was mixed by vortexing and centrifuged at 14 Krpm for 30 minutes. The precipitate portion was taken up in 1 ml of PBS-N_3, dialyzed aganist PBS-N_3 overnight, centrifuged and its hemagglutination titer was determied. The supernatant was dialyzed PBS-N_3 overnight and titered. The results are shown in Table 7.

(7) Chondroitinase ABC, keratanase and heparinase II treatments

(7-a) Chondroitinase ABC (71)

Chondroitinase ABC (Proteus vulgaris, 10 units, Sigma) was taken up in 4 ml of 0.1 mg/ml of BSA (bovine serum albumin). One ml of this enzyme solution was diluted with 3 ml of 0.1 mg/ml of BSA and divided into five portions (each portion had 0.5 units/0.8 ml). 5.080 mg of DL-HHMW was taken up in 1.016 ml of substrate buffer (250 mM Tris-HCl, 300 mM NaOAc and 0.5 mg/ml BSA, pH 8.0) to make 5 mg/ml. 3.865 mg of chondroitin sulfate B was taken up in 0.773 ml of substrate buffer to make 5 mg/ml. The protocol of this experiment is described below :

Tube # 1 : 200 µl of chondroitin sulfate B + 800 µl of 0.1 mg/ml BSA

2 : 200 µl of chondroitin sulfate B + 800 µl of chondroinase ABC (#1 & #2 served as standard assays for chondroitinase ABC)

3 : 200 µl of substrate buffer + 800 µl of chondroitinase ABC

4 : 200 µl of DL-HHMW + 800 µl of 0.1 mg/ml BSA

5 : 200 µl of DL-HHMW + 800 µl of chondroitinase ABC

6 : 200 µl of DL-HHMW + 800 µl of chondroitinase ABC which had been heated in a boiling water bath for 30 minutes.

7 : 200 µl of DL-HHMW + 800 µl of chondroitinase ABC. The mixture was heated in a boiling water bath for 30 minutes.

These seven samples were incubated at 37^{0}C for 2 hours, heated on the the boiling water bath for 10 minutes, centrifuged at a Beckman microfuge for 3 minutes to remove particles and dialyzed

against PBS-N_3 overnight. Tubes 3-7 were titered. The metachromatic spectra for tubes 1,2 and 4-7 were determined by the method of Chandrasekhar et al (47). The results are shown in Table 10.

(7-b) Keratanase (endo -ß-galactosidase) treatment (72)

Keratanase (Pseudomonas, 15 units, Sigma) was taken up in 300 µl of a special 0.1 M Tris-HCl buffer, pH 8.0. The latter consisted of 1.21 g of Tris, 0.024 g of NaOAc, 0.01 g of bovine serum albumin, 0.372 g of EDTA and 0.125 g of N-ethylmaleimide dissolved in dH_2O, the pH adjusted to 8.0 with HCl and the volume made up to 100 ml with dH_2O. Just before the experiment, pepstatin was added to a final concentration of 0.36 mM (previously dissolved in methanol to a concentration of 7.2 mM). 10.6220 mg of DL-HHMW was dissolved in 200 µl of dH_2O (53.11 mg/ml). 1 mg of keratan sulfate was dissolved in 100 µl of dH_2O (10 mg/ml). The protocol for this experiment is described below :

Tube * 1 : 50 µl of keratan sulfate (10 mg/ml) + 20 µl of 0.1 M special Tris-HCl buffer

* 2 : 50 µl of keratan sulfate (10 mg/ml) + 20 µl of keratanase (1 unit) in buffer (* 1 & 2 served as standard assays for keratanase)

* 3 : 50 µl of 0.1 M special Tris-HCl buffer + 20 µl of keratanase (1 unit) in buffer

* 4 : 50 µl of DL-HHMW + 20 µl of 0.1 M special Tris-HCl buffer

* 5 : 50 µl of DL-HHMW + 20 µl of keratanase (1unit) in buffer

* 6 : 50 µl of DL-HHMW + 20 µl of heated keratanase in buffer (1 unit, keratanase had been heated in the boiling water bath for 30 minutes)

* 7 : 50 µl of DL-HHMW + 20 µl of keratanase (1 unit) in buffer. The mixture was heated in the boiling water bath for 30 minutes.

These seven samples were incubated at 37^{0}C for 2 hours, heated

on the boiling water bath for 10 minutes, centrifuged in a Beckman microfuge for 3 minutes to remove particles, diluted to 500 µl with deionized water and dialyzed against PBS-N_3 overnight. Tubes 3-7 were titered. The metachromatic spectra for tubes 1,2 and 4-7 were determined by the method of Chandrasekhar et al (47). The results are shown in Table 10.

(7-c) Heparinase II treatment (73)

Heparinase II (Flavobacterium heparium, 10 units, Sigma) was taken up in 0.5 ml of 0.1 M NaOAc, pH 7.0 and divided into five equal portions (2 units/0.1 ml). 5.120 mg of DL-HHMW was dissolved in 0.2048 ml of dH_2O (25 mg/ml). 7.280 mg of heparin sodium [General Biochemical Inc. (Chagrin Falls, Ohio)] was taken up in 0.2912 ml of dH_2O (25 mg/ml). The protocol for this experiment is described below :

Tube # 1 : 0.04 ml of heparin sodium + 0.03 ml of 10 mM $Ca(OAc)_2$ + 0.23 ml of 0.1 M NaOAc

* 2 : 0.04 ml of heparin sodium + 0.03 ml of 10 mM $Ca(OAc)_2$ + 0.13 ml of 0.1M NaOAc + 0.1 ml of heparinase II (2 units)(* 1 & 2 served as the standard assays for heparinase II)

* 3 : 0.03 ml of 10 mM $Ca(OAc)_2$ + 0.17 ml of NaOAc + 0.1 ml of heparinase II (2 units)

* 4 : 0.04 ml of DL-HHMW + 0.03 ml of 10 mM $Ca(OAc)_2$ + 0.23 ml of 0.1 M NaOAc

*5 : 0.04 ml of DL-HHMW + 0.03 ml of 10 mM $Ca(OAc)_2$ + 0.13 ml of 0.1 M NaOAc + 0.1 ml of heparinase II (2 units)

*6 : 0.04 ml of DL-HHMW + 0.03 ml of 10 mM $Ca(OAc)_2$ + 0.13 ml of 0.1 MNaOAc + 0.1 ml of heated heparinase II (2 units, heparinase II was heated on the boiling water bath for 30minutes)

* 7 : 0.04 ml of DL-HHMW + 0.03 ml of 10 mM $Ca(OAc)_2$ +

0.13 ml of 0.1 M Na OAc + 0.1 ml of heparinase II (2 units), this mixture was heated on the boiling water bath for 30 minutes

These seven samples were incubated at 37^{0}C for 2 hours, heated on the boiling bath for 10 minutes, centrifuged in a Beckman microfuge for 3 minutes to remove particles, diluted to 500 µl of deionized water and dialyzed against PBS-N_3. Tubes 3-7 were titered. The metachromatic spectra for tubes 1,2 and 4-7 were determined by the method of Chandrasekhar (47). The results are shown in Table 10.

(8) Desulfation

The procedure of Nagasawa et al (74) which involved N-acetylation and desulfation was used. (i) Desulfation with dimethyl sulfoxide (DMSO) containing methanol (mainly N-desulfation) : DL-HHMW (8.280 mg in 8.280 ml of dH_2O) was passed through a Dowex 50 W-8X (20-50 mesh) column (diameter 0.8

cm and height 13 cm, which had been preequilibrated with 0.1 M aqueous pyridine). The column was washed with two bed volumes of 0.1 M aqueous pyridine. The pyridinium salt of DL-HHMW thus obtained, was lyophilized, dissolved in 15 ml of dimethylsulfoxide (DMSO) : methanol (v/v) = 9 : 1. The sample was kept in a wired-shut glass-stoppered flask containing 4 Å molecular sieve (~ 1ml) and heated on the steam bath for 21 hours. The reaction mixture was cooled down, centrifuged in a table-top centrifuge at medium speed for 3-5 minutes and the supernatant was diluted with an equal volume of water (15 ml), the pH adjusted to 9.0 with 0.1 M NaOH, dialyzed against running tap water for 6 hours and then dialyzed against deionized water overnight. This dialyzed solution was supposed to contain partially desulfated sample.

(ii) N-acetylation and desulfation with DMSO containing methanol (to remove residual, bound sulfate group): The dialyzed solution from the first desulfation treatment was lyophilized and taken up in 3 ml of dH_2O, 0.30 ml of methanol and 1.8 ml (packed volume) of

Dowex-1 (carbonate form), kept in the cold room until the temperature of the solution was 0-5^{0}C and then 2.0 ml of acetic anhydride was added. The mixture was shaken for 2 hours and centrifuged in the table-top centrifuge. The supernatant was removed and the pellet washed with 1 ml of dH_2O three times. The supernatant and washes were combined and dialyzed against running water for several hours and then dialyzed against dH_2O overnight. This N-acetylated, presumbly partially-desulfated sample was converted to the pyridinium salt by the method which was described above. It was lyophilized, treated with 4 ml of DMSO : methanol (V/V) = 9 : 1 and heated on the steam bath for 2 hours. The mixture was cooled down, diluted with 4 ml of dH_2O, then pH adjusted to 9.0 with 0.1 N NaOH, dialyzed against running water for several hours, and then dialyzed against dH_2O overnight and lyophilized The lyophilized sample was taken up in 4 ml of PBS-N_3 and the titer was determined. The results are shown in Table 10.

Experiments with EATC

(1) Effects of hemagglutinins on EATC growth in mice (in vivo)

EATC were obtained from mice 2 weeks after they had been injected with an EATC suspension prepared from the frozen state. The concentration of EATC was determined as described on page 36 and diluted with sterile PBS to get 10^7 cells/ml.

LMW (11 ml, titer $\geq 2^{12}$, prepared as described on page 41), HMW (11ml, titer $\geq 2^{12}$, prepared as described on page 42) and PBS-0.001% Triton X-100 (PBS-T) were sterilized by passing through 45 µm Millipore filter units into sterile bottles. The CM extract from coelomocytes (11 ml in PBS-T, titer $\geq 2^{12}$) was sterilized by heating on the steam bath for 1 hour.

The protocol used in this experiment was similar to that of Eckhardt, Malone and Goldstein (75). Nine groups of mice (male CF_1), 5 mice in each group, weighing 29-45 grams, were used. These were injected for 10 days as follows.

Group #	Treatment	Day 0	Day 1-4	Day 7-11
# 1	PBS +EATC	0.2 ml PBS +0.1 ml EATC	0.1 ml PBS	0.1 ml PBS
# 2	PBS	0.3 ml PBS	0.1 ml PBS	0.1 mlPBS
# 3	LMW +EATC	0.2 ml LMW + 0.1 ml EATC	0.1 ml LMW	0.1ml LMW
# 4	LMW	0.2 ml LMW + 0.1 ml PBS	0.1 ml LMW	0.1 ml LMW
# 5	HMW +EATC	0.2 ml HMW + 0.1 ml EATC	0.1 ml HMW	0.1 ml HMW
#6	HMW	0.2 ml HMW + 0.1 ml PBS	0.1 ml HMW	0.1 ml HMW
#7	CM +EATC	0.2 ml CM + 0.1 ml EATC	0.1 ml CM	0.1 ml CM
#8	CM	0.2 ml CM + 0.1 ml PBS-T	0.1 ml CM	0.1 ml CM
# 9	PBS-T	0.2 ml PBS-T + 0.1 ml EATC	0.1 ml PBS-T	0.1 ml PBS-T

PBS-T : PBS-0.001% Triton X-100.

All samples were injected intraperitoneally, using sterile 1 ml syringes and 25 gauge sterile disposable needles. The entire

procedure was performed under sterile conditions. Tumor cell growth was monitored by weighing the mice daily on a triple beam balance. Survival time was recorded in days. The experiment was terminated on day 74, at which point postmortems were performed on surviving mice. The results are in Figures 21-22.

(2) Effects of hemagglutinins on Ehrlich ascites tumor cells (EATC) (in vitro)

Frozen Ehrlich ascites tumor cells that had been stored in 40 % glycerol were thawed at room temperature. The cells were washed three times with 10 ml of PBS followed each time by centrifugation in an International Table-Top Clinical centrifuge for 3 minutes and the supernatants were discarded. The packed cells were diluted 1 :10 in PBS.

(2-a) Light micrographic studies

Aliquots of 50 µl of PBS and 50 µl of the EATC suspension were added to each well of one row of a microtiter plate. This row

served as control. For the serially-diluted agglutinin samples, aliquots of 50 µl of PBS were added to each well of a row of the microtiter plate. A 50 µl of the LMW sample was added to the first well. From this well 50 µl was removed and added to the next well. This procedure was repeated for each succeeding well thus making each well more dilute by a factor of 2. To each of these wells 50 µl of tumor cells were added. The plate was sealed, shaken for 3 minutes on a Tektator Plate Shaker (Scientific Products) and incubated at room temperature for 30 minutes. Samples were removed from each well and examined by light microscope at 400 X magnification over a two-hour period, using the "hanging drop technique". Light micrographs were taken as described on page 16.

The same procedure was repeated for incubation of HMW and CM extract (prepared in PBS-T) with tumor cells. However, the control for the CM extract was PBS-T. The results are shown in Figures 23-27.

(2-b) Scanning electron micrographic studies

EATC (treated with PBS or PBS-T, or Nereis agglutinins) were transferred with a sterile pipette to a plastic petri dish containing several pieces of glass coverslips. After a period of adjustment for the cells (usually 1-2 hours), the medium from the petri dish was pipetted to allow a small level of medium to remain over the coverslips and cells. The cells were fixed by gradually flooding the bottom of the petri dish and coverslips with the fixative (2% glutaraldehyde, 0.13 M phosphate buffer, pH 7.3, in 0.13 M sucrose) for 1-2 hr at room temperature. After rinsing several times with 0.13 M phosphate buffer, pH 7.3 in 0.13 M sucrose, the coverslips were placed in a small wire mesh container and dehydrated in a graded series of ethanol-H_2O solutions through to two changes of absolute ethanol. The cells were transferred from absolute ethanol through a graded series of ethanol-amyl acetate mixtures to absolute amyl acetate. The samples were dried by the critical point method, mounted and coated with gold-palladium, then observed

with a high resolution, field emission scanning electron microscope (Cambridge S-4). This work was done by Deborah Brathwaite and Carlene Broderick under the supervision of Dr. Osinchak. The results are shown in Figures 28-32 and are representative of the whole field.

RESULTS

The results of fractionation of Nereis coelomic fluid

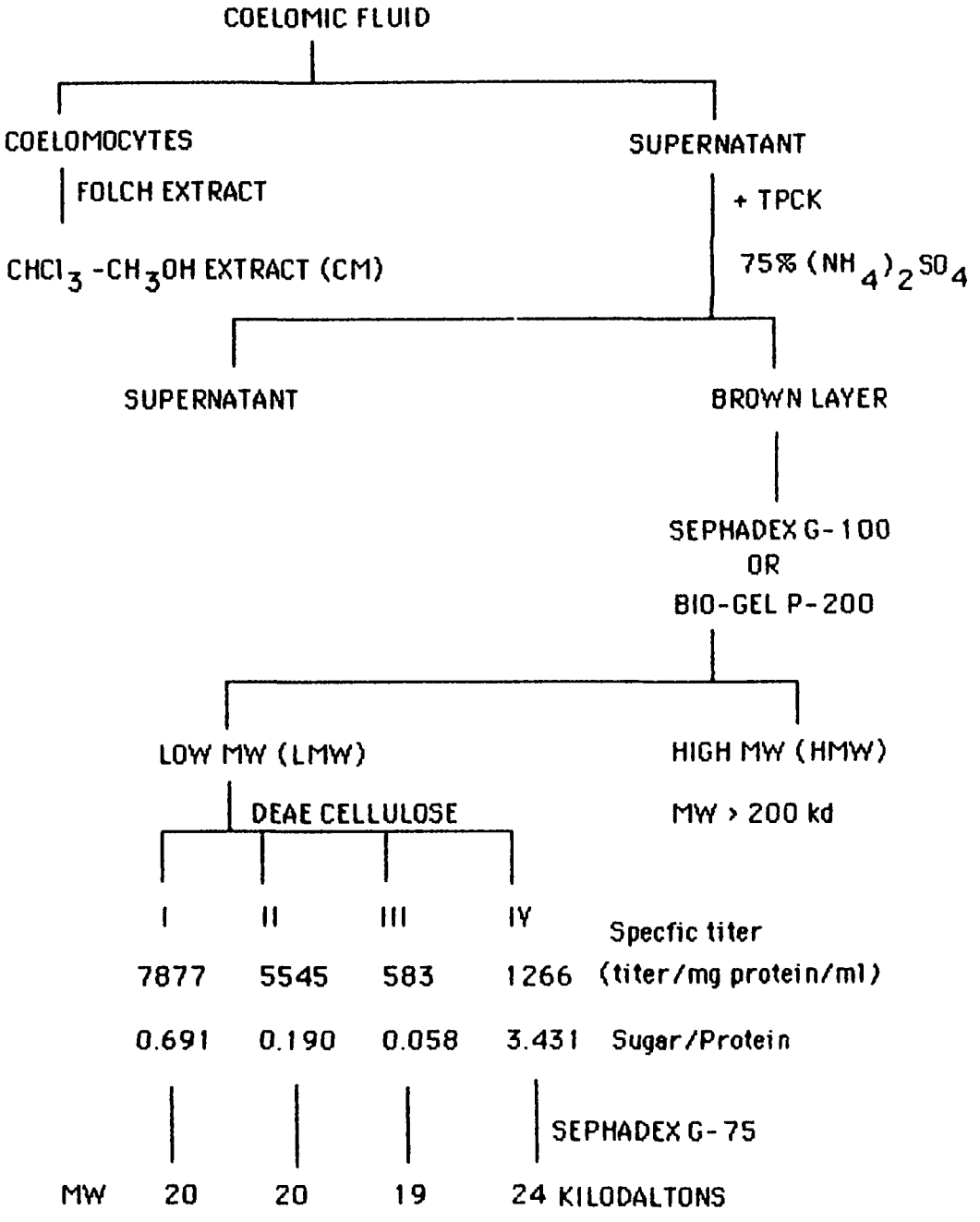
The coelomic fluid from Nereis virens was separated into the particulate matter (mainly coelomocytes) and the supernatant by centrifugation. The supernatant was made 1 mM TPCK and brought to 75% saturated with ammonium sulfate. A brown oily layer, containing the active material, separated and floated to the surface (Scheme 1). This brown oil was further fractionated by Sephadex G-100 or Bio-gel P-200 chromatography into a high molecular weight fraction (HMW, MW > 200 kd) at void volume and a low molecular weight fraction (LMW, MW ~19 kd) (Figures 1 & 2).

LMW was fractionated by DEAE cellulose chromatography into four active fractions (I-IV). They proved to be glycoproteins and their molecular weights were 20 kd, 20 kd, 19 kd, 24 kd respectively (see Scheme 1 for method of isolation and Figure 3 for DEAE chromatography of LMW agglutinin, showing protein and hexose determinations).

The HMW elution profile on Sepharose 4 B showed that the hemagglutination activity of HMW was spread over a wide molecular weight range (Figures 4 & 5) and its activity was correlated with the concentration of hexose (Figure 4). The HMW fraction was heated (HHMW) and further fractionated by delipidation to give DL-HHMW and lipid agglutinin (Scheme 2).

A chloroform-methanol (CM) extract was obtained from the coelomocytes by Folch extraction (Scheme 3). The CM extract was further treated with acetone-10% $MgCl_2$ and divided into acetone-insoluble (AI) and acetone-soluble (AS) fractions (See Scheme 4).

FRACTIONATION OF NEREIS COELOMIC FLUID



Scheme 1

Figure 1 Sephadex G-100 Chromatography of 75 % $(NH_4)_2SO_4$ Precipitate of Nereis Coelomic Fluid Supernatant

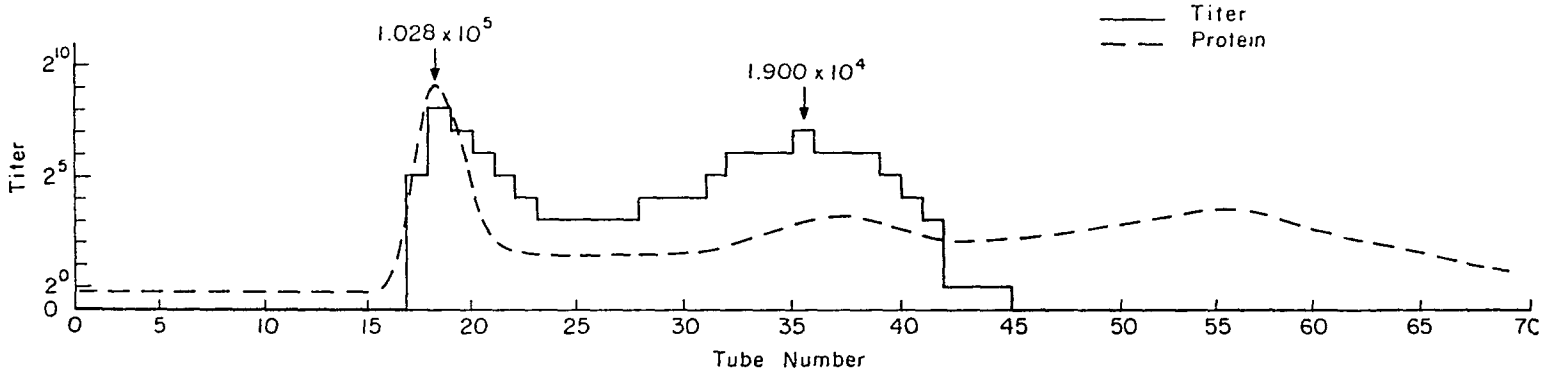


Figure 2 Bio-gel P-200 Chromatography of 75 % $(NH_4)_2 SO_4$ Prepicitate of Nereis Coelomic Fluid Supernatant

