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Title: BACTERICIDAL AND PHYSICO-CHEMICAL PROPERTIES OF A LECTIN DERIVED FROM SPRING CHINOOK SALMON (ONCORHYNCHUS TSHAWYTSCHA) OVA

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Dr. C. L. Fryer

Unfertilized ova from spring chinook salmon (Oncorhynchus tshawytscha) were examined for the presence of classical salmonid immunoglobulin. Immunodiffusion techniques in which rabbit anti-salmonid immunoglobulin was reacted against an ova homogenate and rabbit anti-ova homogenate was reacted against immune chinook serum failed to detect classical-type antibodies within these eggs.

A lectin with bactericidal properties was isolated from spring chinook salmon ova. This protein agglutinated human type B and rabbit erythrocytes, but not human type A, O or sheep red cells. Hemagglutination was inhibited by D-galactose and L-rhamnose. The protein was purified by affinity chromatography and by gel filtration on Bio-Rad P300. The purified lectin contained a minor (0.8%) carbohydrate moiety. A rabbit antiserum was prepared against the protein and used to determine whether ova from coho salmon (O. kisutch), pink salmon (O. gorbuscha), chum salmon (O. keta),

kokanee salmon (O. nerka), steelhead trout (Salmo gairdneri), Lahonten cutthroat trout (S. clarki henshawi) or fall chinook salmon (O. tshawytscha) contained a similar protein. All species tested had an immunologically identical protein within their ova.

Purified spring chinook salmon ova lectin was bactericidal for two serotypes of Vibrio anguillarum, Pasteurella piscidida, Aeromonas hydrophila and Flexibacter columnaris, but not for Yersinia ruckeri, A. salmonicida, Edwardsiella tarda, Cytophaga psychrophila or the agent of bacterial kidney disease, Corynebacterium sp. In addition to these known bacterial fish pathogens, 19 species of bacteria commonly associated with humans were tested; none of the human-associated agents were inhibited by the lectin.

Proteins immunologically identical to the spring chinook salmon ova lectin were purified from ova of seven other salmonids and found to possess little, if any, activity against the bacterial fish pathogens. Even the protein purified from fall chinook salmon ova had minimal bactericidal activity.

Less than 1.0 μg/ml of the purified spring chinook ova lectin was required for complete inhibition of V. anguillarum growth. A 1 hr incubation of the lectin with the bacteria at 22 C was sufficient for 100% growth inhibition. Microscopic examination of the incubation mixture showed that after 20 min, approximately 50% of the bacteria were no longer motile. No cell lysis was observed.

The bacterial inhibition property was stable after heat treatment at 80 C for 6 hr or 100 C for 1 hr. Lyophilization of the protein did not reduce bactericidal activity.

The chinook lectin was compared with a plant lectin, ricin, for bactericidal activity and competitive binding of V. anguillarum cells. Although ricin had a carbohydrate specificity similar to the chinook protein, it was not toxic for V. anguillarum; however, it did compete for binding sites on the bacterium.

Coho salmon which received 125 μg of purified spring chinook ova lectin intravenously were not protected against subsequent challenge by V. anguillarum.

Bactericidal and Physico-chemical Properties of a Lectin Derived from Spring Chinook Salmon (Oncorhynchus tshawytscha) Ova

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INTRODUCTION

The protection of fish from infectious diseases is a major interest of the aquaculture industry; increased survival rates are rewarded by higher economic returns. Immunization of the fish represents a promising disease control measure. Studies have shown that the administration of certain bacterial and viral antigens to salmonid fish induced a protective immunity (Fryer et al., 1976a) and two of these bacterins are currently licensed and commercially available in the United States (Fryer et al., 1977). Although the salmonid antibody has been extensively characterized (Cisar and Fryer, 1974), other aspects of the fish immune state have received little attention and are less understood. One such area of study concerns the protective mechanisms that very young fish may acquire from the maternal adult through the ova. The immunization of pre-spawning adults may induce the passive transfer of antibodies to the ova. Since this could represent an economical method for protection of large numbers of salmon fry, this project was initiated.

The purpose of this study was to determine if maternal antibody, or other protective substances, were transmitted to the ova by salmonid fish. The objective of this research was to provide further

information on the immune mechanisms of salmonids. Information of this nature will ultimately enable aquaculturists to provide the most effective and economical protection to the cultured species and add to knowledge concerning the immune mechanisms of fishes.

Chinook salmon (Oncorhynchus tshawytscha) ova were examined for the presence of the classical-type salmonid immunoglobulin by immunological and anti-bacterial methods. A hemagglutinin protein with specific bactericidal properties was isolated and characterized from these ova. Eggs from six additional species of salmonids including coho salmon (O. kisutch), steelhead trout (Salmo gairdneri), Lahonten cutthroat trout (S. clarki henshawi), kokanee salmon (O. nerka), pink salmon (O. gorbuscha) and chum salmon (O. keta) were tested for the presence of this substance. The protein was purified and information obtained concerning the molecular weight and chemical composition of the molecule, hemagglutination and the sugar specificity of this reaction, the antigenic relationship of the protein isolated from seven salmonids, and the specific bactericidal activity of the protein when isolated from each salmonid species.

LITERATURE REVIEW

This review presents information pertinent to the discussion of maternal contributions to the immune protection of salmonid fry. Recent advances in the study of the immune response of fish are summarized, and information regarding the passive transfer of maternal antibody in other vertebrates is presented for comparative purposes. Additionally, certain studies concerned with receptor-specific agglutinins, also known as lectins, are discussed.

Immune Response of Fish

An extensive review of the immune response in fish was recently published by Corbel (1975). Therefore, only selected references pertinent to the structural and functional aspects of the fish immunoglobulin, and more recent advances concerning the immunization of fish are discussed here.

Salmonid fish respond to antigenic stimulation and produce a 17 S macroglobulin composed of four IgM-like subunits (Cisar and Fryer, 1974). This immunoglobulin, prepared against Aeromonas salmonicida, was found to be a very sensitive agglutinating antibody; only 0.006 μg of antibody N/ml was required for visible agglutination of A. salmonicida cells. Although certain studies suggest that catfish and goldfish may respond with low and high molecular weight

antibodies (Kuhns and Chuba, 1968; Uhr, Finkelstein and Franklin, 1962), investigations with salmonid fish have indicated that no low molecular weight immunoglobulin is present (Hodgins, Weiser and Ridgeway, 1967; Cisar and Fryer, 1974). As presently described, the lungfish (Dipnoi) are the lowest phylogenetic order of vertebrates with two distinct classes of immunoglobulins (Marchalonis, 1969). Sharks produce two types of antibodies (18S and 7S), but they are antigenically identical and both considered IgM-like molecules (Grey, 1969).

Environmental temperature has a direct effect on the rate of antibody synthesis in teleosts (Avtalion, 1969); this was especially noted in salmonid fish which frequently experience marked differences in temperature due to climatic changes and during their ocean migration (Fryer et al., 1976b). In general, an enhancement in the rate and amount of antibody production by coho salmon immunized against A. salmonicida occurred as temperature increased from 3-15 C. Voss, Groberg and Fryer (1978) noted that the catabolism of coho salmon immunoglobulin also was temperature-related; a decrease in the half-life of serum antibody from 56 to 37.5 hr was observed when fish held at 6 C were compared to those maintained at 18 C. The rate of absorption of passively administered coho immunoglobulin from the peritoneal cavity was temperature-related (Groberg, Voss and Fryer, 1978). O'Leary, Cisar and Fryer (1978) reported that

the agglutination activity of coho immunoglobulin was significantly diminished when serum was heated at 55 C for 60 min, and completely absent at 65 C for 30 min. Rabbit immunoglobulin activity, in comparison, was unaffected by heating at 55 C for 120 min, though somewhat diminished at 65 C for 60 min. In general they found a 10 C difference in the temperature sensitivities of the coho antibody as compared to the rabbit immunoglobulin.

Since salmonids produce antibodies in response to antigenic stimulation, efforts have been made to immunize these fish against certain infectious agents (Fryer et al., 1976a; Fryer et al., 1977). Of four immunogens (Flexibacter columnaris, A. salmonicida, Vibrio anguillarum and infectious hematopoietic necrosis virus-IHNV) tested, the two latter agents appeared most promising for mass immunization of salmonids. Although fish did respond immunologically to F. columnaris, four months of continuous antigen administration was required to elicit protective levels of antibody. Certain studies have described successful immunization with A. salmonicida bacterins (Klontz and Anderson, 1970); however much difficulty has been encountered in obtaining consistent protection against this pathogen (Fryer et al., 1976a).