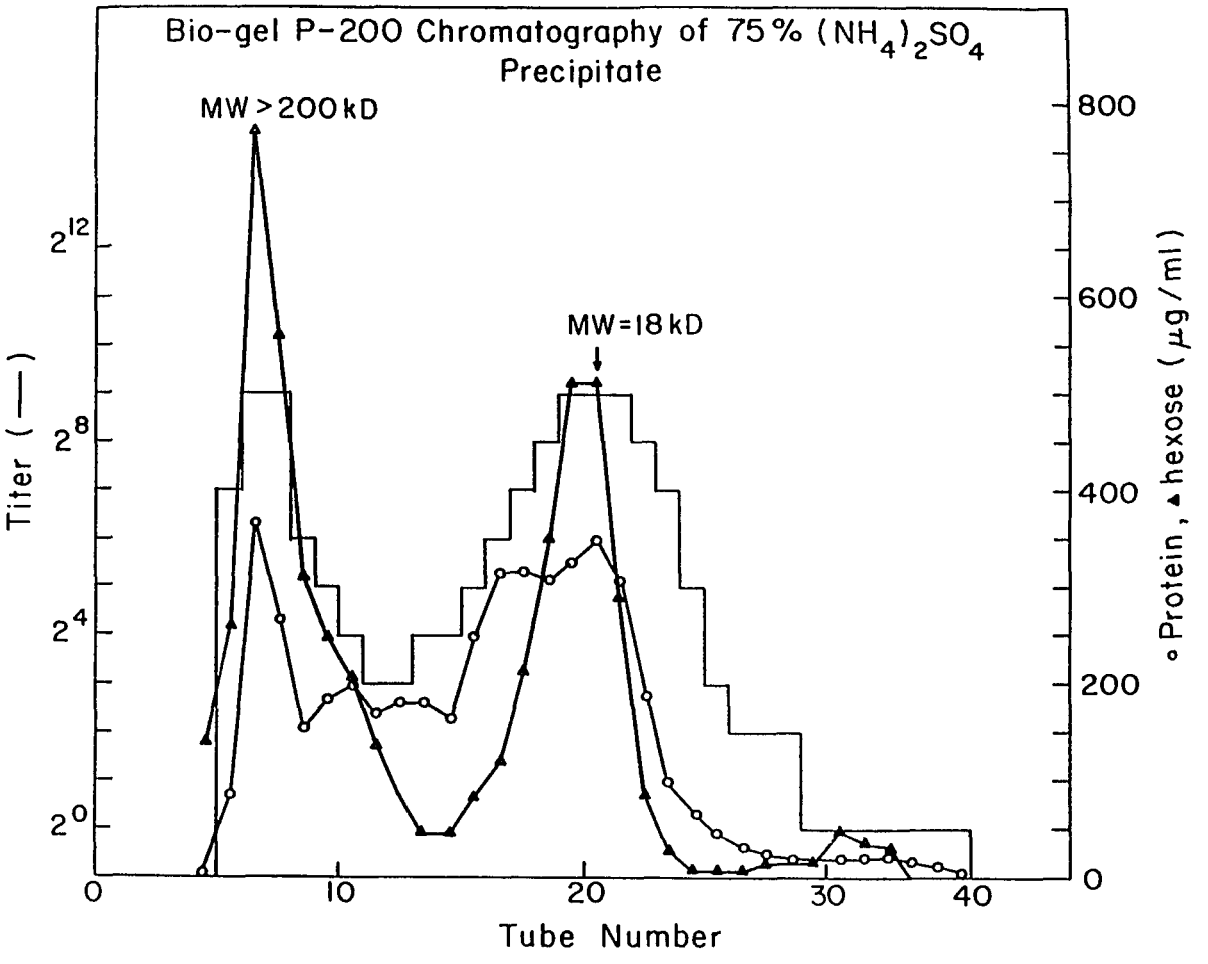


Figure 3 DEAE-Cellulose Chromatography of Low MW Agglutinin

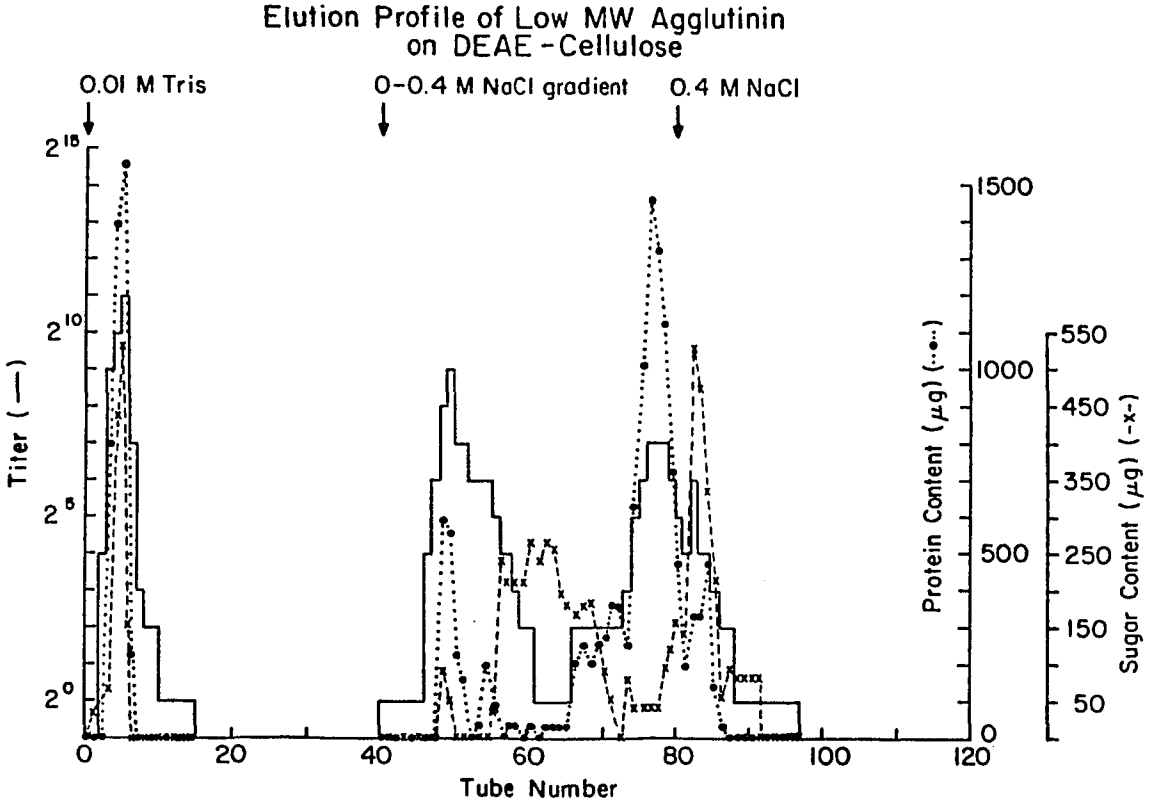
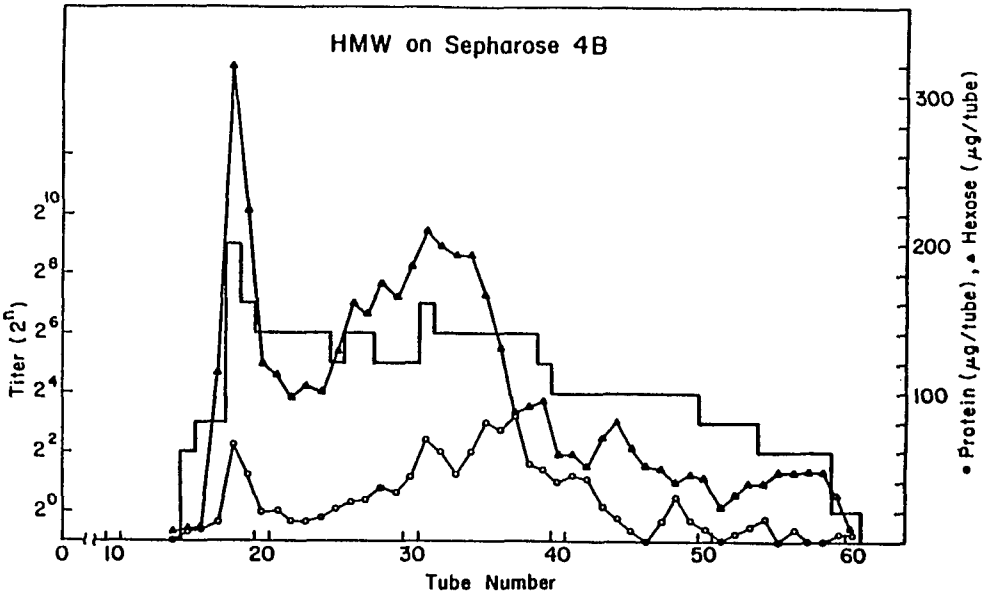
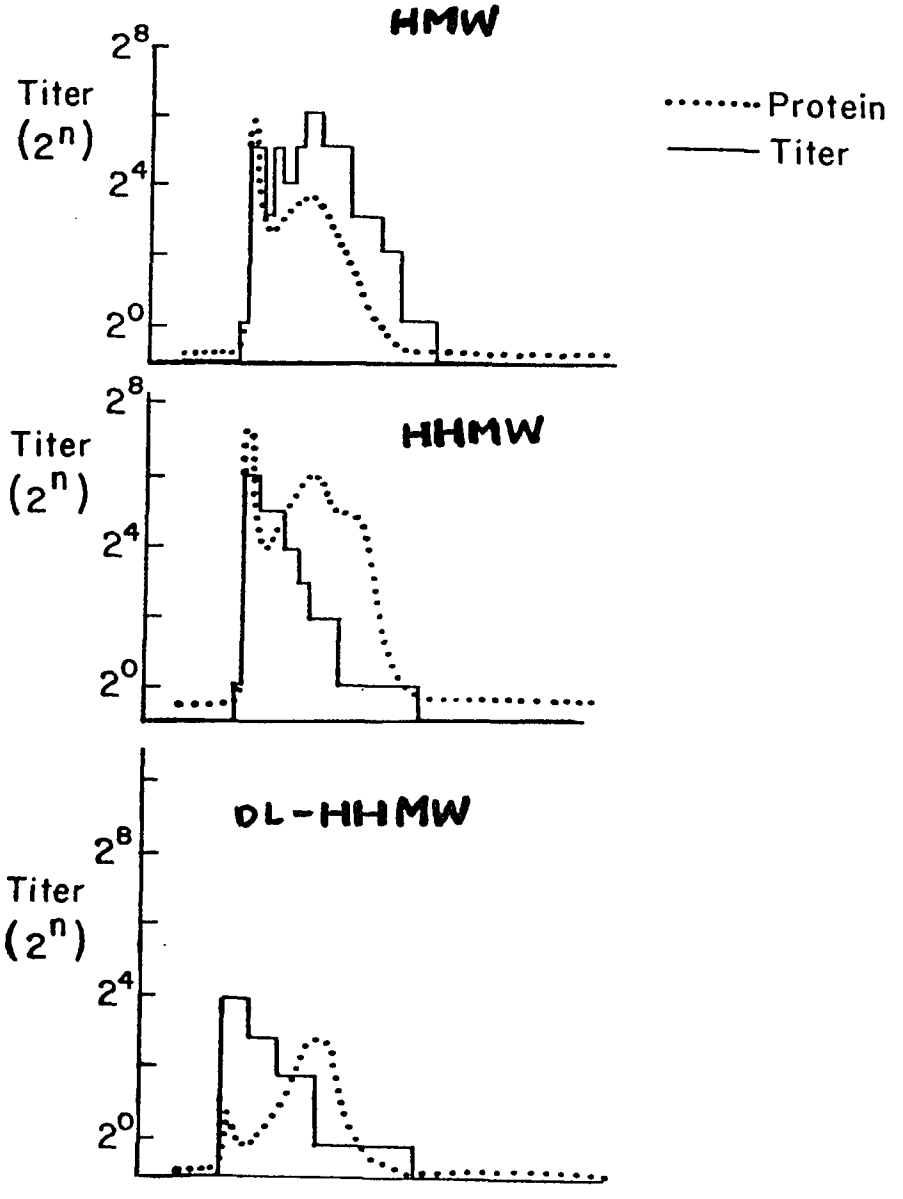


Figure 4 Sepharose 4 B Chromatography* of HMW

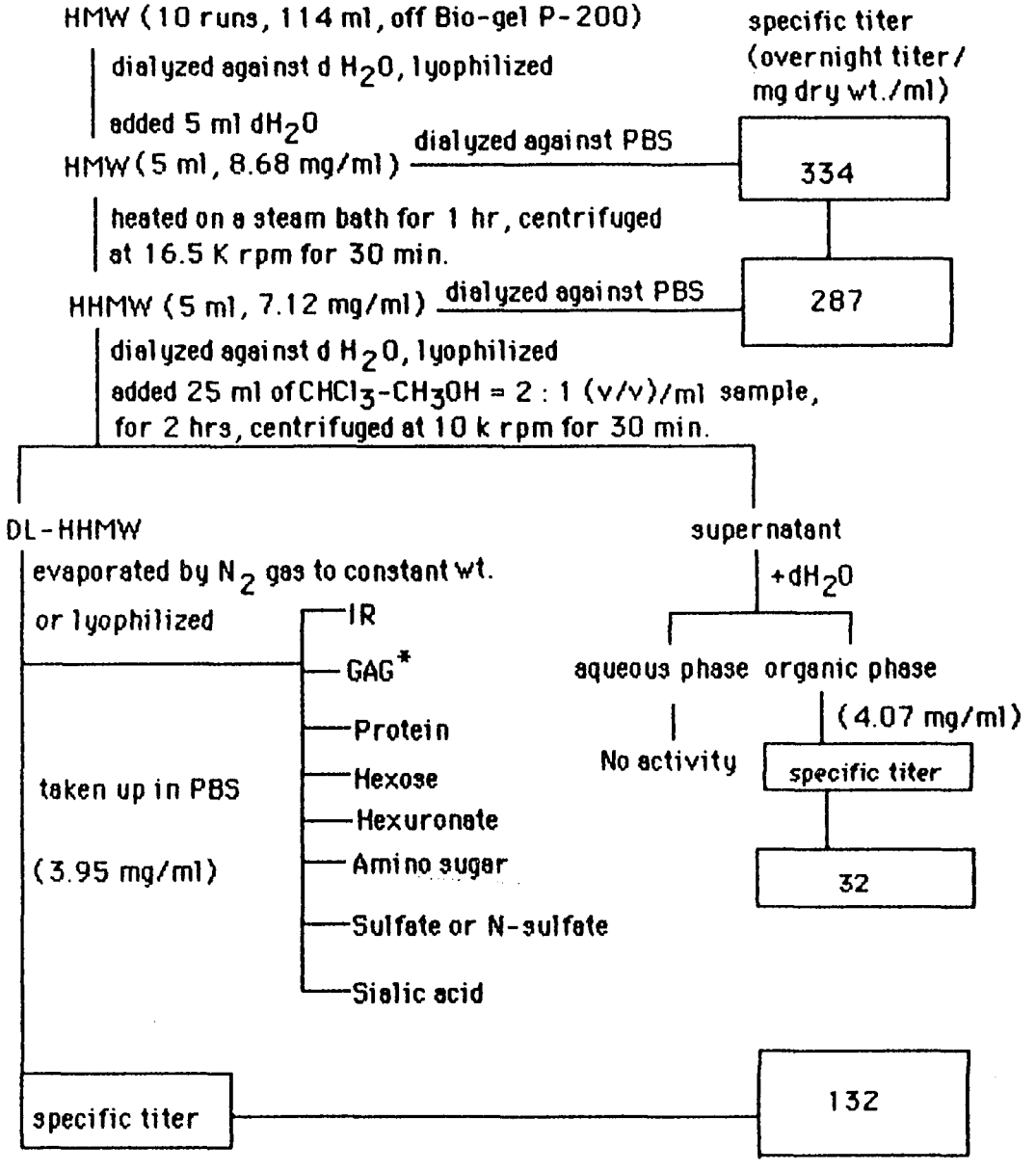


* Scales for protein and hexose are the same.

Figure 5 Sepharose 4 B Chromatography of HMW, HHMW and DL-HHMW



FRACTIONATION OF HMW FROM NEREIS COELOMIC FLUID SUPERNATANT

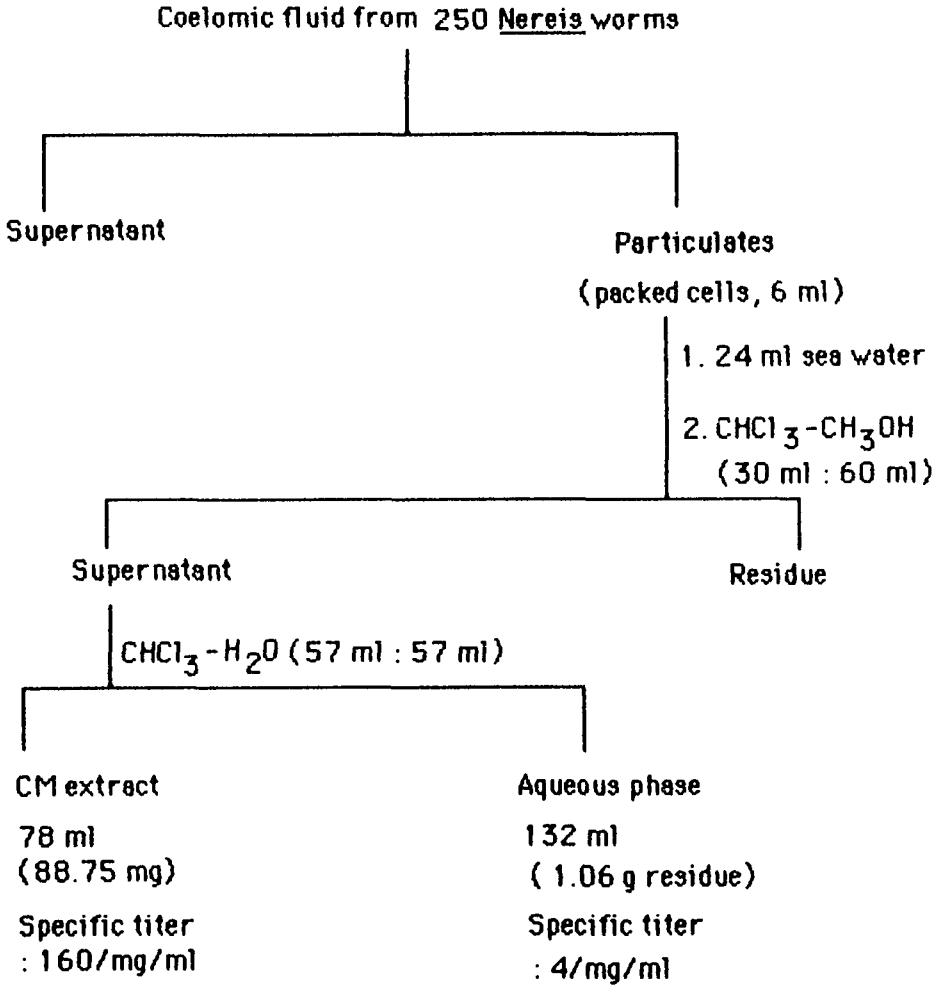


IR : infrared spectrum

GAG* : glycosaminoglycan

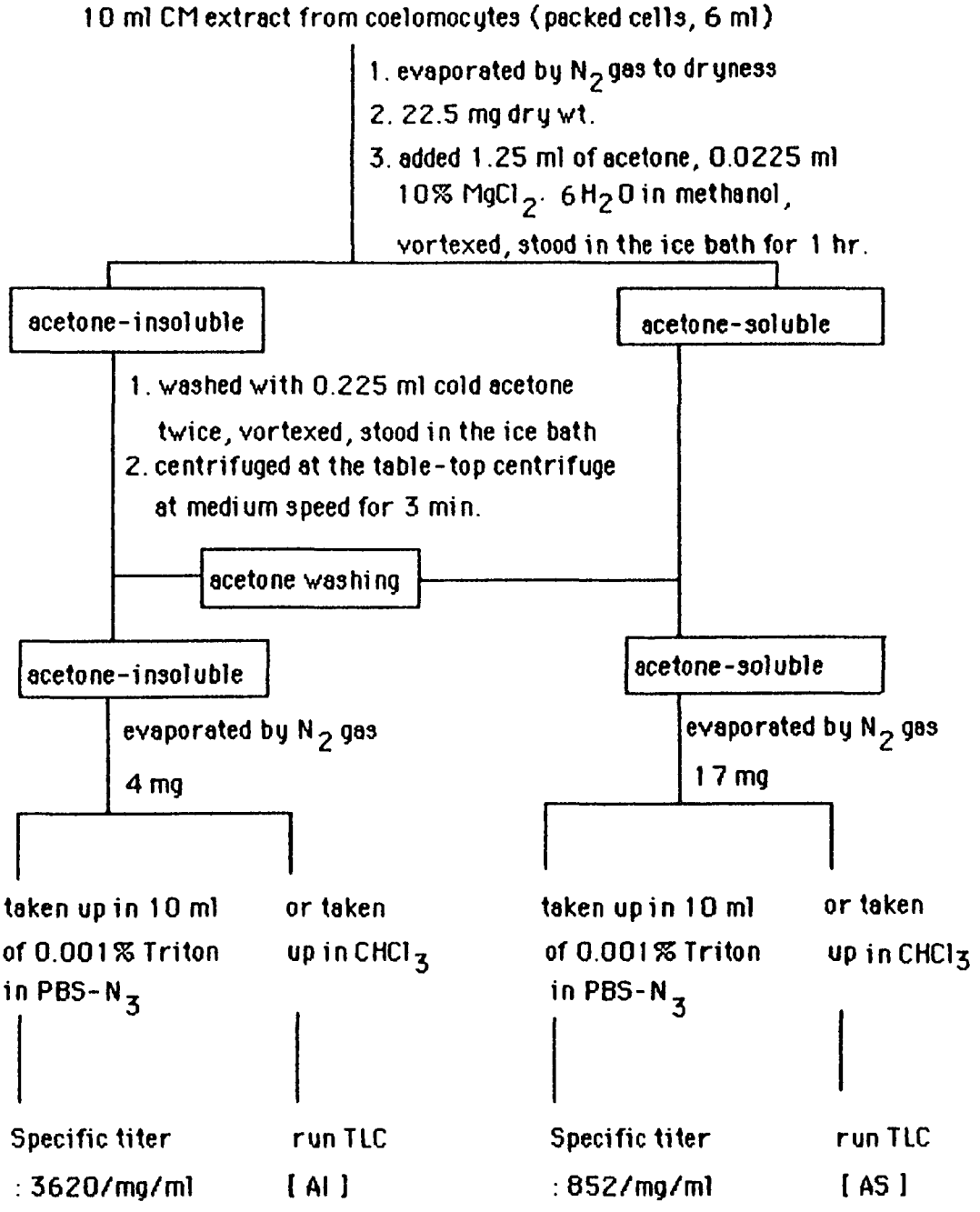
Scheme 2

FOLCH EXTRACTION



Scheme 3

FRACTIONATION OF CM EXTRACT FROM NEREIS COELOMOCYTES



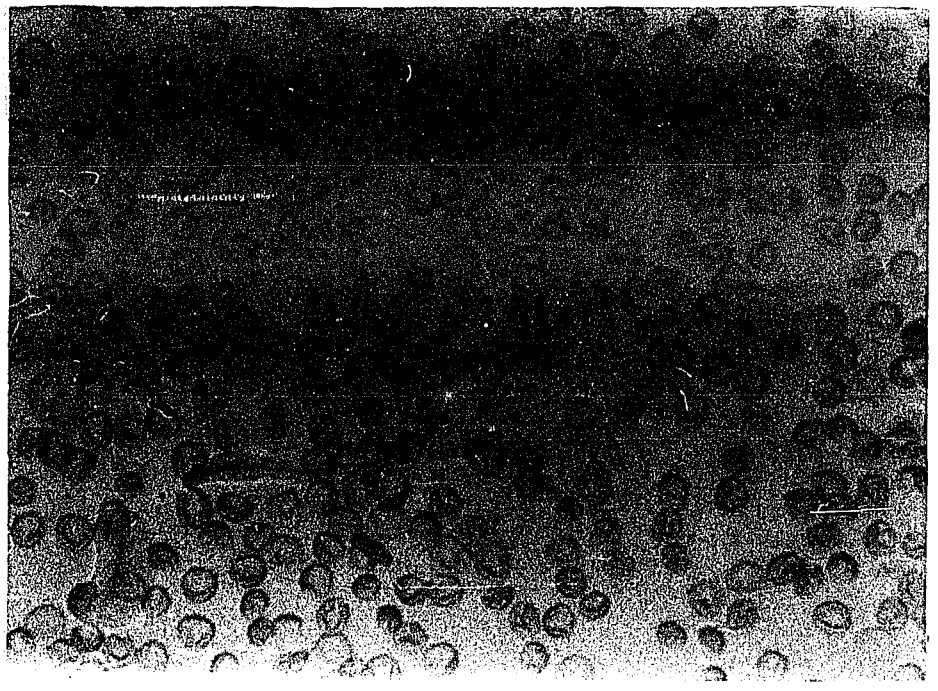
Scheme 4

The nature of the agglutination of rat RBC by Nereis agglutinins

Agglutination of rat RBC by LMW, HMW and CM (acetone-insoluble and -soluble fractions) was visualized under the light microscope (Figures 6-10). Rat RBC started to clump (rosettes formed) immediately after adding Nereis agglutinins. The clumps hampered visibility so the field was moved to an area with a sparser cell population to detect whether cells had fused or agglutinated in chains or agglutinated in rosettes. Mixtures of chains & rosettes were observed. No fusion was observed. Agglutination was found to be time- and concentration-dependent and to involve "cell clumping" but not "cell fusion".