Immunization studies with V. anguillarum have provided encouraging results; both orally and parenterally-administered bacterins conferred excellent protection in immunized animals

when their mortalities were compared to those which occurred in unvaccinated control fish similarly challenged (Rohovec, 1974). This study also demonstrated that oral immunization afforded protection at water temperatures from 4-21 C. Of special interest was the observation that agglutinating antibody was not detected in the serum of orally immunized animals, although injected fish did form antibody. Apparently the protection afforded by the oral route was due to another immune mechanism; possibly cellular immunity or secretory antibodies were involved.

Vibrio anguillarum bacterins have been field tested at selected locations. Antipa (1976) reported that injected preparations of killed cells conferred excellent protection on treated chinook salmon; tests with coho salmon were equivocal due to a poor challenge by the pathogen. Gunnels, Hodgins and Schiewe (1976) used orally and parenterally-administered bacterins for field tests in Puget Sound, Washington, and found that immunized chinook salmon were not significantly protected. This failure may have been due to the presence of serologically different strains of V. anguillarum.

Tebbit (1976) reported successful immunization of kokanee salmon with a partly attenuated strain of IHNV, a viral pathogen of salmonids. Simple addition of the virus to aquaria water which contained the salmon protected them against subsequent challenge with high levels of virulent wild type IHNV from which the altered

strain was derived. Tebbit also showed that immunity lasted at least 110 days and protected animals against virus isolates from four geographical locations.

Interest in the immunization of economically important food fish has stimulated efforts to develop more effective methods of vaccine delivery to the animals for mass immunization (Fryer et al., 1977). Although intraperitoneal injection of the immunogen elicits a potent antibody response, it is impractical for handling large numbers of fish. Rohovec (1974) found that incorporation of V. anguillarum bacterin into the food conferred excellent protection against subsequent challenge. Since the oral route requires no additional effort beyond feeding the fish, it provides an attractive means for delivery of vaccine to the animals. Amend and Fender (1976) reported that immersion of fish into a hyperosmotic solution which contained bovine serum albumin (BSA) resulted in high serum levels of BSA. The lateral line system of the animals appeared to play an important role in uptake of the protein. They suggested this method might effectively deliver immunogens to fish. Tebbit (1976) demonstrated that vacuum infiltration, a method developed by Wildlife Vaccines, Inc., Wheat Ridge, Colorado, immunized fish against IHNV. His studies showed, however, that simple addition of attenuated virus to the aquarium water which contained the experimental animals provided better protection against subsequent challenge with

virulent virus. Another promising technique, spray vaccination, was described by Gould (1977). Vibrio anguillarum bacterin delivered with a spray gun apparatus protected fish against challenge by this virulent fish pathogen.

Passive Transfer of Maternal Antibody

Very little work has been done to determine whether maternal immunity is transmitted to salmonid fry. This is unfortunate since this passive transfer may provide a method for protection of large numbers of fry from diseases commonly associated with very young animals, yet only require the immunization of a relatively small number of adult fish prior to spawning.

Fidler, Clem and Small (1969) found 19S IgM in the sera of newborn nurse sharks (Ginglymostoma cirratum). They concluded, however, that this immunoglobulin was of fetal, and not maternal origin since the 7S IgM of sharks would most likely be the molecular species transmitted to young animals. No 7S immunoglobulin was found in newborn sera.

An interesting method of transmission of maternal immunity to fry was described for the Amazonian discus fish (Symphysodon discus) by Hildemann (1962). The newly-hatched fry fed almost exclusively on a mucus secretion derived from epidermis of the parental skin. When eggs were hatched in the absence of adults,

the fry died within a week even though abundant planktonic food was provided. When a broad spectrum antibiotic was added to the aquarium, fry survived in the absence of the parents. The fry, extremely susceptible to fatal infection by microorganisms, were protected by either adult body mucus or the antibiotic. Unfortunately, no structural work was done to determine if the transferred immunity was due to antibody or, if so, whether the immune protein was identical to serum immunoglobulin.