Figure 6 Light micrographs of untreated rat RBC, 400 X

(a) 0 min



(b) 30 min

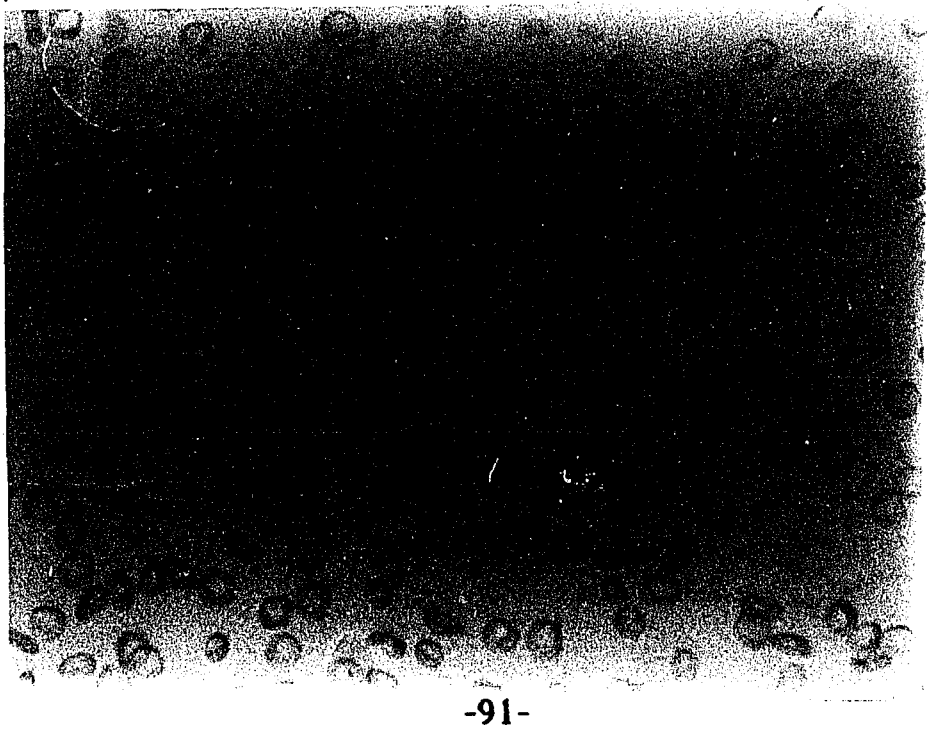
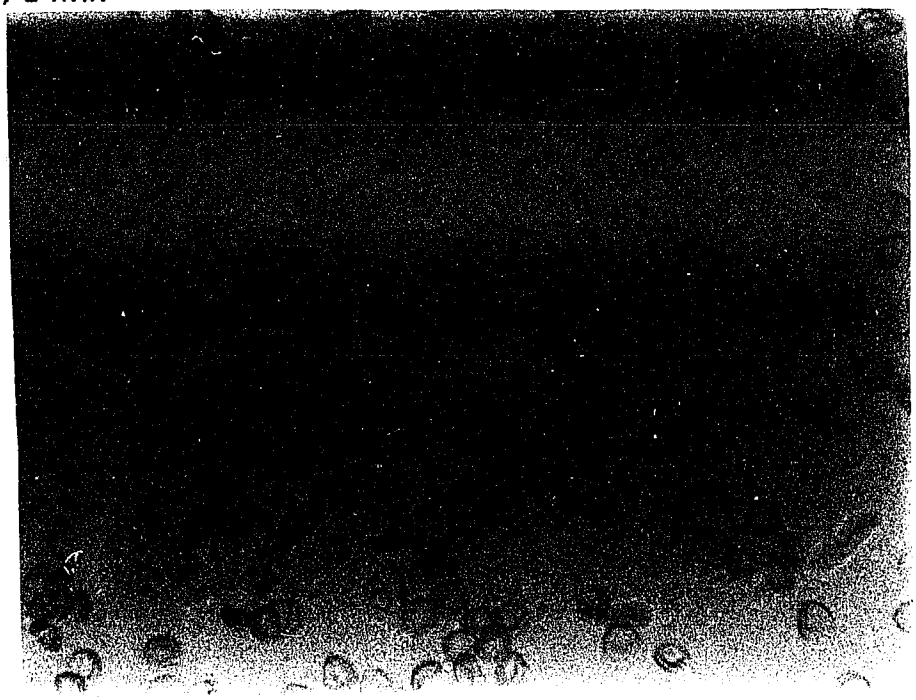


Figure 7 Light micrographs of rat RBC treated with Nereis LMW agglutinin (300µg/ml), 400 X

7-(a) 5 min



7-(b) 30 min

7-(c) 1 hr 30 min

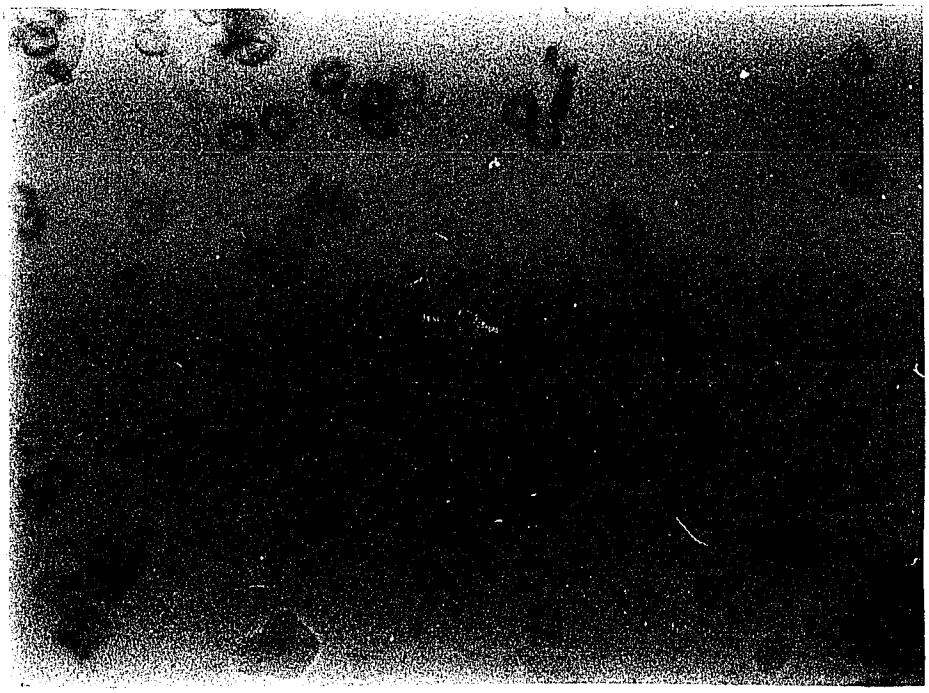
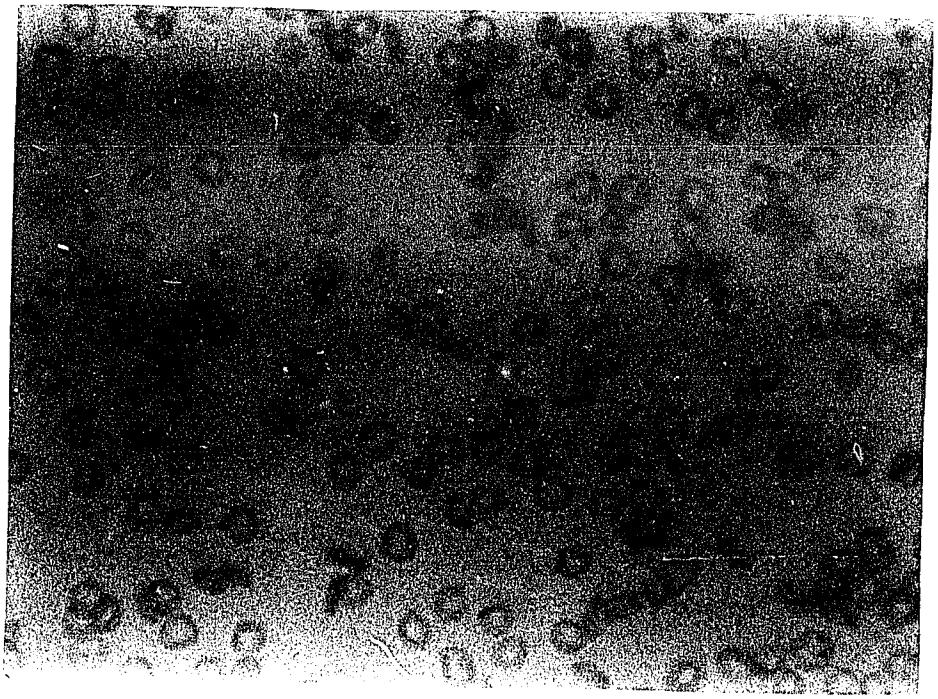


Figure 8 Light micrographs of rat RBC treated with Nereis HMW agglutini(1.061 mg/ml), 400 X

8-(a) 5 min



8-(b) 30 min

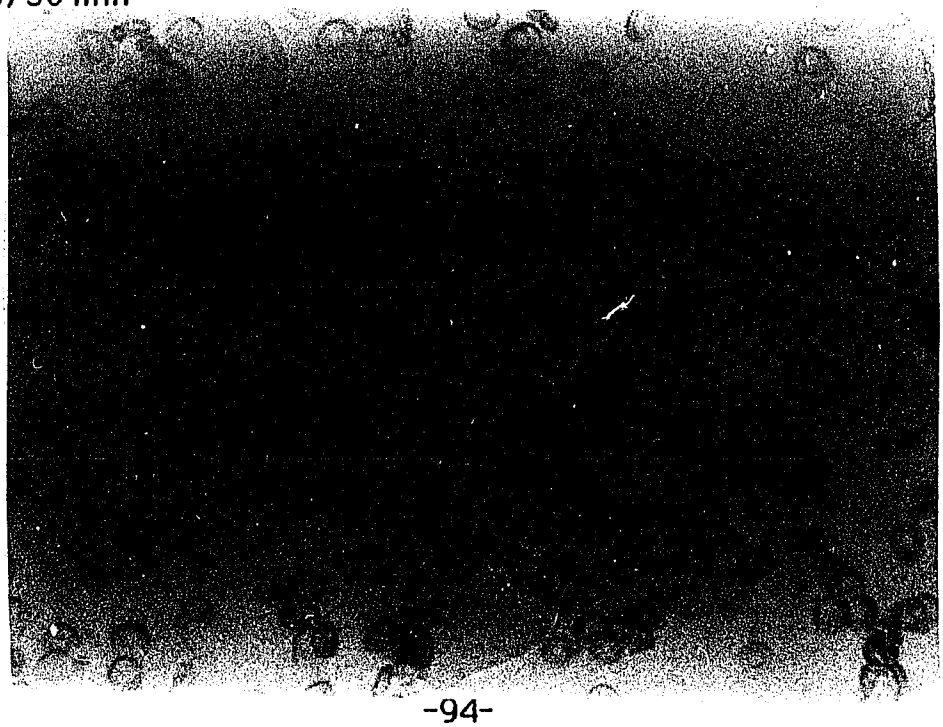
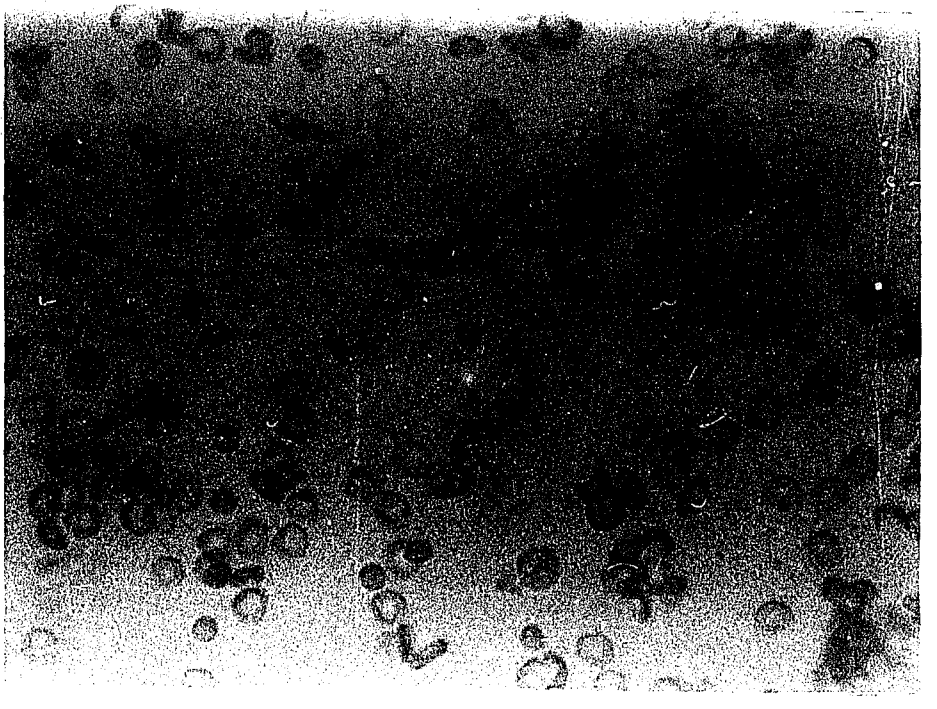
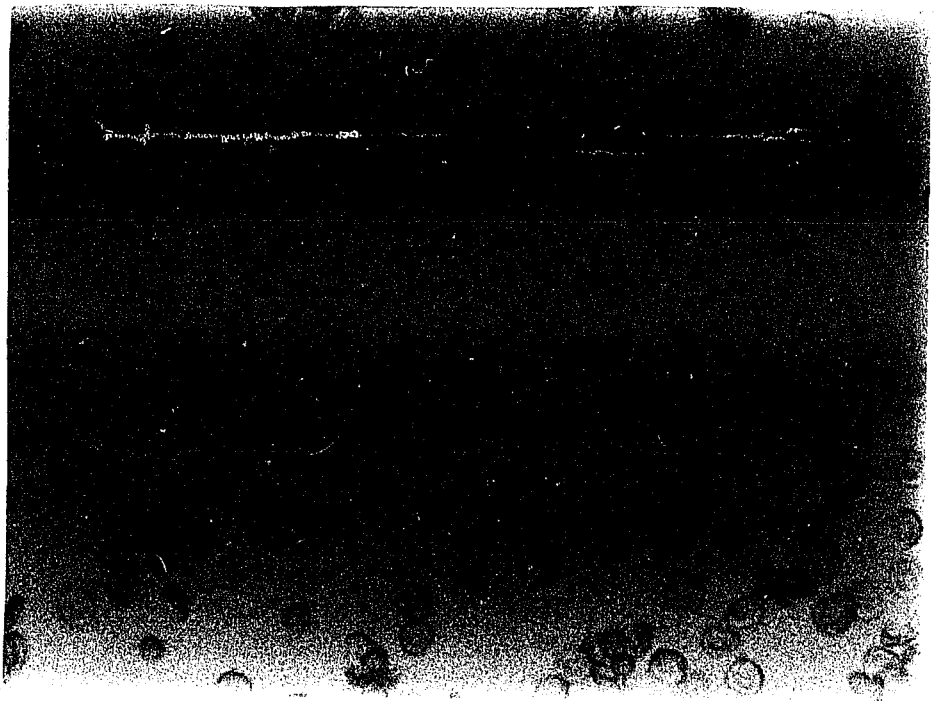


Figure 9 Light micrographs of rat RBC treated with Nereis acetone-insoluble lipid agglutinin (135 µg/ml), 400 X

9-(a) 5 min



9-(b) 30 min



9-(c) 1 hr

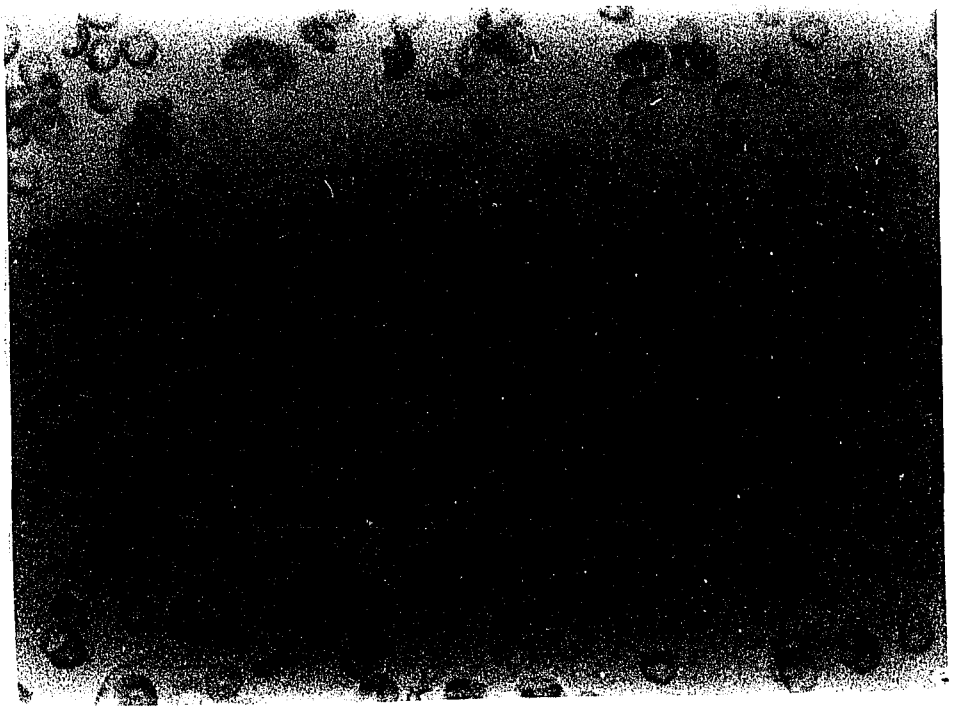
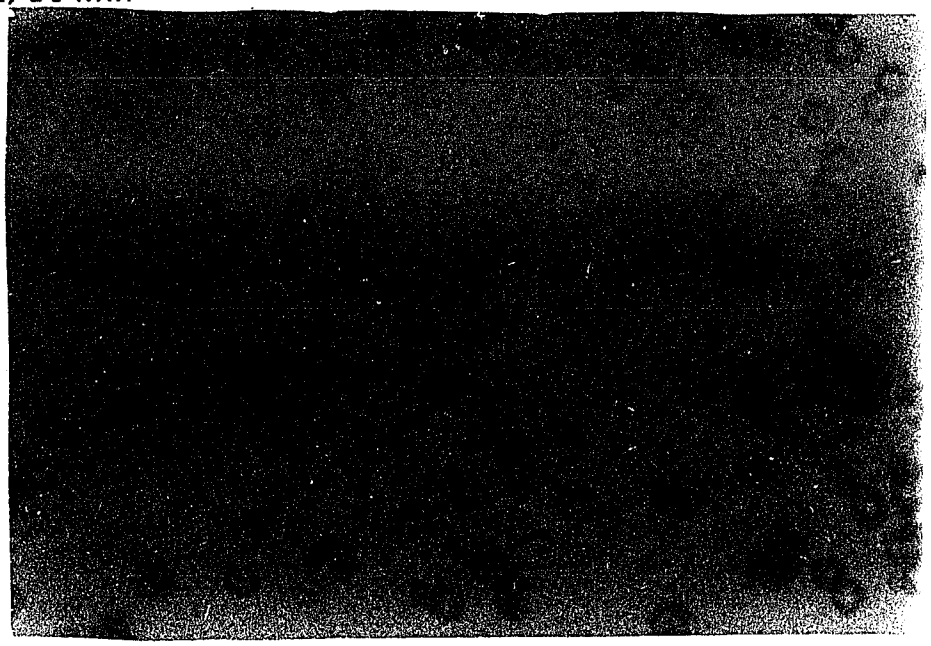
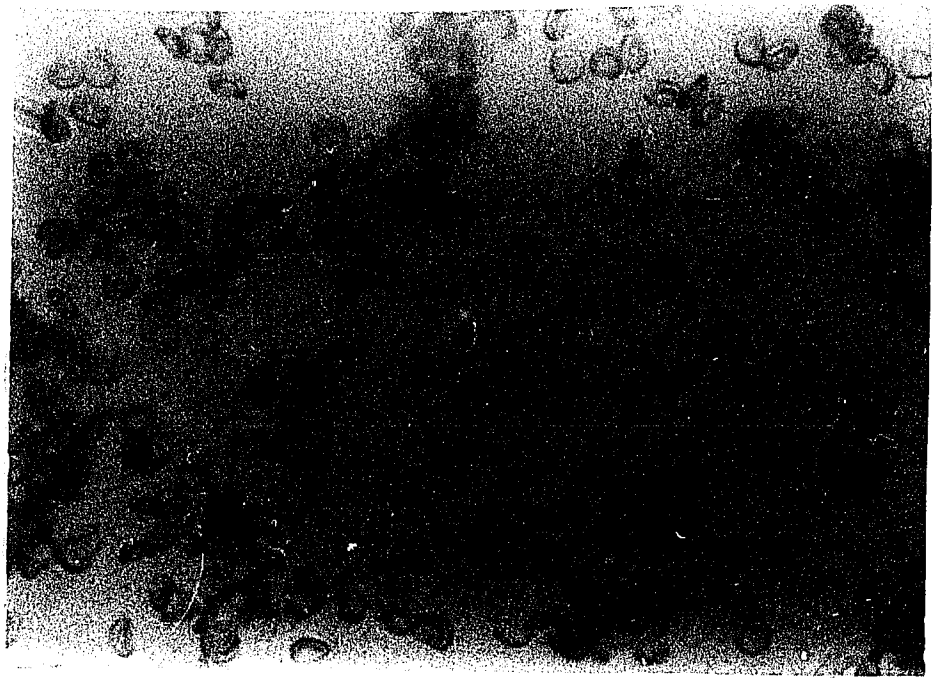


Figure 10 Light micrographs of rat RBC treated with Nereis acetone soluble lipid agglutinin (294 µg/ml), 400 X

10-(a) 30 min



10-(b) 1 hr 30 min



RBC specificity

Tables 1 and 2 show the RBC specificity profiles. (a) The order of RBC specificity for LMW I-III was rat >> chicken. The order of RBC specificity for LMW IV was rat > human (O) = rhesus monkey = African green monkey = steer > guinea pig > sheep. (b) The order of RBC specificity for HMW was rabbit > rat >> chicken. (c) The order of RBC specificity for CM was rat > rabbit > African green monkey > rhesus monkey = human (O) = guinea pig > chicken (Table 1). The acetone-insoluble material from CM was resolved in one dimensional TLC. TLC bands I-III had significant agglutinin activity against rat > rabbit > chicken (but for bands IV-V, rat > rabbit). The CM acetone-soluble TLC band II had significant agglutinin activity against rat > rabbit > chicken = guinea pig (but for band I, rat > rabbit > chicken) (Table 2).

Table 1 Erythrocyte Specificities for Nereis Agglutinins

	CM[1]	HMW[2]	LMW FRACTIONS[3]			
			I	II	III	IV
MW[4] (kd)			20	20	19	24
RBC[5]	RELATIVE TITER[6]					
Human (O)	2^5	0	0	0	0	2^5
Rhesus monkey	2^5	0	0	0	0	2^5
African green monkey	2^6	2^5	0	0	0	2^5
Steer	0	0	0	0	0	2^5
Goat	0	0	0	0	0	0
Horse	0	0	0	0	0	0
Sheep	0	0	0	0	0	0
Rabbit	2^9	2^{15}	0	0	0	0
Rat	2^{12}	2^{12}	2^{12}	2^{12}	2^{12}	2^{10}
Guinea pig	2^5	0	0	0	0	2^4
Turkey	0	0	0	0	0	0
Goose	0	0	0	0	0	0
Chicken	2^2	2^2	2^2	2^2	2^2	0
Pigeon	0	0	0	0	0	0

1 : Chloroform-methanol extract.
2 : High molecular wt. fraction.
3 : Low molecular wt. fractions off DEAE column.
4 : Molecular weight
5 : Red blood cell.
6 : As defined on page 20-21.

Table 2 Erythrocyte Specificities for Nereis Lipid Agglutinins Derived from CM

	Relative Titer[a]						
	Acetone-insoluble					Acetone-soluble	
TLC bands[b]	I	II	III	IV	V	I	II
R_f[c]	0	0.11	0.13	0.39	0.97	0	0.95
RBC[d]							
rat	2^8	2^7	2^8	$2^{3.5}$	2^5	$2^{4.5}$	2^8
sheep	0	0	0	0	0	0	0
goat	0	0	0	0	0	0	0
horse	0	0	0	0	0	0	0
guinea pig	0	0	0	0	0	0	2^2
chicken	2^3	2^3	$2^{2.5}$	0	0	2^2	2^2
dog	0	0	0	0	0	0	0
rabbit	$2^{5.5}$	$2^{4.5}$	$2^{4.5}$	$2^{1.5}$	$2^{3.5}$	2^2	2^6
steer	0	0	0	0	0	0	0

Acetone-insoluble and -soluble fractions from CM extract treated with acetone-10% $MgCl_2$.

(a) overnight titer, titer defined on page 20-21.

(b) The TLC extracts were prepared as described on page 48-50.

(c) R_f value in $CHCl_3$-CH_3OH-acetone-acetic acid- water = 65 : 10 : 20 : 10 : 3 (v/v) as developing solvent system.

(d) 5% suspension in phosphate-buffered saline, pH 7.2 containing 0.01% sodium azide.

Inhibition profiles

Inhibition profiles are shown in Tables 3 & 4 (pp104-105). The activities of LMW I and II were inhibited by bovine submaxillary mucin (BSM) > fetuin (type III) > thyroglobulin (TG) > ovine submaxillary mucin (OSM) = desialylated OSM (DsOSM) = yeast invertase (YI) > (or =, for LMW II) mannan = mannose-1-phosphate [Na^+ and cyclohexylammonium (CH^+) salts and LMW II was also inhibited by the K^+ salt]. The activity of LMW III was inhibited by BSM = fetuin (type III) > TG > hog gastric mucin (HGM) > YI = mannan = mannose-1-phosphate (CH^+). LMW IV was inhibited by BSM = HGM = fetuin (type III)= mannose-1-phosphate (CH^+ salt) > TG > DsOSM = YI = mannan = mannose-1-phosphate (Na^+ and K^+ salts)(see Table 3).

The activity of HMW was inhibited by fetuin (type III) > BSM > DsOSM > OSM. (see Table 3)

The acetone-insoluble material from CM was resolved by one-dimensional TLC. Bands I-II were inhibited by BSM (type I, bound sialic acid 5 %) > HGM = fetuin (type III) > BSM (type IS, bound sialic acid 12 %). Band III was inhibited by BSM (type I) = BSM (type

IS) > fetuin (type III) > HGM. Band IV was inhibited by BSM (type I) = BSM (type IS) = HGM = fetuin (type III) > mannose-1-phosphate (CH^+ salt). Band V was inhibited by BSM (type I) = BSM (type IS) = HGM > mannan > fetuin (type III) (see Table 4).

The CM acetone-soluble TLC band I was inhibited by BSM (type I) = BSM (type IS) = HGM = mannan > fetuin (type III). Band II was inhibited by BSM (type I) > HGM > BSM (type IS) > mannan > fetuin (type III) (see Table 4). Higher sialic acid content did not make BSM a better inhibitor.

From the above results, BSM was shown to be a good inhibitor of hemagglutination activity of all the components of Nereis virens coelomic fluid. Since BSM is known to contain protein and sugar, its composition could be modified by certain treatments that could change its protein and sugar composition. This modification and the concurrent change in BSM's inhibitory properties suggested which parts of the BSM molecule were involved in inhibition. In Table 5 are the results, on the inhibitor activity of heating BSM, or of heating it with acid or of treating it with mercaptoethanol. It was

found that (a) heating BSM for 2 hours lowered its ability to inhibit hemagglutination activity for all fractions (CM, HMW and LMW). (b) treating BSM with 0.12 N HCl and 80^0C inhibited the hemagglutination activity of CM and HMW to a lesser extent than the intact BSM or the heated BSM but was a better inhibitor of LMW. (c) mercaptoethanol caused very little or no change in the inhibition property of BSM.

Table 3 Inhibitory Effects of Glycoproteins, Complex Polysaccharides and Monosaccharides* on the Titer of Nereis Agglutinins against Rat RBC

Inhibitors[a]	LMW[b]				HMW
	I	II	III	IV	
BSM (1mg/ml)	+++++[c]	+++++	++++	++++	+++
HGM (1mg/ml)	-[d]	-	++	++++	-
OSM (1mg/ml)	++	+	-	-	+
DsOSM (1mg/ml)	++	+	-	+	++
Fetuin (type III, 1mg/ml)	++++	++++	++++	++++	++++
YI (1mg/ml)	++	+	+	+	-
TG (1mg/ml)	+++	++	+++	+++	-
Mannose-1-PO_4					
K^+(5mg/ml)	-	+	-	+	-
Na^+(5mg/ml)	+	+	-	+	-
CH^+(5mg/ml)	+	+	+	++++	-
Mannan,12F-3975 (10mg/ml)	+	-	-	-	-
M3604,92F-3901 (20mg/ml)	+	+	+	+	-
33F-3878, (2mg/ml)	+	+	-	+	-

a- BSM : Bovine submaxillary mucin. HGM : Hog gastric mucin.
YI : Yeast invertase. TG : Thyroglobulin
CH^+ : Cyclohexylammonium salt. OSM : Ovine submaxillary mucin.
DsOSM : Desialylated OSM.

b- LMW I-IV samples are the active peaks (peak I-IV)eluated from DEAE column

c- "+" is measure of inhibitory effect. i.e. + means lowering in titer by 2^2, ++ means lowering in titer by 2^3, +++ means lowering in titer by 2^4, ++++ means by lowering in titer by 2^5 and +++++ means lowering in titer by 2^6.

d- "-" means no effect.

*- Glucose, galactose, mannose, fucose, rhamnose,D-galacturonic acid, glucosamine, D-mannose-6 phosphate, N-acetylglucosamine, N-acetylgalactosamine, N-acetyl -ß-D-mannosamine-H_2O, phenyl-ß-D-galactoside, 1-O-methyl-alpha-D-galactopyranoside, 1-O-methyl-alpha-D-glucopyranoside, and 1-O-methyl-ß-glucopyranoside,up to 0.2 M, have no inhibitory effect.

Table 4 The Inhibitory Effects of Glycoproteins, Complex Polysaccharides and Monosaccharides on the Titers of Lipid Agglutinins Against Rat RBC.

	Relative Titer(10 min)						
	Acetone-insoluble[a]					Acetone-soluble[a]	
TLC bands	I	II	III	IV	V	I	II
R_f[b]	0	0.11	0.13	0.39	0.97	0	0.95
Inhibitors							
Control[c]	$2^{7.5}$	2^6	2^6	$2^{3.5}$	$2^{3.5}$	2^3	2^7
BSM[d]	2^3	0	0	0	0	0	0
BSM[e]	$2^{6.5}$	2^2	0	0	0	0	2^4
HGM	2^6	2^4	2^2	0	0	0	2^3
Fetuin[f]	2^6	2^4	2^3	0	2^2	$2^{1.5}$	$2^{5.5}$
Mannan	$2^{8.5}$	2^7	$2^{6.5}$	$2^{3.5}$	2^3	0	$2^{4.5}$
Control[c]	$2^{11.5}$	$2^{6.5}$	$2^{6.5}$	2^6	0	$2^{2.5}$	2^7
mannose-1-PO_4[g]	2^{12}	2^7	2^7	$2^{4.5}$		2^2	2^8
mannose-1-PO_4[h]	2^{12}	$2^{9.5}$	$2^{9.5}$	2^6		$2^{3.5}$	$2^{8.5}$
mannose-1-PO_4[i]	2^{12}	$2^{7.5}$	2^8	$2^{5.5}$		2^2	$2^{7.5}$

(a) Acetone-insoluble and -soluble fractions from CM extract treated with acetone-10% $MgCl_2$.

(b) R_f in $CHCl_3$-CH_3OH-acetone-acetic acid- water = 65 : 10 : 20 : 10 : 3 (v/v) as developing solvent system. (c)medium in all cases was 0.001% Triton X-100 in phosphate buffered saline,pH 7.2 containing 0.01% sodium azide. (d) Type I (Sigma) bound sialic acid 5%. (e) Type IS (Sigma) bound sialic acid 12%. (f) Type III (Sigma). (g) dicyclohexylammonium salt. (h) potassium salt. (i) sodium salt. The concentration of each inhibitor was 1 mg/ml except mannan (10 mg/ml) and mannose-1-PO_4 (5 mg/ml).

Table 5 The Inhibitory Effects of BSM and Modified BSM on the Titers of Hemagglutinins from Nereis virens against Rat RBC

Medium	CM[a]	HMW[b]	LMW[c]
	Specific titer[d]		
PBS-N_3 (Control)	110	1418	3160
BSM-Control	2	44	395
BSM (heated)[e]	10	177	1580
BSM (heated with acid)[f]	55	251	99
BSM (mercaptoethanol)[g]	5	44	395

(a) The concentration of CM was 1.64 mg/ml.
(b) The concentration of HMW was 0.722 mg/ml.
(c) The concentration of LMW was 0.162 mg/ml.
(d) Specific titer was expressed as overnight titer/ mg/ml.
(e) BSM solution was heated on a steam bath for 2 hr, then dialyzed against PBS-N_3, pH 7.2.
(f) BSM solution was treated with 0.12 N HCl, heated on 80°C water bath for 1 hr, and centrifuged. The supernatant was dialyzed against PBS-N_3, pH 7.2.
(g) BSM solution (8 mg/ml PBS-N_3) was dialyzed against 0.1 M Tris, pH 8.0 containing 0.2 M 2-mercaptoethanol for 48 hours at 4°C, then dialyzed against PBS-N_3, pH 7.2 for 48 hours at 4°C.

The effects of cations on the hemagglutination activities for Nereis agglutinins

From the results of Table 6, it can be seen that although the hemagglutination activities for HMW and LMW samples were not affected by the presence of EGTA (ethylene-bis-(beta-aminoethylether)-N,N'-tetraacetic acid), but Mg^{++} lowered HMW activity. The results also showed that the hemagglutination activity of CM extract was inhibited by the presence of EGTA and this inhibition could be restored by Ca^{++} or Mg^{++}.

The effects of heat, protease, phenol, polyvinylpolypyrrolidone (PVPP), periodate and TCA denaturation treatments on the titers of Nereis agglutinins against rat RBC (Table 7)

(a) LMW (or CM) before and after treatment with insoluble PVPP had the same hemagglutination activity but PVPP slightly lowered the activity of HMW. (b) Four LMW agglutinins (I-IV peaks off DEAE) were heat-sensitive, phenol-sensitive and periodate-sensitive but their activities did not decrease after treatment with insoluble

protease. (c) HMW was heat-insensitive and periodate-insensitive but phenol-sensitive. When the HMW sample was treated with 10% TCA (trichloroacetic acid), the precipitate and supernatant both retained hemagglutination activity. (d) CM activity was heat-insensitive but phenol-sensitive and periodate-sensitive.

Effects of rabbit antiserum to coelomic fluid on the hemagglutination titers of Nereis hemagglutinins against rat RBC

The results are shown in Table 8. (a) Rabbit antiserum to coelomic fluid lowered the hemagglutination titers of HMW, CM, heated CM, LMW III and LMW IV agglutinins. (b) Heated antiserum lost its ability to inhibit agglutination. (c) Rabbit antiserum did not inhibit the hemagglutination activity of heated HMW (HHMW), delipidated & heated HMW (DL-HHMW), LMW I and LMW II agglutinins. (d) Titers of LMW I and LMW II were not lowered by rabbit antiserum.

Table 6 The Effects of Cations on the Titers of Hemagglutinins from Nereis virens against Rat RBC

Medium	CM[a]	HMW[b]	LMW[c]
	Specific titer*		
PBS-N_3	156	177	2731
1mM EGTA	39	177	2731
10mM EGTA	39	177	2731
1mM $CaCl_2$	156	177	2731
1mM $MgCl_2$	156	89	2731

* Specific titer : overnight titer/mg/ml.
(a) The concentration of CM was 6.56 mg/ml.
(b) The concentration of HMW was 0.722 mg/ml.
(c) The concentration of LMW was 1.50 mg/ml.

Table 7 The Effects of Heat, Protease, Phenol, Polyvinyl-polypyrrolidone (PVPP), Periodate and TCA Denaturation Treatments on the Titers of Nereis Agglutinins against Rat RBC

	Relative Titer (overnight)					
			LMW			
Treatment	CM	HMW	I	II	III	IV
Control	2^{11}	2^{10}	2^{12}	2^{12}	2^{11}	2^{10}
Heated[a]	2^{12}	2^{11}	0	2^{3}	2^{4}	2^{3}
Control	n.d.	n.d.	2^{12}	2^{11}	2^{9}	2^{8}
Protease[b]	n.d.	n.d.	2^{12}	2^{10}	2^{9}	2^{8}
Heated protease[b]	n.d.	n.d.	2^{12}	2^{11}	2^{10}	2^{9}
Control	2^{11}	2^{11}		$2^{11.5}$ h		
Phenol[c]	2^{6}	$2^{8.5}$		$2^{3.0}$		
Control	$2^{10.5}$	$2^{9.5}$		2^{12}		
PVPP[d]	$2^{10.5}$	2^{8}		2^{12}		
Control	2^{9}	2^{9}		2^{9}		
Periodate[e]	$2^{7.5}$	$2^{8.5}$[f]		$2^{6.5}$		
Control	n.d.	$2^{9.5}$		n.d.		
10 % TCA[g]	ppt :	2^{8}		n.d.		
	supernatant :	$2^{5.5}$				

n.d. : not determined.

a : Heated on a steam bath for 1 hour.

b : Method 5 (page 62) in the degradation or denaturation treatments was followed. The insoluble protease was from Streptomyces griseus.

c : Method 2 (page 58-59) in the degradation or denaturation treatments was followed.

d : Method 3 (page 59) in the degradation or denaturation treatments was followed.

e : Method 4-a (page 59-60) in the degradation or denaturation treatments was followed.

f : Method 4-b (page 60) in the degradation or denaturation treatments was followed. Periodate (up to 0.04 M) did not affect the titer of HMW.

g : Method 6 (page 63-64) in the degradation or denaturation treatments was followed.

h : This was done on unfractionated LMW.

Table 8 Effects of Rabbit Antiserum to Coelomic Fluid on the Hemagglutination Titers of Nereis Hemagglutinins against Rat RBC

Sample	Treatment : Heated antiserum[1]	Titer(overnight) PBS	Antiserum
PBS (Control)	0	0	0
HMW	2^9	$2^{2.5}$	$2^{9.5}$
Heated HMW (HHMW)[2]	2^9	2^{10}	2^{10}
Delipidated & Heated HMW (DL-HHMW)[3]	2^8	2^8	2^8
CM	2^8	2^4	2^8
Heated CM[4]	$2^{8.5}$	2^6	2^9
LMW I	$2^{4.5}$	$2^{5.5}$	2^7
LMW II	2^6	$2^{6.5}$	$2^{7.5}$
LMW III	2^5	2^1	2^8
LMW IV	$2^{4.5}$	0	$2^{7.5}$

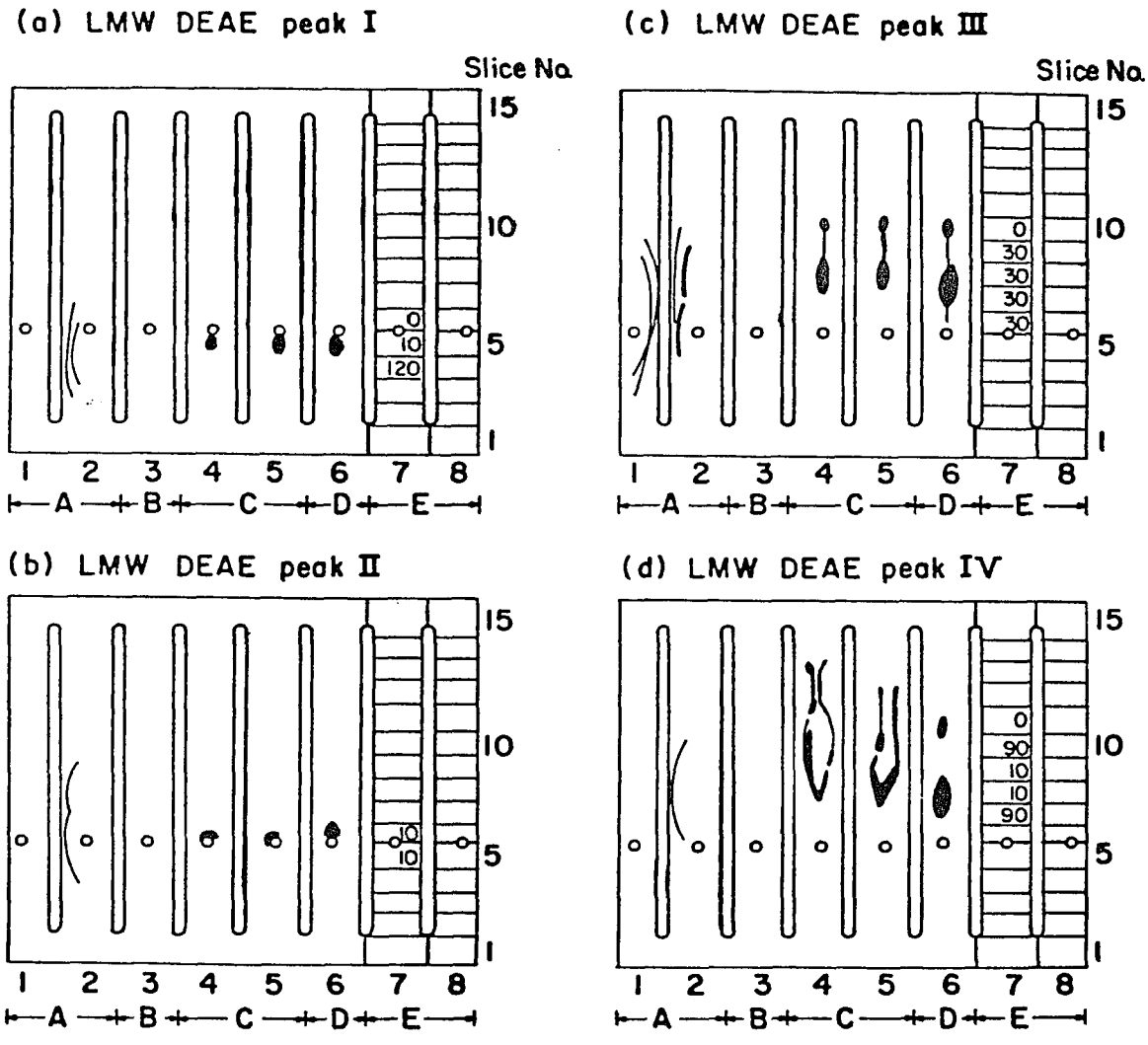
1 : Rabbit antiserum was heated on a steam bath for 1 hour.
2 : HHMW was prepared by the method (b-2) in the preparations
3 : DL-HHMW was prepared by the method (b-3) in the preparations.
4 : CM extract was heated on a steam bath for 1 hour.

The results of immunoelectrophoresis of Nereis agglutinins

(a) Rabbit antibody to coelomic fluid gave two precipitin bands below the origin for LMW I. The hemagglutination activity coincided with the precipitin line. The active region of LMW I stained for protein and sugar (Figure 11 (a), page 114). (b) LMW II gave two precipitin lines near the origin with rabbit antiserum. The activity coincided with the bottom precipitin band. The active region of LMW II stained for protein and sugar (Figure 11 (b)). (c) LMW III gave two precipitin bands near the origin with antiserum. The top precipitin line gave hemagglutination activity. It was also found that another region had agglutination activity but did not give a precipitin line with rabbit antiserum. The active regions all stained for protein and sugar (Figure 11 (c)). (d) Antiserum gave one precipitin band near the origin with LMW IV. The hemagglutination activity occurred in the region which coincided with the precipitin line and another region having no precipitin line. The active regions all stained for protein and sugar (Figure 11 (d)). (e) HMW gave two precipitin bands near the origin with rabbit antiserum.

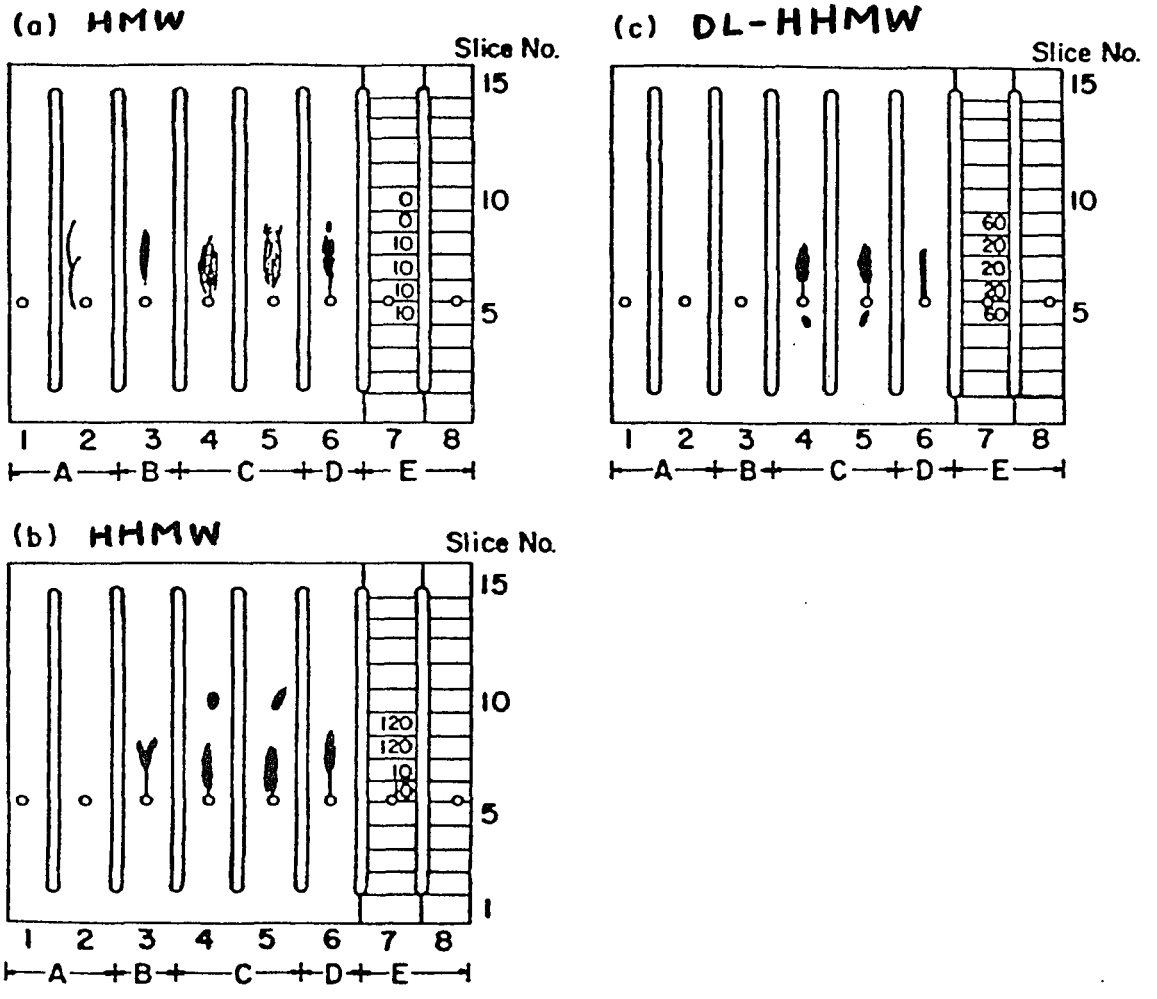
No precipitin bands appeared for HHMW and DL-HHMW but hemagglutination activity remained in the same region, which also stained for protein and sugar. The active regions of HMW and HHMW but not DL-HHMW also stained for lipid. Heating had destroyed the antigenic determinant but not the activity. Delipidation caused the disappearance of lipid staining in DL-HHMW and of an active faster-moving lipid-positive spot and the appearance of an active proteoglycan spot which had moved away from the origin in the opposite direction (Figure 12(a)-(c)). (f) CM agglutinin gave no precipitin line with rabbit antiserum. The active smear regions stained for lipid, sugar and also with Coomassie blue (Nakamura et al (76) had reported that Coomassie brillant blue could stain lipid on thin-layer plates). The stain may be due to lipid or to protein.

Figure 11 Immunoelectrophoresis of LMW Agglutinins



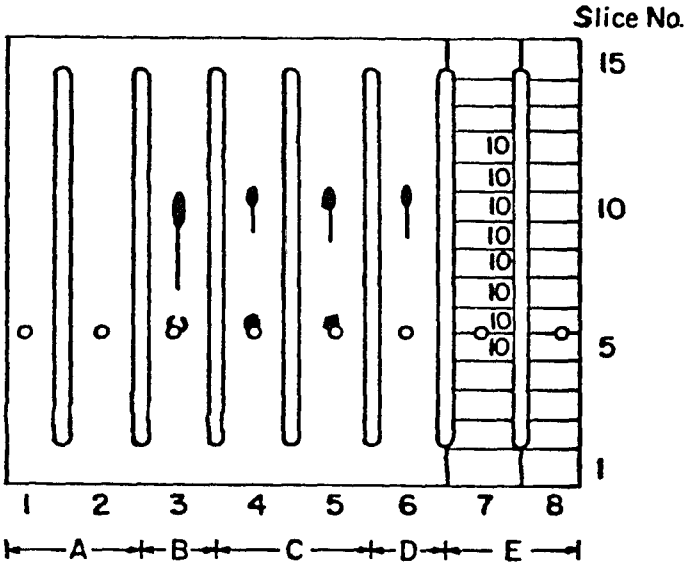
Wells 1 & 8: Tracking dye (except LMW III plate, well 1 contains LMW III)
Wells 2-7 : LMW prep
Part A : Immunodiffusion with rabbit antiserum to coelomic fluid
Part B : Lipid stain
Part C : Carbohydrate stain
Part D : Protein stain
Part E : 0 means activity overnight. The other numbers designate the time elapsed in minutes before activity was observed.

Figure 12 Immunoelectrophoresis of HMW Agglutinins



Wells 1 & 8: Tracking dye
Wells 2-7 : HMW prep
Part A : Immunodiffusion with rabbit antiserum to coelomic fluid
Part B : Lipid stain
Part C : Carbohydrate stain
Part D : Protein stain
Part E : 0 means activity overnight. The other numbers designate the time elapsed in minutes before activity was observed.

Figure 13 Immunoelectrophoresis of CM Agglutinin



Wells 1 & 8: Tracking dye
Wells 2-7 : CM
Part A : Immunodiffusion with rabbit antiserum to coelomic fluid
Part B : Lipid stain
Part C : Carbohydrate stain
Part D : Protein stain
Part E : 0 means activity overnight. The other numbers designate the time elapsed in minutes before activity was observed.

Studies on HMW

The composition and activity of HMW, HHMW and DL-HHMW

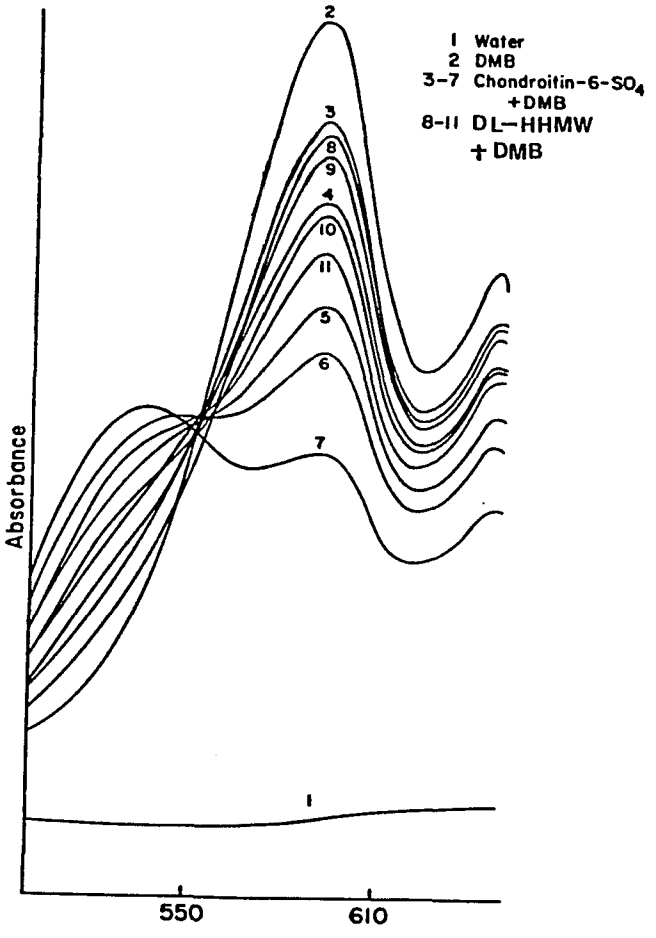
The results of Table 9 showed that heating lowered the titer of HMW slightly and delipidation of HHMW lowers the specific titer further. The extracted lipid showed some activity (Scheme 2). Quantitative analyses (Table 9) showed that sialic acid and N-sulfate were absent in all three fractions. There were only traces of phosphorus and pentose. Hexose, protein, hexuronic acid, hexosamine and sulfate (as O-ester) were present. Hexuronic acid and hexosamine were present in equal amount. A metachromatic reaction was observed for DL-HHMW with the cationic dye, dimethylmethylene blue in the presence of guanidinium chloride (Figure 14). Hyaluronic acid and DNA do not react under these conditions (47). The infrared (IR) and Fourier transform infrared (FTIR) analyses of DL-HHMW (Figures 15), showed a hexose-6-sulfate ester [having absorption at 1243 cm^{-1} (sulfate) and 813 cm^{-1} (equatorial-6-sulfate)] and amide bands [having absorption at 1661 cm^{-1} (amide I) and 1536 cm^{-1} (amide II)]. The latter is probably due to the protein moiety as well as to N-acylhexosamine.