Certain egg-laying vertebrates transmit maternal immunity by way of the ovarian follicle into the yolk of the developing egg. Antibodies then are absorbed from the embryonic yolk-sac and transmitted to the circulation of the embryo (Buxton, 1952). A similar process occurs in the tortoise (Brambell, 1970). Antitoxins, antiviral antibodies and bacterial agglutinins are transmitted from the circulation of the adult to the yolk of chicken ova. Buxton (1952) reported that yolk agglutinin titers against Salmonella pullorum were nearly equal to titers of the maternal sera. Paterson et al. (1962) found that precipitins against BSA appeared in eggs four days after the appearance of the antibody in the serum, and reached maximum levels 5-6 days after the peak serum levels were attained.

The transmission of maternal immunity to the young mammal may occur before birth, after birth, or at both times depending upon the species (Table 1). Horses, pigs and ruminants have no prenatal

transmission, but the young of these animals acquire postnatal immunity from maternal colostrum by absorption of antibodies across the gut. The young of these mammals must receive the colostrum within 48 hr after birth for transmission of immunity; after that time the antibodies are enzymatically degraded and not transported across the walls of the digestive tract. Rats, mice, guinea pigs and rabbits acquire maternal antibodies prenatally by way of the yolk-sac; rats and mice also obtain postnatal immunity from colostrum up to 20 days after birth. Maternal immunity is transmitted to young cats and dogs postnatally by way of colostrum; however, this only occurs from 1-2 days after birth. Primates transmit maternal antibodies across the placenta rather than through a yolk-sac, and most immunity is acquired during the prenatal period. Postnatal transmission of antibodies in the colostrum may aid the young primate in prevention of enteric infections; it is unlikely that proteins of this molecular size are transmitted across the gut wall and to the newborn circulation.

The transmission of maternal antibodies was shown to be a selective process in humans (Franklin and Kunkel, 1958); 19S IgM, though present in maternal sera, is not transmitted to cord sera. Protein transmission is apparently due to molecular specificity rather than size, since 19S α_2-globulin is transmitted to cord sera. Studies have also shown that homologous IgG is preferentially transmitted when compared to heterologous IgG (Brambell, 1970). Among

Table 1. Time of transmission of passive immunity.[a]

Species	Transmission of passive immunity		
	Prenatal	Postnatal	
Horse	0	+++	(24 hr)
Pig	0	+++	(24-36 hr)
Ox, goat, sheep	0	+++	(24 hr)
Wallaby (Setonix)	0	+++	(180 d)
Dog, cat	+	++	(1-2 d)
Fowl	++	++	(5 d)
Hedgehog	+	++	(40 d)
Mouse	+	++	(16 d)
Rat	+	++	(20 d)
Guineapig	+++	0	
Rabbit	+++	0	
Man, monkey	+++	0	

[a]From Brambell, 1970.

ruminants, IgG is selectively transferred to colostrum; in fact, IgG levels in colostrum exceed that of the maternal serum (Murphy et al., 1964).

Receptor-Specific Proteins (Lectins)

Since hemagglutinating proteins have been described from salmonid ova (Todd, 1971) and at least one of these agglutinins has been shown to be bactericidal for certain fish pathogens (Voss, Fryer and Banowetz, 1978), selected references concerning these substances, also known as lectins, are presented below.

Lectins, sometimes referred to as receptor-specific proteins, are proteins or glycoproteins which agglutinate erythrocytes or certain other cells by specific reaction with carbohydrate groups (Sharon and Lis, 1972). Some lectins have other unique biological and chemical properties; for instance, certain are specific in their reactions with human blood groups and are used for blood-typing. Other lectins act as mitogens in that they stimulate the transformation of lymphocytes from small "resting" cells to large actively growing cells. Some of these proteins preferentially agglutinate transformed tissue culture cells and are used to study cellular surfaces. Many lectins exhibit two or more of the above properties.