Table 9 Composition and Activity of HMW Before and After Heating (HHMW) and Delipidation (DL-HHMW)

Preparation	HMW (%)	HHMW (%)	DL-HHMW (%)
Protein[1] (mg/ml)	1.505 (17)	0.896 (13)	0.460 (12)
Hexose[2] (mg/ml)	2.040 (14)	1.785 (25)	1.20 (30)
GAG[3] (mg/ml)	1.33 (15)	1.30 (18)	0.88 (22)
Pentose[4] (mg/ml)	0.072 (1)	0.060 (1)	0.044 (1)
Hexuronate[5] (mg/ml)	0.575 (7)	0.520 (7)	0.356 (9)
Sialic acid[6]	0	0	0
Amino sugar[7] (mg/ml)	n.d.[12]	n.d.	0.329 (8)
N-sulfate[8]	n.d.	n.d.	0
Sulfur[9]	n.d.	n.d.	(2.4)
Phosphorus[9]	n.d.	n.d.	(0.5)
Sulfate[10] (mg/ml)	0.744 (9)	0.500 (7.0)	0.277 (7.0)
Dry weight (mg/ml)	8.68	7.12	3.95
Titer	$2^{11.5}$	2^{11}	2^{9}
Specific titer[11]	334	287	132

[1] BioRad method [Bradford, 1976].
[2] Phenol-sulfuric method [Dubois, et al., 1956].
[3] Dimethylmethylene blue method [Chandrasekhar, 1987]. Using chondroitin sulfate B as a standard.
[4] Orcinol method [A. H. Brown, Arch. Biochem. 11, 269 (1946)].
[5] Carbazole method of Dische and of Bitter and Muir [Chaplin and Kennedy, 1986].
[6] Jourdian's and Warren's methods [Chaplin and Kennedy, 1986].
[7] Morgan-Elson method [Chaplin and Kennedy, 1986].
[8] Z. Dische and E. Borenfreund, J. Biol. Chem. 184, 514 (1950).
[9] Schwarzkopf Microanalytical Laboratories (Woodside, N.Y.).
[10] Carney method [Chaplin and Kennedy, 1986].
[11] Overnight titer divided by dry weight (mg/ml).
[12] Not determined.

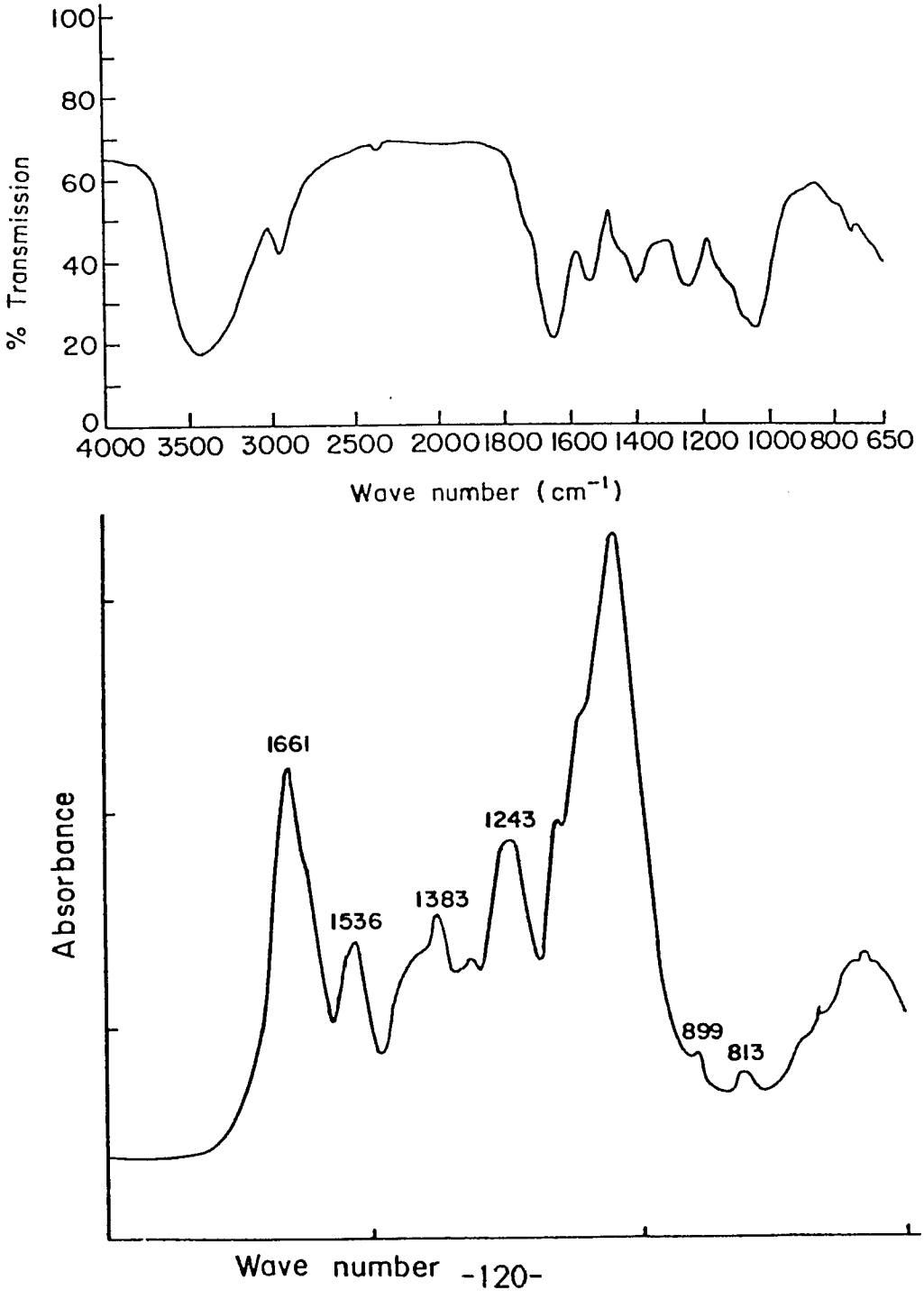
Figure 14 Reaction of Proteoglycan with Dimethylmethylene Blue (DMB)



* The concentrations of chondroitin-6-SO_4 in curves 3-7 were 1 µg/ml, 2 µg/ml, 3µg/ml, 4 µg/ml and 5 µg/ml respectively.

The concentrations of DL-HHMW in curves 8-11 were 31 µg/ml, 62 µg/ml, 93 µg/ml and 123 µg/ml respectively.

Figure 15 Infrared (IR, top) and Fourier transform infrared (FTIR, bottom) spectra of DL-HHMW



The effects of enzyme and chemical treatments on titer and metachromatic spectrum of DL-HHMW (Table 10)

When DL-HHMW was treated with three known GAG (glycosaminoglycan) depolymerization enzymes (chondroitinase ABC, keratanase and heparinase II) neither the titer nor the metachromatic spectrum of DL-HHMW changed while the standards chondroitin sulfate A or B or C, keratan Sulfate and heparin (sodium) respectively showed diminution of the DMB reaction. Periodate, pronase and desulfation treatments did not change the titer of DL-HHMW.

Studies on CM

Analyses of spots eluted from 2D TLC of acetone-insoluble (AI) from Nereis coelomocytes

(1) Group-specific reagents

AI was resolved by two dimensional thin layer chromatography on silica (2D TLC) (Figure 17). The individual spots, visualized with I_2, were characterized with group-specific spray reagents (Table

11). Spot 3, which had a specific titer of 517/mg/ml contained phosphate, choline and vicinal -OH. Spot 10-11 which had a specific titer of 567/mg/ml contained phosphate, $-NH_2$ and vicinal -OH but no reducing sugar. The position of spot 10-11 in two dimensional TLC plate was close to that of dioeloylphosphatidylethanolamine (DOPE).

(2) Infrared spectra (IR) (Figure 18 and Table 11)

The IR spectrum of spot 3 showed that it had C=C (3020 cm^{-1}), fatty acid ester(1730 cm^{-1}), phosphate [the absorption band is very broad, which included P=O (bonded, 1270 cm^{-1}), P-O-C and $P-O^-$(1100 cm^{-1})]. The IR spectrum of spot 10-11 showed that it had ester (1730 cm^{-1}), ammonium (1580 cm^{-1} and 3100 cm^{-1}), and phosphate [P=O bonded (1270 cm^{-1}) and $P-O-C^-$ near 1100 cm^{-1}] group and one or more long carbon chains (730 cm^{-1}).

Analyses of spots eluted from 2D TLC of acetone-soluble (AS) from Nereis coelomocytes

The result of 2D TLC of AS is shown in Figure 19. The individual spots, visualized with I_2, were characterized by group-specific spray reagents and the results are shown in Table 12. The most active spots were near the origin. Spot 1 had -NH and vicinal -OH. This work was done by Ioannis Patrikios.

Table 10 The Effects of Enzyme and Chemical Treatments on Titer and Metachromatic Spectrum of Heated & Delipidated HMW (DL-HHMW) against Rat RBC

Treatment	Relative Titer[e]	Metachromatic spectrum
- Periodate	$2^{9.5}$	N.D.[f]
+ Periodate (0.1M $NaIO_4$, 96 hrs)	$2^{10.5}$	N.D.[f]
- Pronase	$2^{9.5}$	N.D.[f]
+ Pronase (1% by wt, 20 hrs)	$2^{9.5}$	N.D.[f]
Control	2^{8}	N.D.[f]
Desulfation[a]	2^{8}	N.D.[f]
Control	$2^{8.5}$	+
Chondroitinase ABC[b] (0.5 units, 2 hrs)	$2^{8.5}$	+
Control	2^{11}	+
Keratanase[c] (1 unit, 2hrs)	2^{11}	+
Control	2^{9}	+
Heparinase II[d] (2 units, 2hrs)	2^{9}	+

a : The procedure of Nagasawa et al (1977) which involved N-acetylation was used. The titer did not change but the IR spectrum changed slightly.

b : The standard, chondroitin sulfate B, is degraded under this condition as judged by loss of metachromicity. The method of Chandrasekhar (1987) was used.

c : The standard, keratan sulfate, is degraded under this condition as judged by loss of metachromicity. The method of Chandrasekhar (1987) was used.

d : The standard, heparin sodium, is degraded under this condition as judged by loss of metachromicity. The method of Chandrasekhar (1987) was used.

e : Overnight titer.

f : Not determined.

Figure 16 Structures of some major glycosaminoglycans

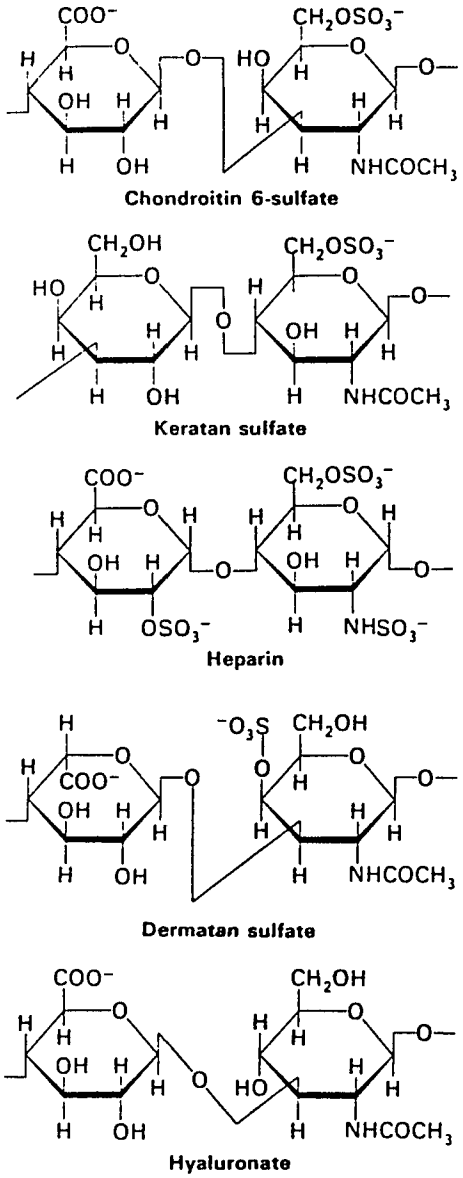
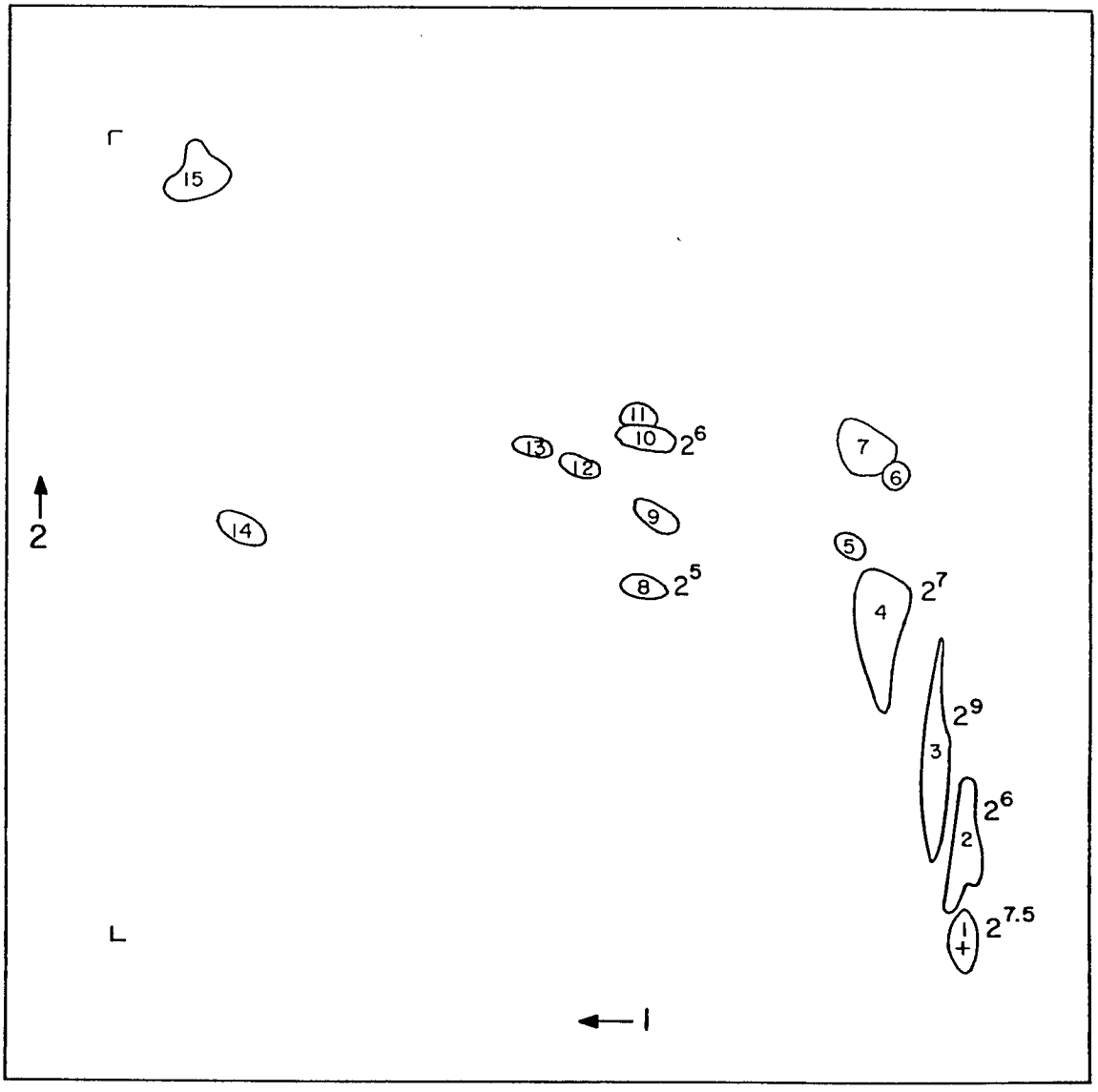


Figure 17 Two Dimensional TLC on Silica Gel of Actone-Insoluble Fraction from CM Extract of Coelomocytes Treated with Acetone-10% $MgCl_2$ (in Methanol)



1. chloroform-methanol-acetone-acetic acid-water [65 : 10 : 20 : 10 : 3 (v/v)]
2. chloroform-methanol- conc. ammonia [65 : 25 : 5 (v/v)]

Table 11 Analyses of spots eluted from 2D TLC of AI from Nereis coelomocytes

Spot No. / Test	1	2	3	4	5	6	7	8	9	10-11	12	13	14	15
I_2 vapor	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phosphate	+	+	+	+	+	+	+	–	–	+	–	–	–	–
Choline	+	+	+	–	–	+	+	–	–	–	–	–	–	–
$-NH_2$	+	+	–	+	+	–	–	+	+	+	+	+	–	+
-NH group	+	+	+ (weak)	+	+	+	+	+	+	+	+	+	–	+
Vicinal-OH	+	+	+	+	–	–	+	–	–	+	–	–	+	+
reducing sugar	+	+	–	+	–	–	+	–	–	–	–	–	–	–
sugar	+	+	–	+	–	–	+	–	–	–	–	–	–	–
Sterol	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Characteristic IR absorption bands (cm^{-1})														
Ester (1730)	+	+	+	+	n.d.	n.d.	n.d.	n.d.	n.d.	+	n.d.	n.d.	n.d.	n.d.
C=C (3020)	–	–	+	–	n.d.	n.d.	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	n.d.
Phosphate (P=O bonded, 1270, P-O-C and $P-O^-$, 1100)	+	+	+ (broad)	+	n.d.	n.d.	n.d.	n.d.	n.d.	+	n.d.	n.d.	n.d.	n.d.
$N^+(CH_3)_3$ (970)	–	+	–	–	n.d.	n.d.	n.d.	n.d.	n.d.	–	n.d.	n.d.	n.d.	n.d.
Titer (overnight)	$2^{7.5}$	2^6	2^9	2^7	n.d.	n.d.	0	$2^{5.5}$	n.d.	2^6	n.d.	n.d.	$2^{3.5}$	$2^{3.5}$
Specific activity (Titer/mg dry wt./ml)	54	96	517	320	n.d.	n.d.	0	226	n.d.	567	n.d.	n.d.	106	106

AI : Acetone insoluble fraction from CM extract treated with acetone-10% $MgCl_2$.

n.d. : not determined.

Spots (# 1-15) are shown in Figure 17.

Figure 18 (a) The IR Spectra of Spot 3 (Top) and Spot 10-11 (Bottom) Eluted from 2D TLC of AI

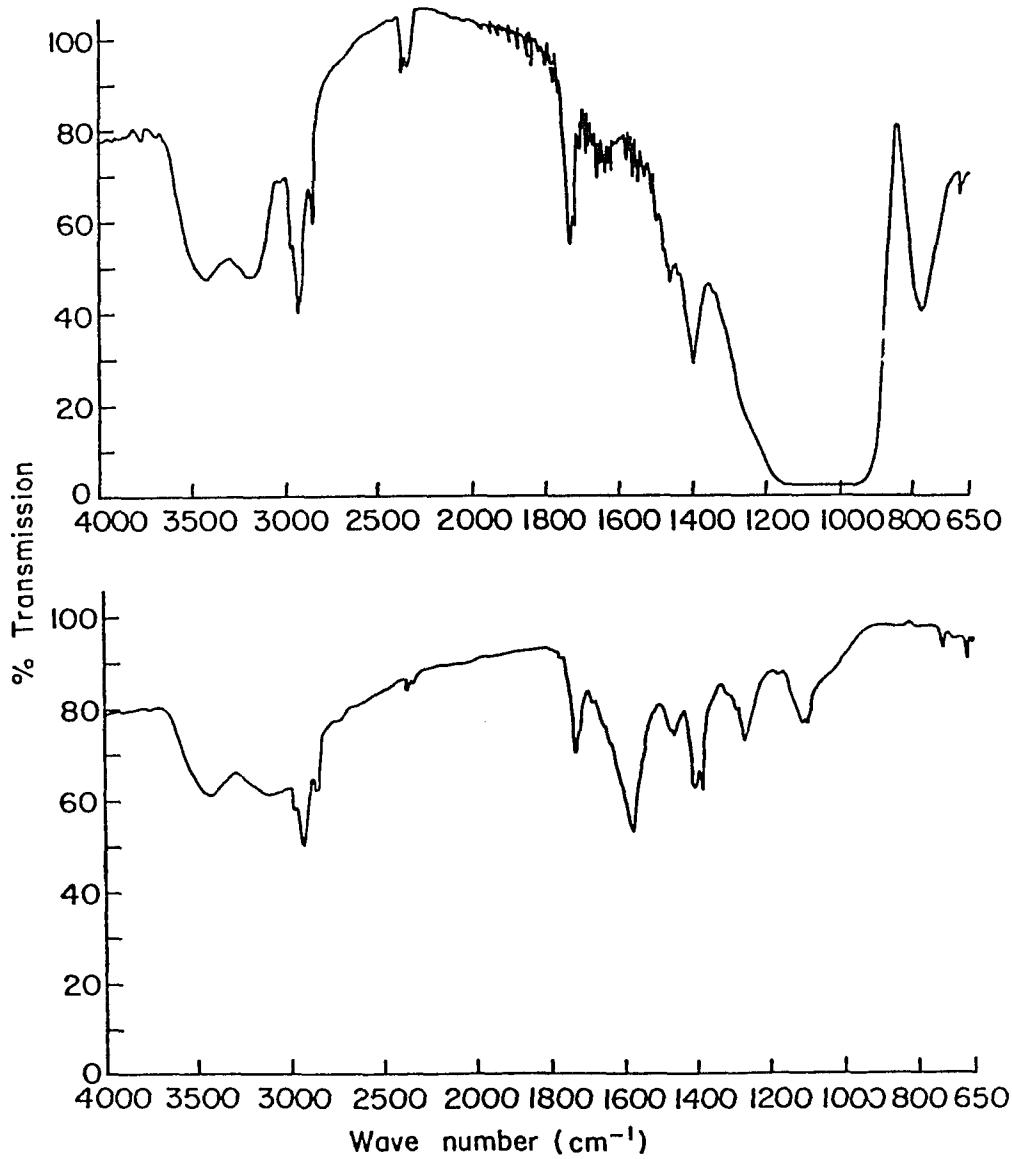


Figure 18 (b) The IR Spectra of Dioleoylphosphatidylinositol (DOPI, top) and Dioleoylphosphatidylethanolamine (DOPE, bottom)

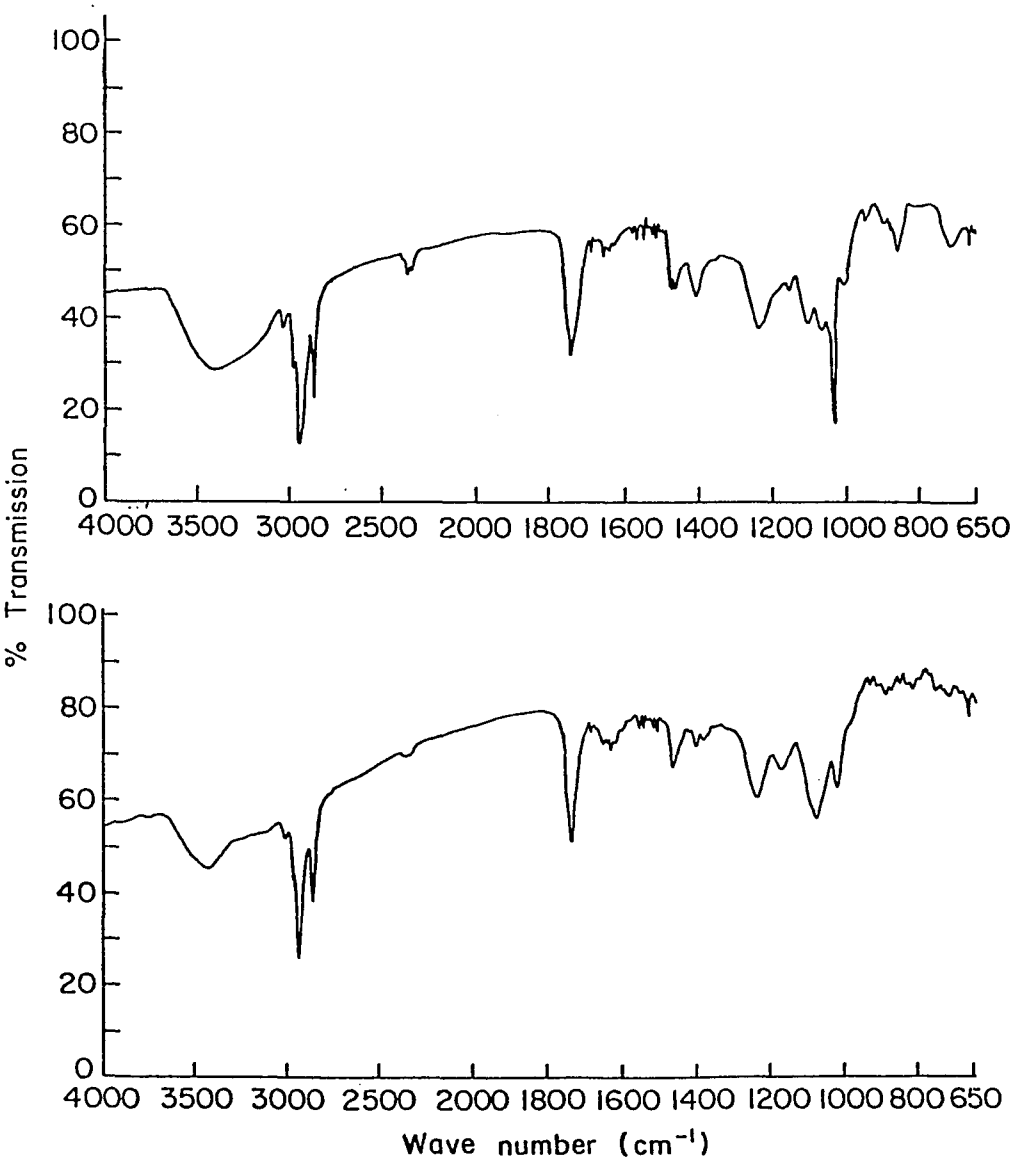
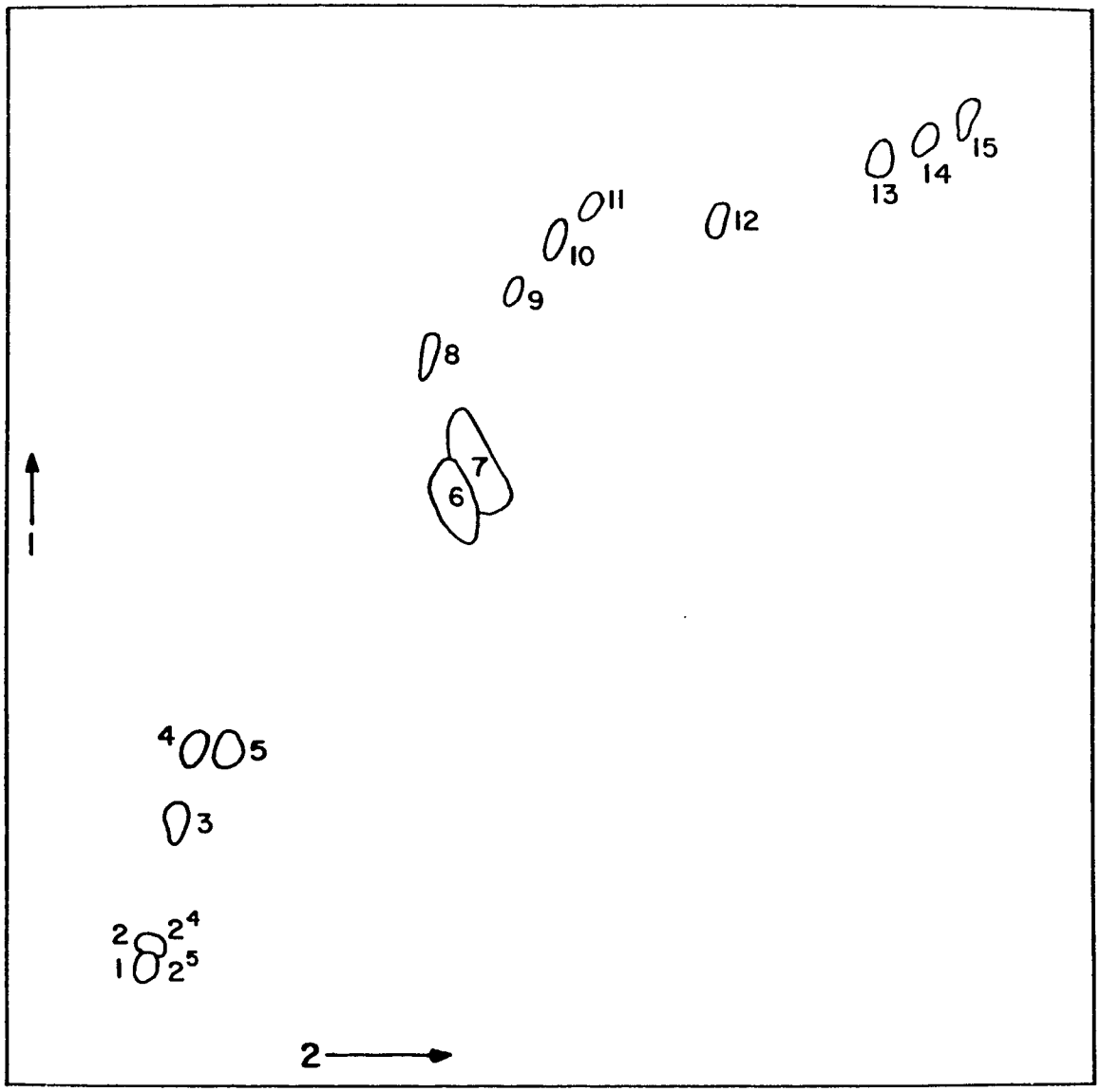


Figure 19 Two Dimensional TLC on Silica Gel of Acetone-Soluble Fraction from CM Extract of Coelomocytes Treated with Acetone - 10% $MgCl_2$ (in Methanol).



1. Hexanes : ethyl ether : acetic acid [80 : 20 : 1 (v/v)]
2. Hexanes : ethyl ether : acetic acid [90 : 10 : 1]

Table 12 Analyses of Spots Eluted from 2D TLC of AS from Nereis coelomocytes

Test \ Spot No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
I_2 vapor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phosphate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$-NH_2$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Choline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-NH group	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
vicinal-OH	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-
Ganglioside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
sugar	-	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ester test	-	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Titer (overnight)	2^5	2^4	$2^{1.5}$	2^2		2^2		2^2	0		0	0	0	0	0

AS : Acetone soluble fraction from CM extract treated with acetone-10% $MgCl_2$.

n.d. : not determined.

Spots (#1-15) are shown in Figure 19.

Experiments with Ehrlich ascites tumor cells (EATC)

(1) In vivo experiments

(a) Dose of cells to inject into test mice (Figure 20)

The three mice injected with 10^7 EATC /mouse, died by day 21. Mice injected with 5×10^5 tumor cells failed to show significant weight gain (attributable to tumor growth) by day 21 (Figure 20). Mice that received 10^6 to 5×10^6 tumor cells showed a very large weight gain in at least one mouse in each group. These data implied that a dose of 10^6 tumor cells would be an optimal dose of EATC to inject into test mice. The mice which received more tumor cells finally gained less weight due to illness (2×10^6, 5×10^6) or did not survive (10^7).

(b) The effect of the Nereis fractions on the growth of Ehrlich ascites tumor cells in CF_1 mice (i) Mice given injections of (10^6) EATC and sterile PBS (group 1), had an average weight gain of about 17 g/mouse by 25 days after tumor inoculation. The increase in body weight, was due to the accumulation of ascitic fluid (Figure 21 (b)). This can be compared with mice given injection of PBS only

(group 2, see Figure 21 (a)), where there was no large weight gain. At the end of the experiment (day 74), only one mouse of group 1 remained alive (Figure 22). This mouse had shown observable signs of tumor growth around day 20; however, by day 28 it started to lose weight. It was sacrified on day 74. There was no ascitic fluid in the peritoneal cavity.

(ii) We used PBS-T with EATC for group 9 because CM extract was dissolved in PBS-T [There was no large weight gain when treated with PBS-T alone (data not shown)]. It showed an average weight gain of about 12 g/mouse 25 days after tumor inoculation. At the end of the experiment (day 74), only two mice remained alive (Figure 22). One of these two surviving mice had abdominal fat and the other mouse showed one large lymph node.

(iii) The mice given injections of the low molecular weight fraction and EATC (group 3) showed an average growth increase of about 7 g/mouse, with all mice in the group surviving to day 74 (Figure 21 (b)). The control group (group 4), which had received the low molecular weight fraction but no tumor cells, did not shown any

side effects. It indicated that the toxicity of the low molecular weight fraction was minimal at this dose level. All the mice in group 3 showed no ascitic fluid and three of these 5 surviving mice in group 3 showed signs of abdominal fat.

(iv) The mice receiving the high molecular weight fraction and tumor cells (group 5), showed an average weight gain on day 42 of 17 g/mouse. The increase in body weight, was due to the accumulation of ascitic fluid (Figure 21 (b)). Only three mice had survived by day 74 (Figure 22). The toxicity of this fraction was also minimal, since control mice injected with the high molecular weight fraction and no tumor cells (group 6), showed no toxic symptoms. The water bottle of the cage of the control group had fallen down on days 4-7, it showed a large weight loss of 7 g/mouse and one mouse was dead on day 7 (Figure 21 (a)). The surviving 3 mice of group 5 when autopsied on day 74 showed signs of fat but no ascitic fluid in the peritoneal cavity.

(v)The mice given the CM extract and tumor cells (group 7), showed an average increase of 5.9 g/mouse by day 14 and then the

growth rate declined (Figure 21 (b)). One mouse died on day 28, the other mouse died on day 35, the third one died on day 37 and the rest of the mice (2 mice) died on day 44 (Figure 22). Postmortem examinations were done . The mouse that died on day 28 showed ascitic fluid in the peritoneal cavity but the rest of the mice did not show any ascitic fluid in the peritoneal cavity or any solid tumor cells. All the control mice injected with CM extract, but no tumor cells (group 8) were healthy (Figure 21 (a)). It indicated that the toxicity of the CM extract was minimal at this dose.

(2) In vitro experiments (Figures 23-32)

(a) Light microscopic studies

The light microscopic studies showed that Nereis hemagglutinins affected Ehrlich ascites tumor cells in vitro. Tumor cells incubated with PBS (Figure 23) or PBS-T (Figure 26) showed single cells throughout the field of vision and there were no changes in these for up to two hours. LMW caused swelling of the tumor cells within two hours (Figure 24). After two hours the cells were ruptured. This

was evidenced by the irregular shaped cells and cell contents that were floating in the field of vision under the microscope. Agglutination of the tumor cells by HMW was observed (Figure 25). Swelling and rupturing later of the tumor cells were observed with CM (Figure 27 (a), 1 hr). However the ruptured cells and their contents seemed to become clumped (Figure 27 (b), 2 hr).

(b) EM studies

The results of EM studies showed that (i) The size of the tumor cell in PBS (Figure 28) or PBS-T (Figure 31) was about 10 µm. (ii) The size of the tumor cell became smaller and some of the cells ruptured in the case of EATC with LMW (Figure 29). (iii) The tumor cells agglutinated when EATC interacted with HMW (Figure 30). (iv) A large cavity formed on the surface of the tumor cell when EATC were treated with CM (Figure 32). The results shown are representative of the whole field in each case.

Figure 20 The Determination of the Optimal Number of EATC Needed for Tumor Growth in Mice

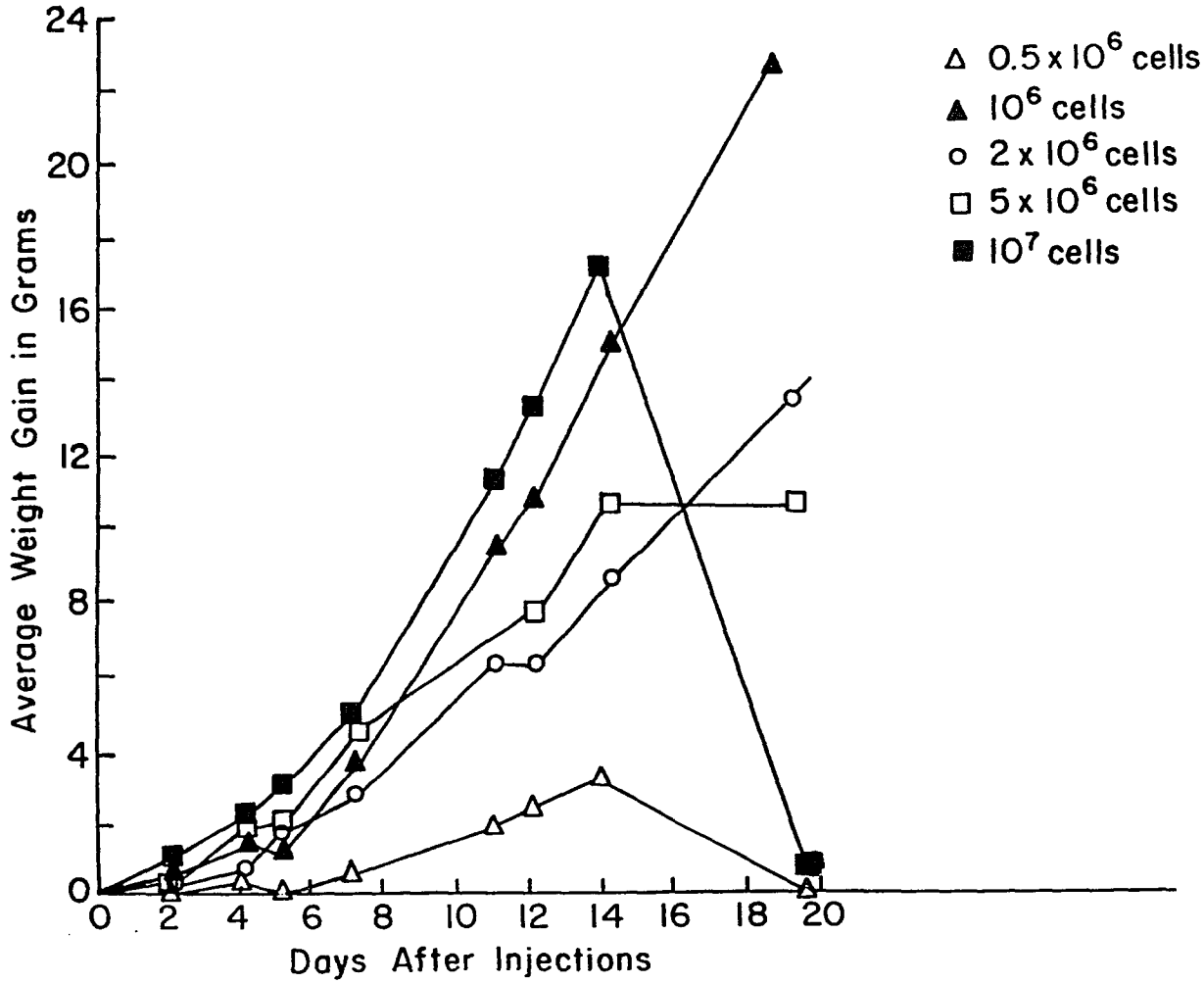


Figure 21 (a) Effects of intraperitoneal injection of Nereis agglutinins or PBS on mice over 70 days

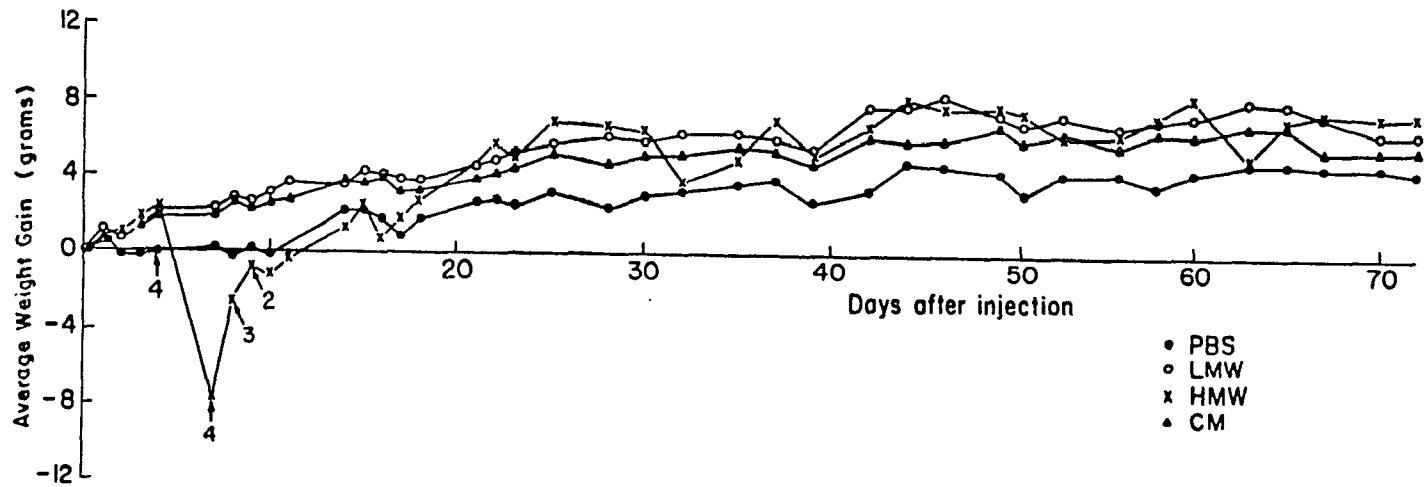


Figure 21 (b) Effects of intraperitoneal injection of Nereis agglutinins, or PBS, or PBS-T on the growth of Ehrlich ascites tumors in mice

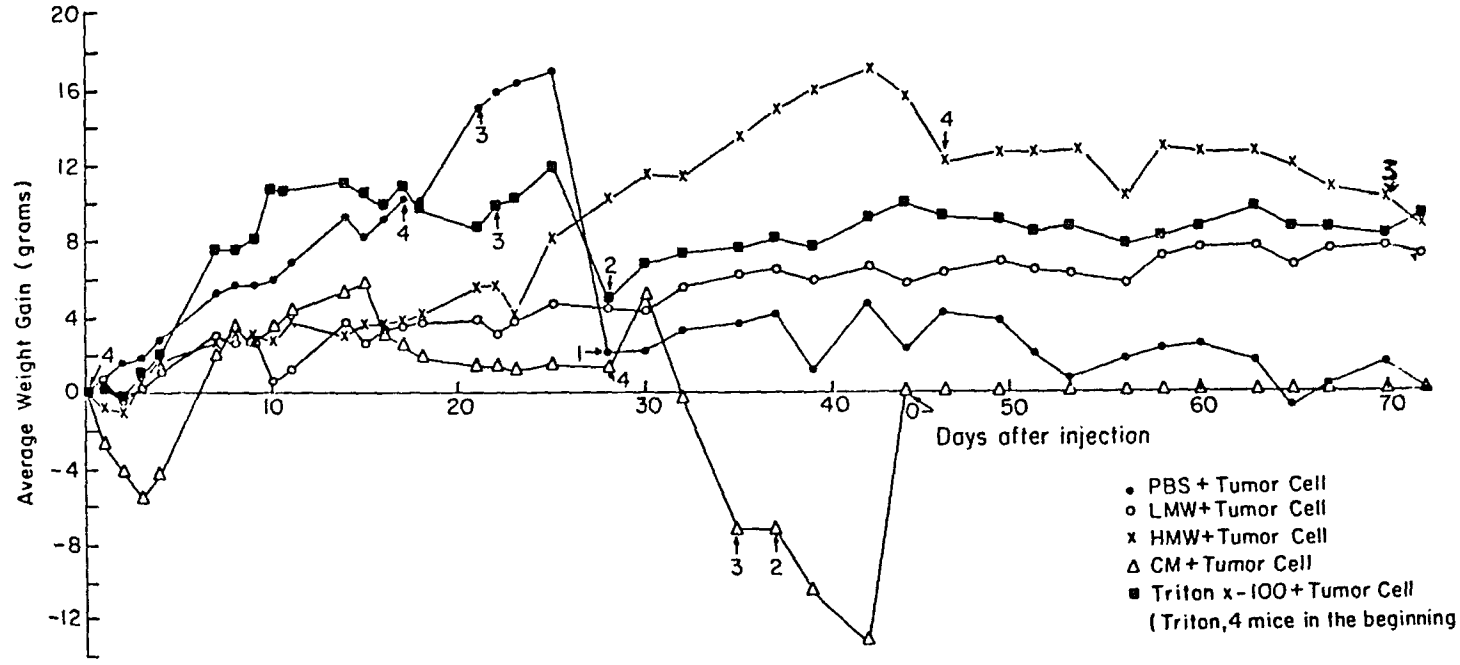


Figure 22 The surviving number of mice given Nereis agglutinins following intraperitoneal injection of Ehrlich ascites tumor cells (10^6 cells)

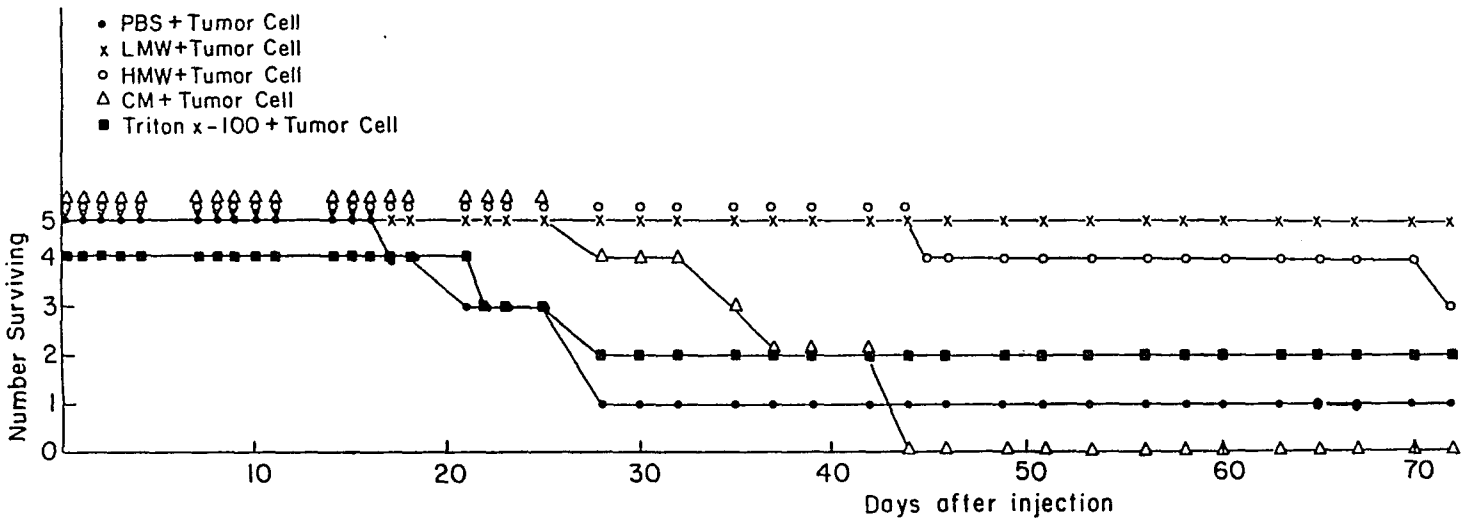
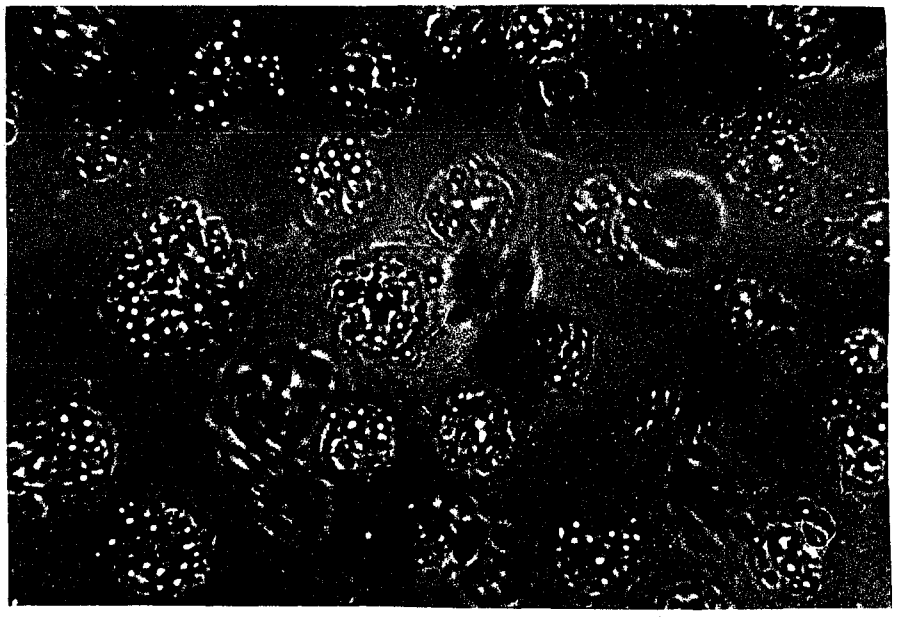


Figure 23 Light micrographs of Ehrlich ascites cells (10^6 cells /ml) treated with PBS

(a) 30 min, 400 X



(b) 2 hours, 400 X

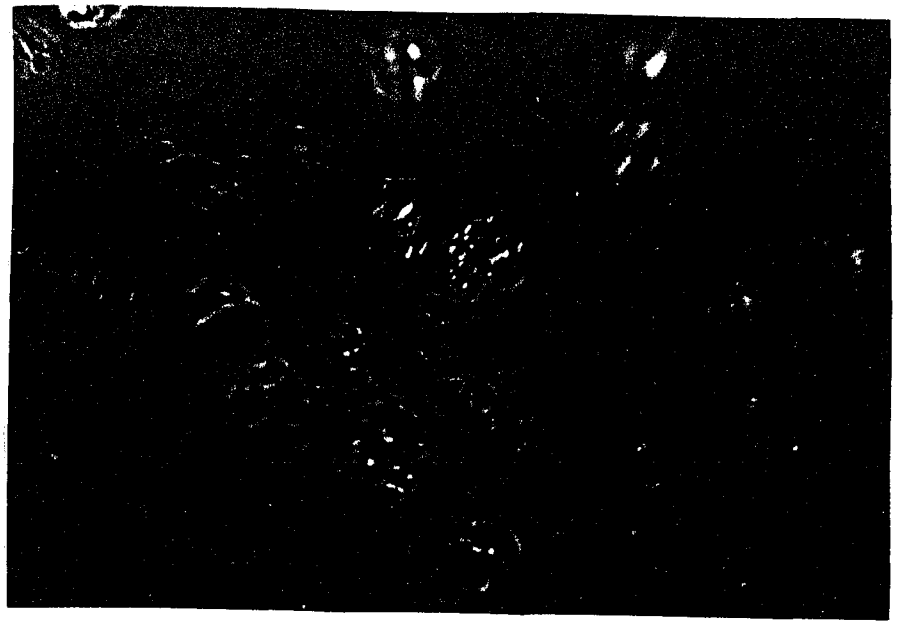


Figure 24 Light micrgraphs of Ehrlich ascites tumor cells treated with Nereis LMW agglutinin (1.60 mg/ml), 30 min, 400 X