Most lectins have been isolated from plant origin; concanavalin A is probably the most well-studied of these proteins (Gold and

Belding, 1975). The function of these substances in plants remains unclear. Specific lectins may serve as recognition proteins responsible for Rhizobium-legume root nodule symbiosis (Bohool and Schmidt, 1974; Dazzo and Brill, 1977). Dazzo and Brill found that Rhizobium trifoli possessed a surface antigen identical to one found on the surface of clover roots, and that a lectin eluted from the roots specifically bound this antigenic group. This was consistent with a model suggested earlier which postulated that the lectin served as a cross-bridging mechanism between bacterium and legume (Dazzo and Hubbell, 1975).

Plant lectins have also been postulated to serve as plant "antibodies," carbohydrate transport proteins, regulators of embryonic cell division or as storage proteins; however, conclusive evidence for any of these functions is lacking and further research concerning the functions of lectins is required (Callow, 1975).

Lectins have been isolated from a wide variety of invertebrates; mollusks and crustaceans provide an especially rich source of these agglutinins (Gold and Belding, 1975). While the role of lectins in invertebrates is unclear, some studies suggest the proteins may serve in clearance of bacteria (Pauley, Krassner and Chapman, 1971; Prokop et al., 1974). Pistole and Furman (1976) described a bactericidal system in the hemolymph of the horseshoe crab, Limulus polyphemus; unfortunately, they reported no structural work which

indicated that this activity was due to the Limulus hemagglutinin described by Finstad et al. (1972).

Hemagglutinins have been isolated from fish, amphibians, reptiles and birds (for a complete review, see Gold and Belding, 1975). In addition, lectins have been isolated from embryonic mammalian neuronal tissue; they are postulated to function as a cell recognition aid in developmental processes (Simpson, Thorne and Loh, 1977; Teichberg et al., 1975).

Salmonid-derived hemagglutinins were found in Salmo gairdneri serum (Hodgins, Weiser and Ridgway, 1967) and in the ova of S. eriox, S. gairdneri, S. salar and S. trutta (Todd, 1971), S. irideus (Anstee, Holt and Pardoe, 1973), Oncorhynchus tshawytscha and O. kisutch (Voss, Fryer and Banowetz, 1978). All the salmonid ova-derived lectins agglutinated human type B erythrocytes, and this activity was specifically inhibited by D-galactose and L-rhamnose. The S. trutta ova lectin was unaffected by 2-mercaptoethanol, stable to lyophilization and strongly active after heat treatment at 80 C for 10 min (Anstee, Holt and Pardoe, 1973).

Although the salmonid ova-derived lectins have been termed "protectins," little is actually known of their function in the fish. Voss, Fryer and Banowetz (1978) isolated and purified a lectin from O. tshawytscha ova that was bactericidal for certain Gram-negative fish pathogens. Dose response and kinetic studies indicated that the

bactericidal activity was very specific. This protein agglutinated rabbit and human type B erythrocytes, had a pI of 4.5, a molecular weight of approximately 122,000 daltons, and bound D-galactose and L-rhamnose monosaccharides. Since these authors did not detect classical salmonid immunoglobulin within the ova, they postulated that the lectin served a protective role in young salmon. However, final proof for this hypothesis awaits further studies.

MATERIALS AND METHODS

Salmonid Ova Sources

The following salmonid ova were obtained from the Oregon Department of Fish and Wildlife. Chinook salmon (Oncorhynchus tshawytscha) eggs were taken from spawning animals at the South Santiam Salmon Hatchery. Coho salmon (O. kisutch) and steelhead trout (Salmo gairdneri) ova were obtained from the Alsea River Trout Hatchery. The kokanee salmon (O. nerka) ova were taken from fish at Suttle Lake and the Lahonten cutthroat trout (S. clarki) eggs were obtained from spawning trout at Mann Lake.

Pink salmon (O. gorbuscha) ova were collected by Mr. David Ransom, Dept. of Microbiology, Oregon State University, from adult salmon in Alaska. Chum salmon (O. keta) ova were obtained from spawning adult fish at Netart's Bay, Oregon.