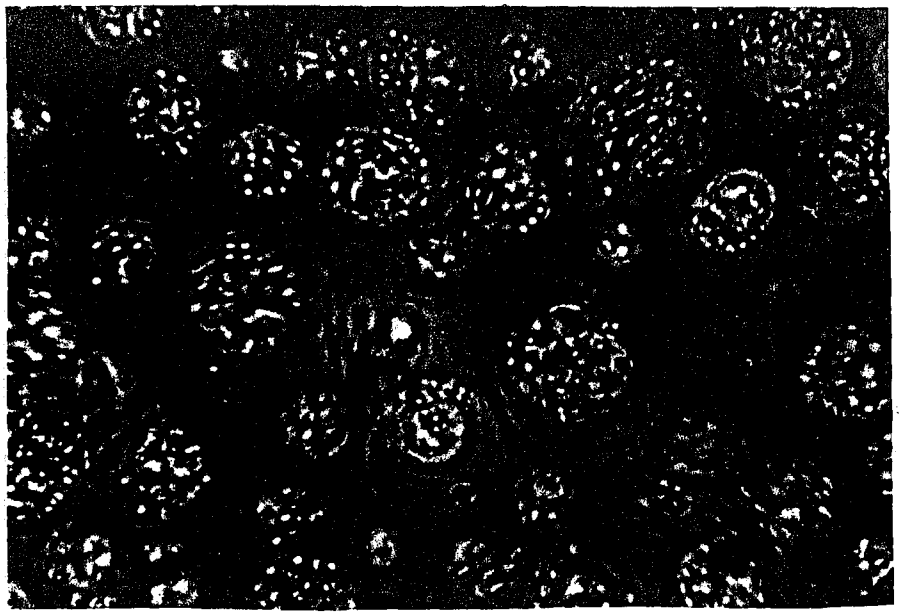


Figure 25 Light micrograph of Ehrlich ascites cells treated with Nereis HMW agglutinin (0.722 mg/ml), 2 hrs, 400 X

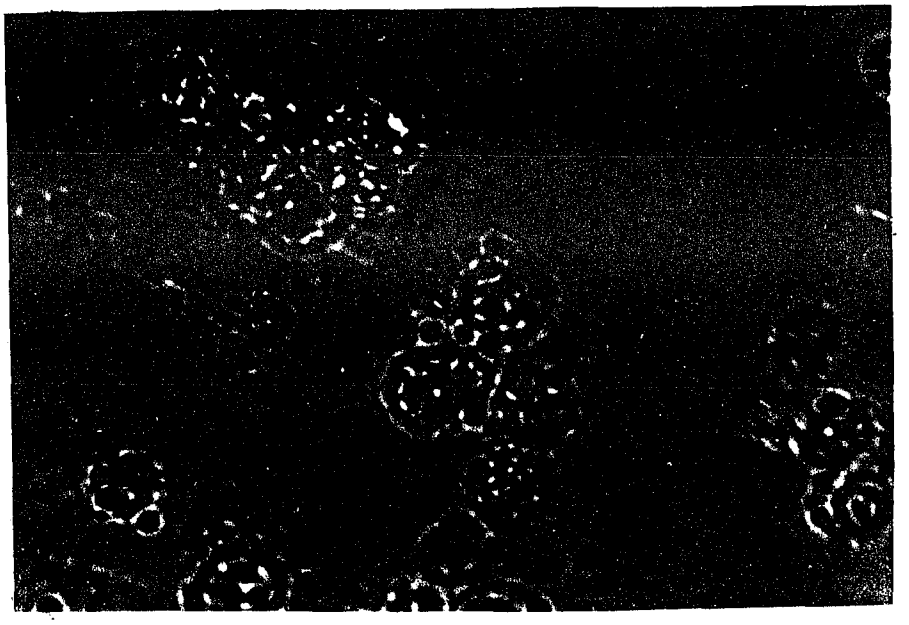


Figure 26 Light mirograph of Ehrlich ascies tumor cells treated with PBS-0.001% Triton X-100 , 1 hr, 400 X

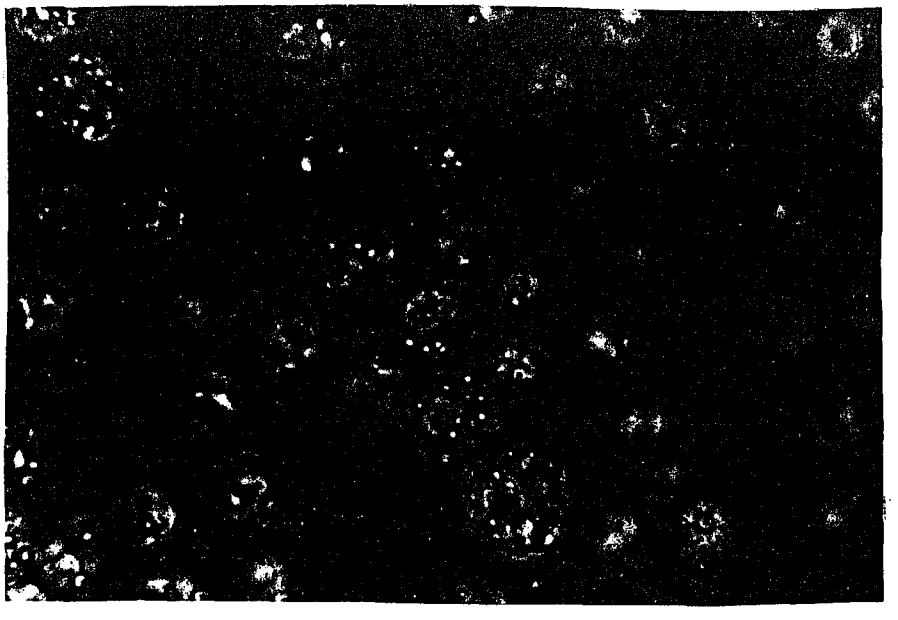
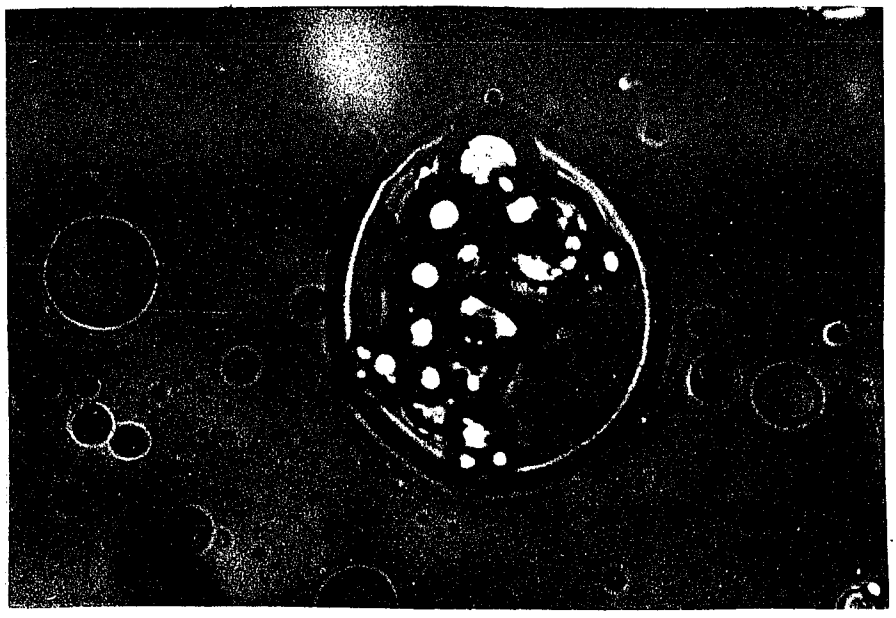


Figure 27 Light micrographs of Ehrlich ascites tumor cells treated with Nereis CM extract (from coelomocytes, 1.64 mg/ml)

(a) 1 hr, 400 X



(b) 2 hrs, 400 X

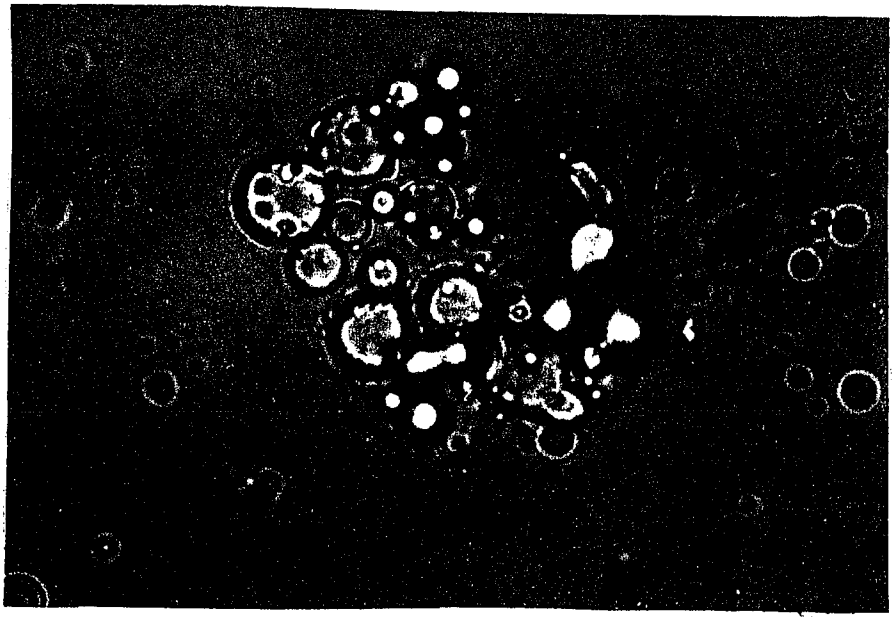


Figure 28 Scanning electron micrograph of EATC and PBS (X 4700)

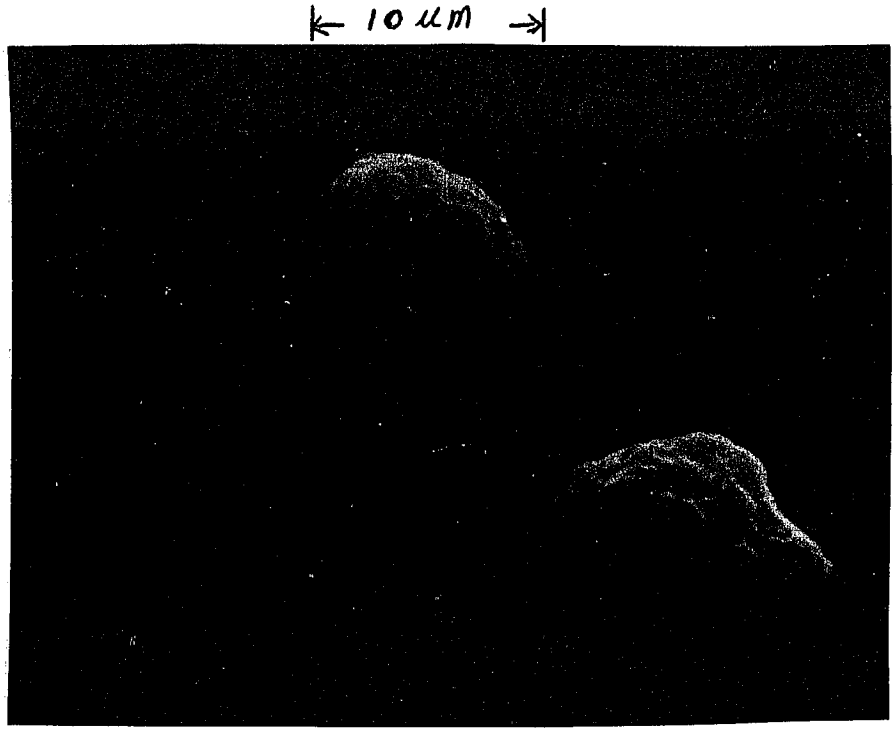


Figure 29 Scanning electron micrograph of EATC and LMW (1.60 mg/ml) (X 4200)

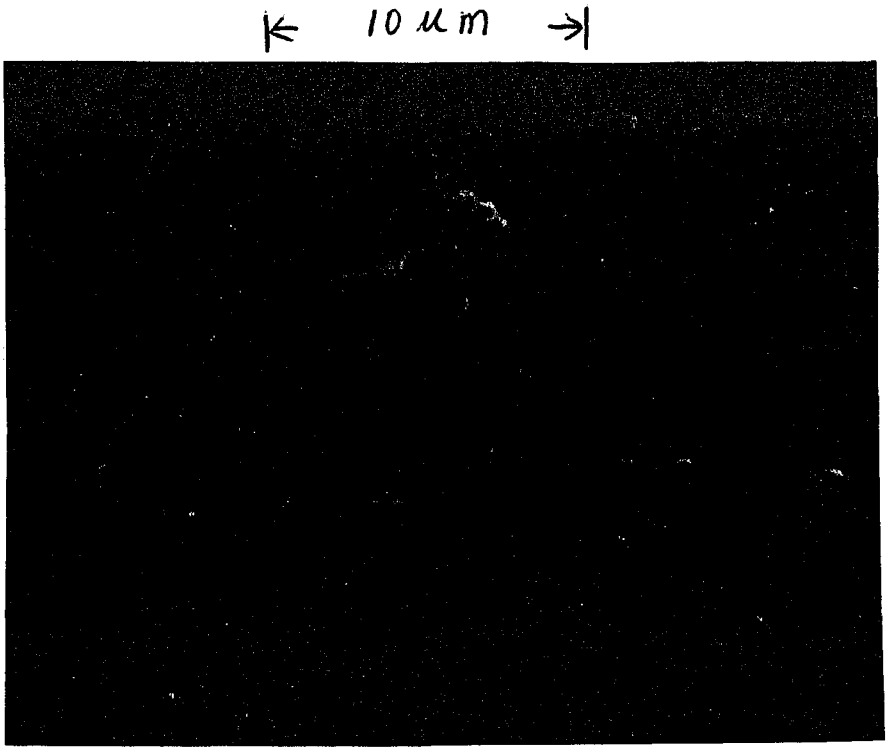


Figure 30 Scanning electron micrograph of EATC and HMW (0.722 mg/ml)(X 1400)

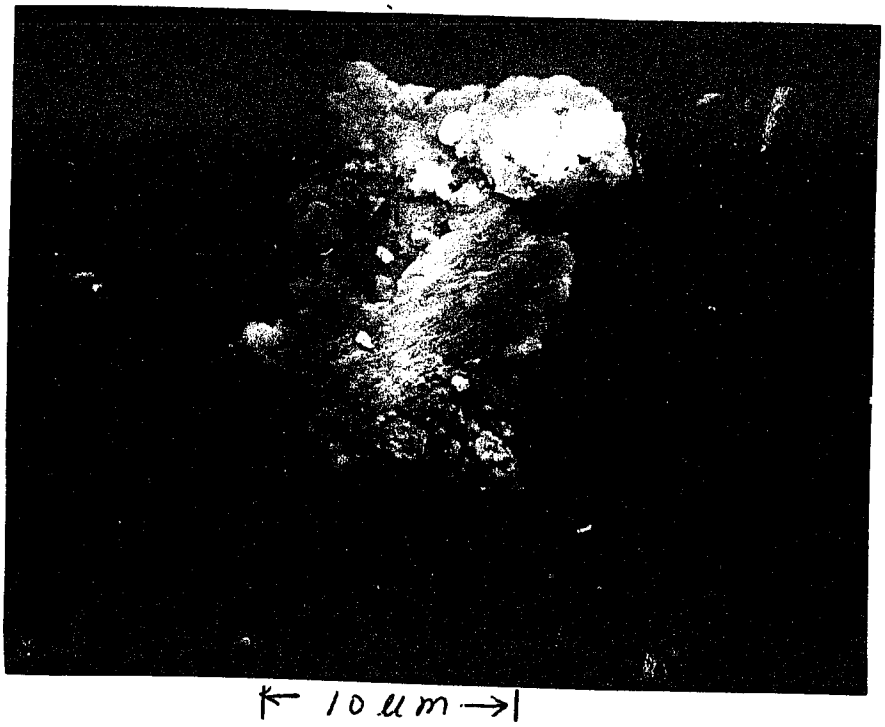


Figure 31 Scanning electron micrograph of EATC and 0.001 % Triton X-100 in PBS (X 4900)

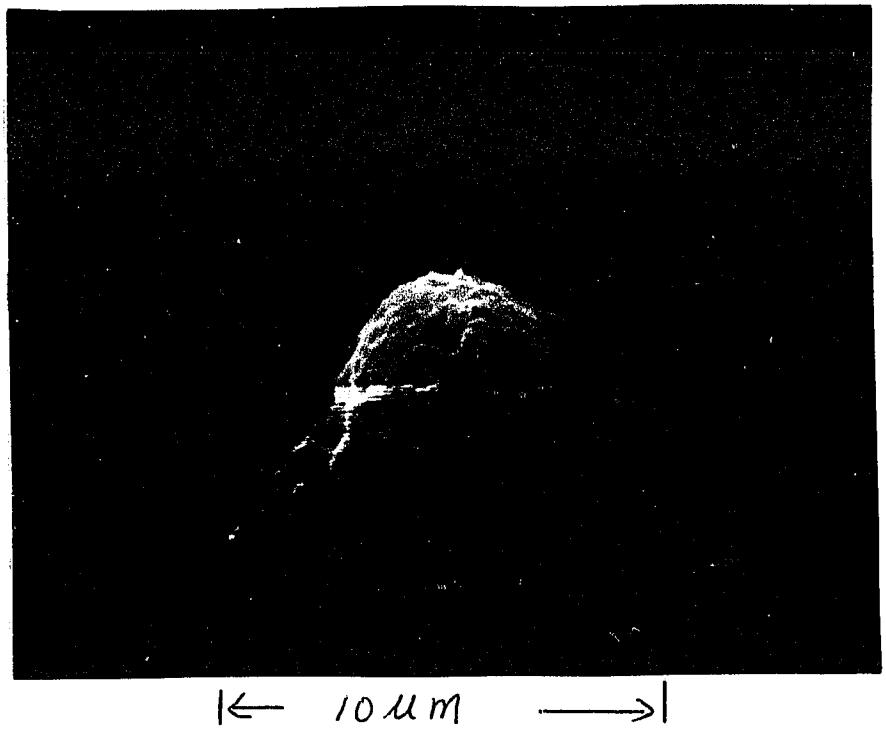
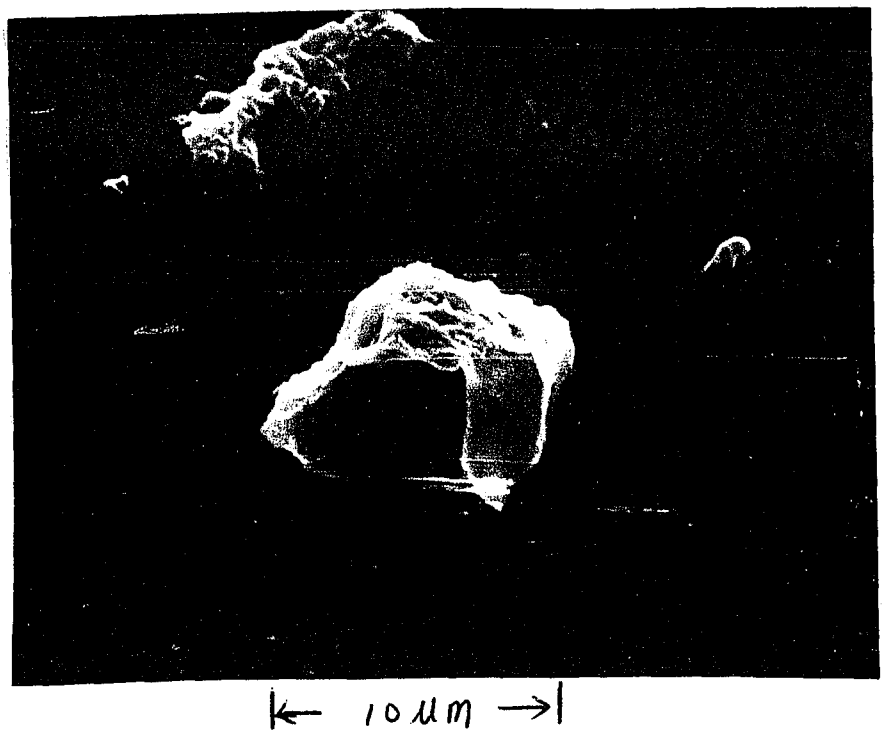


Figure 32 Scanning electron micrograph of EATC and CM extract (from coelomocytes, 1.64 mg/ml)(X 4500)



Studies of hemagglutination activity of PE with different C-18 fatty acid side chains (Table 13)

This study was initiated for several reasons. (1) There are conflicting reports in the literature about the hemagglutininating activity of different PE's. Forbes and coworkers ((34) reported that egg PE and DOPE agglutinated mouse RBC. Kawai (33) reported that PE was not an agglutinin. (2) One of Nereis lipid agglutinins behaves like PE on 2D-TLC. Pure synthetic PE would be a good model system for this activity. We found that DOPE (Avanti Polar Lipids) showed no agglutination activity after heating a chloroform solution to dryness on a steam bath for 1 hour and dissolving in 80% ethanol. However heating DOPE in the presence of trace amounts of water for 1 hour (specific titer = 4096/mg/ml) or dry for 3 or 6 hours (specific titer; 4096/mg/ml and 32768/mg/ml respectively) produced a powerful agglutinin. DEPE (heated with water for 1 hr), DLPE (heated with or without water for 1 hr) and DLNPE (heated with or without for 1hr) had hemagglutination activities, while DSPE did not have hemagglutination activity after

any of these treatments. Standard MOPE caused cell lysis but DOPA had a specific titer of 1448/mg/ml. TLC of the DOPE samples on silica in $CHCl_3$-CH_3OH-acetone-acetic acid-H_2O = 65 : 10 : 20 : 10 : 3 (v/v) showed the same spot with the same R_f as for the unheated inactive sample (R_f= 0.328). In this solvent, MOPE sample showed the same R_f as the unheated inactive sample in $CHCl_3$-CH_3OH-conc. ammonia = 65 : 25 : 5 (v/v) (R_f = 0.558). DOPA stayed near the origin (R_f = 0.108) but MOPE had an R_f = 0.225. Silica gel TLC of mixtures of standards showed the same R_f's as the individual components chromatographed swparately. The IR spectra of the inactive and active DOPE samples were the same. The inactive and active materials from DLPE and DLNPE gave positive periodate-Schiff tests while the active materials from DEPE and DOPE gave weak responses.

^{13}C NMR spectra of DOPE (heated with water) and DOPE (N_2)

Figure 33 shows the numbering system for DOPE and ^{13}C chemical shift assignments. Figure 34 shows that the spectrum of the modified active DOPE was slightly different from that of the standard. The signals at 63.9 ppm due to -CH-O-P- in O-acyl glycerol (sn-1), 70.3 ppm due to -CH-O-CO-R, in sn-2-esterified glycerol, 173.1ppm and 173.4 ppm due to R-CO-acyl ester carbonyl group were diminished as compared to neighboring signals of some magnitude. A new signal at 67 ppm due to -CH-OH, sn-2 in glycerol appeared. The peaks at129.7 ppm (due to C_9 at = bond) and 130 ppm (due to C_{10} at = bond) slightly changed.

Table 13 Analyses of DOPE, DOPA, MOPE, DSPE, DEPE, DLPE, and DLNPE

Sample	Specific titer*	R_f on silica TLC	
		Solvent A[a]	Solvent B[b]
DOPE (N_2)[c]	0	0.328	0.558
DOPE (Δ) 1 hr[d]	0	0.328	0.558
3 hr	4096	n.d.	n.d.
6 hr	32768	n.d.	n.d.
(Δ +H_2O) 1 hr[e]	4096	0.328	0.558
DSPE (N_2)[c]	0	n.d.	n.d.
(Δ) 1 hr[d]	0	n.d.	n.d.
(Δ + H_2O) 1 hr[e]	0	n.d.	n.d.
DEPE (N_2)[c]	0	n.d.	n.d.
(Δ) 1 hr[d]	0	n.d.	n.d.
(Δ + H_2O) 1 hr[e]	2048	n.d.	n.d.
DLPE (N_2)[c]	0	n.d.	n.d.
(Δ) 1 hr[d]	2048	n.d.	n.d.
(Δ + H_2O) 1 hr[e,f]	2048	n.d.	n.d.
DLNPE (N_2)[c]	0	n.d.	n.d.
(Δ) 1 hr[d]	512	n.d.	n.d.
(Δ + H_2O) 1 hr[e,f]	128	n.d.	n.d.
DOPA	1448	0.582	0.108
MOPE	cell lysis	0.082	0.225

* : Overnight titer/mg/ml.

a : Solvent A system was chloroform-methanol-acetone-acetic acid -water = 65 : 10 20 : 10 :3 (v/v).

b: Solvent B system was chloroform-methanol--conc ammonia = 65 : 25 : 5 (v/v).

c : PE was evaporated by N_2 and made 1 mg/ml of solution in 80 % ethanol.

d : PE was heated on a steam bath for 1 hr or heated on Multi Blok heater (American Scientific Products) for 3 or 6 hours and made in 1 mg/ml of solution in 80 % ethanol.

e : PE was heated with traces of water on a steam bath for 1 hour and dissolved in 80% ethanol at a concentration of 1 mg/ml.

f : Heated samples were not all soluble in 80% ethanol. The ppt was centrifuged off and the clear supernatants were titered.

Figure 33 Structure of DOPE with number assignments and ^{13}C NMR assignments (77, 78)

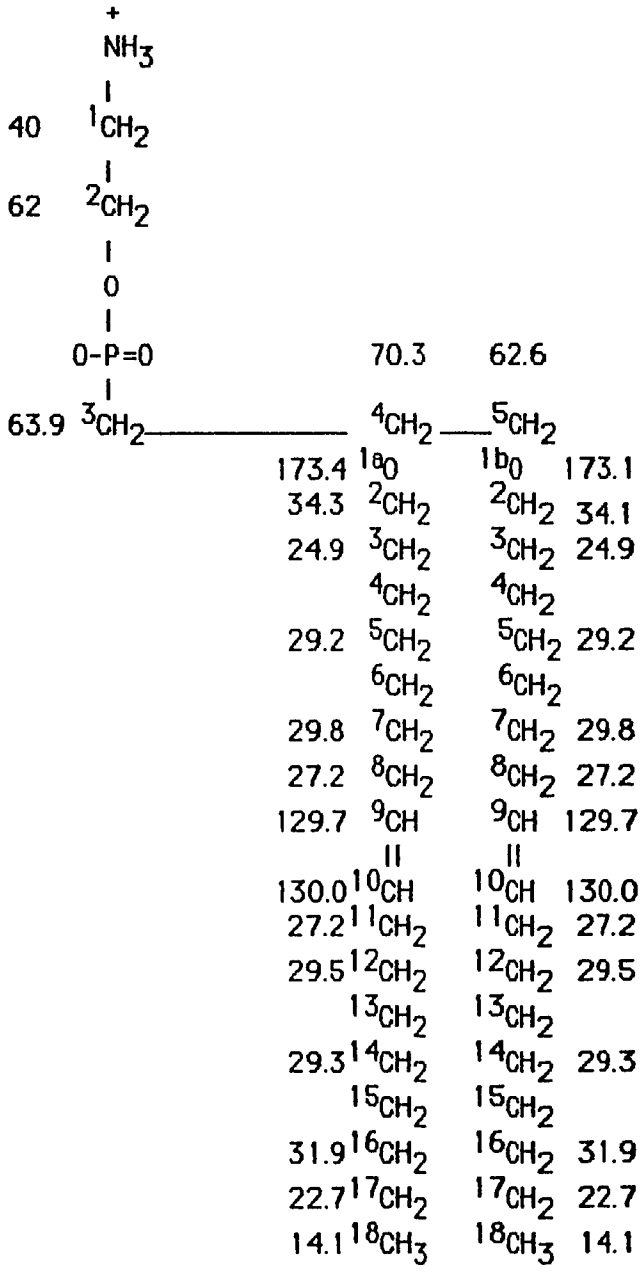
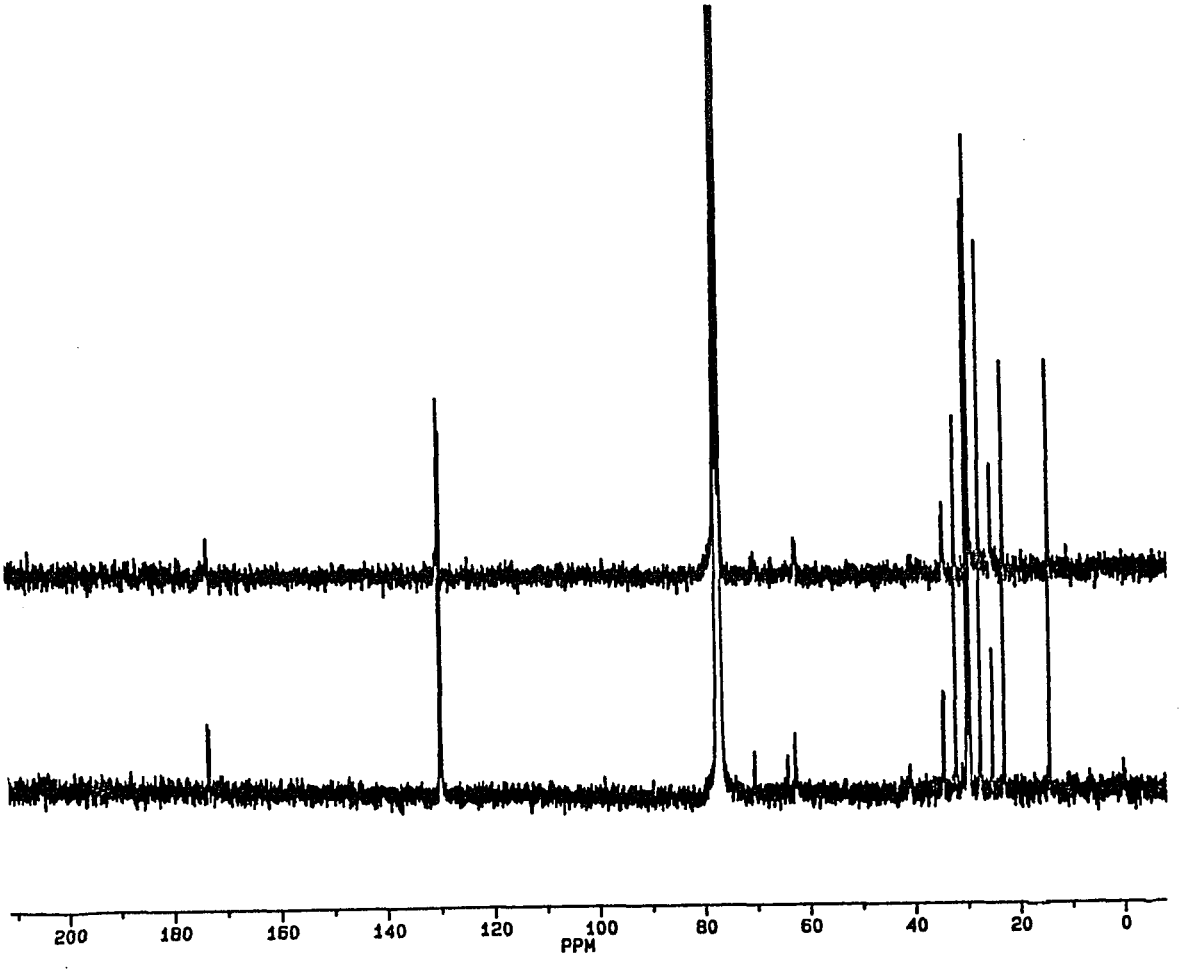


Figure 34 ^{13}C NMR Spectra of Dioleoylphosphatidylethanolamine* (DOPE, heated with water; top) and DOPE* (N_2, bottom)



*: 5 mg of DOPE (heated with water) was dried by lyophilizer/ or DOPE (N_2) was dried by rotatory evaporator and taken up in deuterochloroform (with TMS, Cambridge Isotope Laboratories (Woburn, MA)) and transferred to the NMR tube (Wilmad Glass Company) and filled up to 4 cm. The 75.469 MHz ^{13}C NMR spectra of DOPE samples were obtained with FTNMR spectrometerNR/300 (IBM instruments Inc.). The spectral width was 17241 Hz and gated broad-band proton decoupling with NOE was used. The delayed time between pulses was 4.000 s. The number of scans collected from 13657 to 10177.

DISCUSSION AND CONCLUSIONS

The coelomic fluid supernatant of Nereis virens was resolved into LMW and HMW hemagglutinins (Scheme1). A lipid extract (CM) from the particulate matter (mainly coelomocytes) also agglutinated RBC (Scheme 1). The nature of the hemagglutination of rat RBC by Nereis agglutinins was time- and concentration-dependent involving "cell clumping" but not "cell fusion" (Figures 6-10). Nereis agglutinins (LMW, HMW and CM) before and after treatment with insoluble PVPP, which is known to be able to remove polyphenolic compounds, showed very little or no change in hemagglutination activity (Table 7). It suggested that the agglutinins from Nereis virens were not polyphenolic compounds.

LMW

LMW agglutinin was Ca^{++}/Mg^{++}-independent because its hemagglutination activity was not affected by the presence of EGTA (a specific chelating reagent for the divalent cation) (Table 6). LMW activity was, however, phenol- & periodate-sensitive

(suggesting vicinal diol structure was required for activity). LMW was further fractionated by DEAE-cellulose chromatography into four active agglutinins (peak I, II, III, IV) with MW. 20, 20, 19, 24 Kd respectively (Scheme 1 and Figure 3). All four agglutinins were heat-sensitive and insoluble protease-resistant (Streptomyces griseus) (Table 7). Each had specific RBC and inhibition profiles. The hexose/protein (mg/ml) ratios of LMW peak I, II, III, IV were 0.691, 0.190, 0.058, 3.431 respectively (see Scheme 1) and suggested that LMW agglutinins were glycoproteins. The only monosaccharide inhibitor for all four agglutinins was mannose-1-phosphate (dicyclohexylammonium salt) (Table 3). They were also inhibited by BSM, fetuin (type III), thyroglobulin, yeast invertase and mannan. Immunoelectrophoresis of each LMW fraction gave precipitin lines which coincided with areas of hemagglutination. The agglutinin bands stained for protein and vicinal diol suggesting carbohydrate content. Based on electrophoretic migration at pH 8.3, LMW I was cationic, LMW II was neutral, LMW III & IV were anionic. Although this experiment

suggested that LMW III and IV corresponded to the same agglutinin, they showed different RBC specificities.

In in vivo experiments, all the mice receiving LMW and Ehrlich ascites tumor cells showed an average growth increase of about 7 g/mouse (Figure 21 (b)), all survived to the end of the experiment and showed no ascitic fluid although postmortem examination showed that three of the five surviving mice had signs of abdominal fat which was not significant. The mice receiving LMW alone (control) did not show any side effects (Figure 21 (a)). These results indicated that LMW protected mice from proliferation of Ehrlich ascites tumor cells. In vitro, LMW swelled EATC within two hours and ruptured them thereafter (Figures 24 qnd 29). It suggested that the permeability of the cell membrane was destroyed by LMW.

HMW

HMW is found to be a Ca^{++}/Mg^{++}-independent agglutinin with strong agglutination activity against rabbit and rat RBC (see Tables

1, 6 and 7). Sepharose 4B chromatography showed that activity is spread over a wide range of molecular weights (Figures 4 & 5). Activity is inhibited in decreasing order by fetuin (type III) > BSM > DsOSM > OSM (Table 3). Its activity is slightly affected by heating and TCA, and not at all by protease (soluble form, Streptomyces griseus) and periodate treatments (Table 7) although quantitative analyses for protein and hexose had shown that HMW contains both. These results suggest that HMW is not a simple glycoprotein. In immunoelectrophoresis using rabbit antiserum to coelomic fluid, HMW gives two precipitin bands near the origin which coincide with areas of hemagglutination activity. Precipitin bands appear neither for HHMW nor for DL-HHMW but hemagglutination activity remains mainly in the same region, which also stains for sugar and protein (Figure 12). The active region of HMW and HHMW, but not DL-HHMW, also stains for lipid. Activity for DL-HHMW includes a spot which is on the other side of the origin. It suggests that heating has destroyed the antigenic determinant. Heating also slightly decreases the specific titer (see Scheme 2) and

delipidation lowers the specific activity further (some activity remains in the organic phase, see Scheme 2).

DL-HHMW contains protein (12%), hexose (30%), hexuronate (9%), hexosamine (8%), GAG (glycosaminoglycan, at least 22%) and sulfate (7%) and a trace of phosphorus and pentose. Neither sialic acid nor N-sulfate are present. Hexuronic acid, hexosamine and sulfate are present in equal amounts (by weight). The total amounts (9% + 8% = 17%, by weight) of hexuronic acid and hexosamines are not equal to that of hexose (30%). These determinations are based on glucose, glucuronic acid and glucosamine. Other sugars might give lower or higher values. For instances, in the hexuronic acid determination, mannuronic acid gives only 17 % the color intensity of glucuronic acid (79). It suggests that DL-HHMW has other kinds of hexuronic acid (not glucuronic acid, the standard) and hexosamine (not glucosamine, the standard) and that the values are probably underestimated. Protein is determined by the Bio-Rad method and gamma-globulin may not be the best standard to use. DL-HHMW reacts with a cationic dye

[dimethylmethylene blue (DMB)] in the presence of guanidinium chloride and gives a metachromatic reaction (Figure 14). The results indicate that DL-HHMW is polyanionic. Here also, the 22% GAG estimate is based on chondroitin sulfate as standard and might be much too low because the intensity of response varies with the GAG. The IR and FTIR (Figure 15) show that DL-HHMW contains hexose-6-sulfate [1243 cm^{-1} (sulfate) and 813 cm^{-1} (equatorial-6-sulfate)] and amide [1536 cm^{-1} (amide II) and 1661 cm^{-1} (amide I), due to protein moiety as well as N-acylhexosamine)]. These results suggest that DL-HHMW is a glycosaminoglycan. Figure 16 shows structures of some typical glycosaminoglycans which contain one molecule of hexuronic acid and one molecule of hexosamine with/without 4-axial or 6-equatorial-sulfate.

When treated with proteoglycan depolymerization enzymes, chondroitinase ABC, keratanase and heparinase II, DL-HHMW does not lose its activity and GAG content as judged by titer and metachromatic reaction respectively. Standard chondroitin sulfate A and B, keratan sulfate and heparin are hydrolyzed by their

respective depolymerization enzymes under the same conditions. Either the protein component is protecting this GAG or this a new kind of GAG or both. I conclude that HMW is a lipid-associated sulfated proteoglycan. Further work will involve identification of the hexosamine and hexuronic acid. The protein will be removed by borohydride reduction, GAG separated on DEAE cellulose chromatography, and then depolymerization enzymatic treaments will be repeated and protein determined separately.

In vivo experiments showed that mice receiving HMW and EATC had an average weight gain on day 42 of 17 g/mouse (Figure 21 (a) & (b)). Three of the five mice which survived to the end of experiment did not show ascitic fluid. These results suggested that HMW delayed the growth of EATC in mice. In vitro, HMW causes the agglutination of EATC (Figures 25 & 30). This may be due to specific interactions of HMW, HHMW and DL-HHMW with cell surfaces or an electrostatic effects between GAG and positive charges on the cell surfaces. The higher titer before delipidation suggests that hydrophobic interactions improve the interaction

and/or that the teriary structure favoring agglutination is stabilized by lipid or that lipid fraction is also an agglutinin and the effects are additive.

CM

The lipid extract from the particulate fraction (mostly coelomocytes) (CM) was heat-sensitive, mannan-insensitive and Ca^{++}/Mg^{++}-dependent and agglutinated rat, rabbit > African green monkey > rhesus monkey = human (O), guinea pig >> chicken RBC (Table 1). In immunoelectrophoresis, CM did not give any precipitin line with rabbit antiserum (Figure 13). The region of activity extended along the length of the gel most of which stained with Sudan Black B. Antiserum lowered the titers of CM and heated CM. It may be that the antiserum contains specific or non-specific binding proteins that react with CM (Table 8) but not in an antibody-antigen fashion.

In In vivo experiments, mice receiving CM and tumor cells showed an average weight gain of 5.9 g/mouse by day 14 and then

the growth rate declined [Figure 21 (b)] and all the mice died on day 44. Only the mouse which died on day 28 showed ascitic fluid in the peritoneal cavity but the rest of the mice did not show any ascitic fluid in the peritoneal cavity or any solid tumors. It might be due to a toxic compound produced by Ehrlich ascites tumor cells in the presence of CM. CM itself is not toxic because the mice injected with CM alone were all healthy. In vitro CM swelled EATC within two hours and ruptured them thereafter. It suggested that CM might be incorporated into the cell membranes and changed their selective permeability.

CM extract was further fractionated into acetone-insoluble (AI) and acetone-soluble (AS) fractions by acetone-10% $MgCl_2$. AI and AS fractions were further resolved by TLC. One (spot 10-11) of the two active spots from AI moved very close to DOPE in 2D TLC and based on specific spray reagents, contains phosphate, $-NH_2$ and vicinal-OH (DOPE gives a negative response to the Schiff test) but no reducing sugar (see Table 11). It is possible that the fatty acid

side chains contain vicinal-OH groups.

The results of spray reagent test for specific functional groups showed that another active spot (spot 3) contains phosphate, choline, fatty acid ester and vicinal-OH. The positive staining result for choline might be due to double bonds. The IR spectrum of spot 3 (Figure 18) has a small peak for C=C (at 3020 cm^{-1}) and dilinoleoylphosphatidylethanolamine (having 2 double bonds) and dilinolenoylphosphatidylethanolamine (having 3 double bonds) also give positive choline tests. Spot 3 has a broad and strong phosphate IR band which is more characteristic of monophosphate than diphosphate ester. These results suggest that spot 3 might be a phosphatidylinositol with unsaturated fatty acid side chains and monophosphate ester groups. The structures of these two active spots are binding studied by mass spectroscopy and, using more material, by ^{13}C-NMR and ^{31}P-NMR.

The similarities of the specificities for I, II, and III shown in Tables 2 and 4 suggest that a compound or moiety common to these TLC bands is responsible for agglutination activity or that certain

red cells are more susceptible to agglutination by some lipids. If the agglutination activity is a function of shape and charge on the molecule, it is surprising that the materials behaving so differently on TLC share these similarities in activity. The hemagglutination activity may be a function of groups on the molecules, the shape of the molecule, charge distribution, state of aggregation or a function of all of these. The rat RBC are clumped not fused. Maybe liposomes are joining cells by multiple interactions. Kawai (32) found that many liposomes of the ornithine-containing lipid of B. pertussis were on the surface of the erythrocytes (observed by EM). Liposomes composed of PE and the ornithine-containing lipid (molar ratio = 3 :1), PE/ cholesterol/ the aminolipid (molar ratio = 1 : 1 : 0.3) had heamgglutination activity while those composed of phosphatidylcholine and the aminolipid/ phosphatidylcholine, cholesterol and the aminolipid showed no activity. Since only certain lipids do this, the surface of the liposome or aggregate would be unique.

AS was subjected to TLC systems appropriate for neutral lipids. The active spots for AS were the spots near the origin. Using different systems, AS was resolved into several components, one of which showed activity and has -NH and vicinal-OH but no phosphate, NH_2 or sugar (Figure 19 & Table 12). The latter might be a long chain amide with a vicinal-OH in a side chain. This possibility is being investigated by Ioannis Patrikios.

Bovine submaxillary mucin (BSM) was a good inhibitor of hemagglutination for all agglutinins from Nereis virens coelomic fluid (see Table 5). When BSM was heated on a steam bath for 2 hrs, which presumably denatured it, it becomes a poorer inhibitor of LMW, HMW and CM. BSM, treated with acid and heated at 80^0C for 1 hour (which causes hydrolysis of sialic acid and presumably some denaturation of protein), becomes a poorer inhibitor of HMW and CM while it becomes a better inhibitor for LMW (Table 5). The results suggest that inhibition by BSM of HMW & CM is partially due to the mucin's protein moiety. There is little or no change in the inhibition ability of BSM when it is treated with mercaptoethanol, suggesting

that disulfide linkages are not required for inhibition. BSM is a sialic acid containing glycoprotein. It contains 35.9% of sialic acid, 25.10% N-acetylhexosamine, 1.4% D-galactose and 1.23% L-fucose. The main prosthetic group in BSM is 6-alpha-D-sialylpyranosyl-N-acetyl galactosamine. The sialic acids of native BSM are N-acetylneuraminic acid, N-glycolylneuraminic acid, N-acetyl-7-O-acetylneuraminic acid, N-acetyl-8-O-acetylneuraminic acid and N-acetyl-7,8-di-O-acetylneuraminic acid. In addition to the main disaccharide prosthetic group, other oligosaccharides are present in BSM. The structure of the oligosaccharides are GalNAc-GalNAc-ol, Glc-GalNAc-ol, L-Fuc-[D-Gal. $GlcNH_2$]-$GalNH_2$-ol and NANA-[D-Gal. $GalNH_2$]-$GalNH_2$-OH. Trypsin break down BSM into a 28 amino acid glycopeptide with the composition ; (GalNAc), $(Ser, Thr)_{9.4}$ $(Ala, Gly)_{8.3}$ $(Pro)_3$ $(Val, Leu, Ile)_{3.5}$ $(Glu, asp)_{2.5}$ $(Asp)_1$. The composition is very similar to that for the original BSM. This similarity implies that BSM is composed of about 150 peptide sequences of 28 amino acids each. Aspartic acid is the N-terminal

amino acid. BSM contains traces of cysteine (80).

DOPE as an hemagglutinin

Because one of the CM agglutinins (10-11) moves like DOPE on Sil G 2D-TLC, we studied pure synthetic DOPE as an agglutinin. In addition, the literature contains contradictory reports about the hemagglutination activity of phosphatidylethanolamine (PE). Kawai (33) found that PE showed no hemagglutination activity. The source and preparation of PE were not given. On the other hand, Forbes et al (34) found that PE's of egg type III (lot 90F-8305, Sigma), of egg (Calibiochem), of E. coli of soybean, and pure [^{14}C] dioleoyl PE (Amersham) agglutinated mouse RBC. We find that fresh commercial DOPE (Avanti Polar Lipids) does not have agglutination activity while samples which have "aged" or been heated with water for 1 hour or been heated dry for 3-6 hours become powerful agglutinins. While DOPA is active, the specific titer of the treated material from DOPE is much higher.

In order to study the effects of unsaturation on the fatty acid

side chain of PE, a series of PE's [distearoyl (DSPE), dielaidoyl (DEPE), dilinoleoyl (DLPE), dilinolenoyl (DLNPE)] were studied in the same way. DEPE, where the 9,10 double bond is trans instead of cis as in DOPE, had hemagglutination activity when treated in the same way as DOPE (Table 13). DLPE, which has 2 double bonds per fatty acid chain, and DLNPE, which has 3 double bonds per fatty acid chain, were activated just by heating for 1 hour without water. However, no hemagglutination activity was observed when DSPE, which has no double bonds, was treated in the same way. It seems that unsaturation is necessary for PE to be converted to an hemagglutinin. As the degree of unsaturation increases, this conversion becomes more facile. At this stage, results suggest that oxidation, possibly with crosslinking, rather than hydrolysis, is responsible for this remarkable generation of hemagglutination activity.

The active material has the same R_f values as DOPE, which is different from those of DOPA and MOPE in two different solvent systems. MOPE and some other lysophospholipids (LPE,LPS, LPC and

LPI), all have R_f values which are lower than those of PE, PS, PC and PI in both solvents and are lysins, while oleic acid, which is also a lysin, moves with the solvent front in solvent A. The active material is yellow while DOPE is colorless. The infrared spectra for DOPE samples are the same except that the peak at 3020 cm^{-1} due to C=C seems relatively smaller in the heated sample. These results suggest that the active material is not a hydrolysis product, DOPA or MOPE or oleic acid but might be an oxidation product or polymerization product. The ^{13}C NMR spectrum of DOPE (heated with water) has very similar peak heights at 129.7 ppm (due to C_9 on = bond) and 130.0 ppm (due to C_{10} on = bond) as that of DOPE. It suggests that the $C_{9,10}$ double bond remains unaltered. In the active sample heated with water, peaks at 63.9 ppm [due to -$\underline{C}H_2$-O-P in O-acyl (sn-1)], 70.3 ppm (due to -$\underline{C}$H-O-CO-R in sn-2-esterified glycerol), 173.1 ppm and 173.4 ppm (due to R-$\underline{C}$O-acyl ester carbonyl group) have diminished. A new signal at

67 ppm (might due to -CH-OH, sn-2 in glycerol) appears suggesting that some hydrolysis has taken place. The next approach will be (a) analysis for aldehyde components for peroxides. (b) enzymatic hydrolysis with phospholipase A_1 or A_2, (c) ^{31}P, ^{13}C and proton NMR of the material isolated from TLC.

Reers & coworkers (81) studied 1-palmitoyl-2-linoleoyl-PE and isolated hydrolysis and oxidation products from aged samples. Lyso-PE was observed, but in addition, two other compounds resulting from oxidation were isolated, both of which still contained the palmitoyl group. Palmitic acid but not linoleic was recoverable by hydrolysis and gas chromatography of the methyl esters. They concluded that the linoleoyl group had been oxidized on the aging of their samples.

Duijun & coworkers (78) showed that egg PE is much more sensitive to oxidation by O_2 at 40^0C than is PC. As judged by fatty acid analysis and malondialdehyde formation, unsaturated, but not saturated fatty acids are destroyed in the process. Analysis of the

head group $-NH_2$ by trinitrobenzenesulfonic acid (TNBS) showed that a decrease in head group $-NH_2$ correlates with loss of the unsaturated fatty acid side chain. In addition, ^{13}C NMR showed a diminished signal for the methylene carbon attached to the head group-NH_2 and the appearance of a new signal attributable to a methylene carbon attached the nitrogen of a Schiff base, i.e. $-CH_2-N=CH-$. This suggests that malondialdehyde, formed in peroxidation, cross-links PE head groups. ^{31}P NMR and small-angle X-ray diffraction were used to study aggregate composition. Low levels of peroxidation stabilized lamellar against hexagonal$_{II}$ aggregates. Further peroxidation introduces a disorder-nonbilayer system possibily an " interwoven bilayer" such as has been shown for cardiolipin.

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