Preparation of Ova

The ova were prepared for experimental use in the following manner. Eggs were placed in an ice bath and homogenized with a Virtis 23 tissue grinder. Phosphate buffered saline (PBS) pH 7.2, 0.15 M was used for dilution of the homogenates; routinely, the ground ova were added to five volumes of PBS, though certain homogenates were diluted with ten volumes of the buffer. These

suspensions were centrifuged at 13,000 x g for 20 min (4 C) and the supernatants harvested and stored at -20 C. Prior to experimental use, ova solutions were thawed, dialyzed against 1000 ml of 0.10M Tris, pH 8.0, and four 1000 ml volumes of PBS (4 C), and passed through a 0.45 μm membrane filter.

Examination of Unfertilized Chinook Salmon Ova for Maternal Antibody

The purpose of this study was to determine if adult female chinook salmon which had known amounts of circulating specific antibody passed these proteins to their ova. Spawning female chinook at the South Santiam Salmon Hatchery were sampled in the following manner. Blood, eggs, ovarian fluid and kidney tissues were collected from each individual and placed in an ice bath. Serum was harvested from whole blood, filter sterilized, and assayed for agglutinating antibodies against Aeromonas salmonicida and the causative agent of bacterial kidney disease (BKD). The microtiter method of Conrath (1972) was used for this assay and employed washed A. salmonicida and BKD organisms (heat-killed at 62 C for 45 min) suspended to an optical density (O.D.) at 520 nm of 0.8 and 1.0 respectively. Serum was also tested for precipitating antibodies against soluble antigens of these same bacteria. Ouchterlony double diffusion in 1.0% Agarose[1]

[1]Sigma Chemical Co., Ann Arbor, Mich.

in PBS was used to react each serum against culture supernatants of A. salmonicida and BKD. Material removed from within the eggs by a tuberculin syringe fitted with a 26 gauge needle was assayed for precipitating antibodies by the same method. The double diffusion method was also used to react each egg sample against rabbit anti-coho immunoglobulin serum (Cisar and Fryer, 1974). Ovarian fluid samples were assayed for agglutinating and precipitating antibodies against A. salmonicida and BKD and also reacted against the rabbit anti-coho immunoglobulin serum. Kidney tissues were inoculated onto Brain Heart Infusion[2] (BHI) agar and Mueller-Hinton[2] agar (supplemented with 0.1% L-cysteine HCl) to detect active A. salmonicida and BKD infections, respectively. Data were collated so that results of egg and ovarian fluid examinations could be correlated with circulating antibody levels found in the blood of each individual.

Characterization of Salmonid Ova Proteins

Dialyzed preparations of ova homogenates were used in these studies except for one electrophoresis experiment which tested the effects of Tris dialysis on a nondialyzed homogenate. The non-dialyzed preparation consisted of a homogenate mixed with ten volumes of PBS.

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[2]Difco Laboratories, Detroit, Mich.

Electrophoresis

Microscope slides (25 x 75 mm) set in immunoframes[3] were covered with molten 1% agarose prepared in barbital buffer (2.46 g/l barbital and 9.75 g/l sodium barbital, pH 8.6). Wells (1 mm diameter) were cut with a punch set[3] and loaded with 25 µl of sample. Electrophoresis was conducted in the deluxe electrophoresis chamber[3] for 2 hr at 200 V with a 0.01M solution of fluorescein used as a marker. When the dye migrated to the anodal edge of the slide, electrophoresis was stopped and the slides dried with a heat gun.[4] The dried gels were stained with 0.25% Coomassie Blue in 9% acetic acid-50% methanol for 5 min and destained with 5% methanol-7% acetic acid.

Hemagglutination

Erythrocytes tested for hemagglutination by the ova proteins were washed three times with PBS and resuspended to 1% in PBS for the assay. Washed cells (50 µl) were added to wells of a disposable microtiter plate[5] which contained 50 µl of the serially diluted protein.

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[3] Gelman Instruments Co., Ann Arbor, Mich.

[4] Master Appliance Corp., Racine, Wis.

[5] Cooke Engineering Co., Alexandria, Vir.

The plates were covered with an adhesive acetate sheet and incubated at 22 C for 18 hr. Hemagglutination titer was recorded as the reciprocal of the final dilution which showed visible agglutination.

The rabbit erythrocytes were collected from a New Zealand white rabbit. Sheep red cells were purchased from Prepared Media, Portland, Ore. The human red cells were provided by the Blood Bank, Good Samaritan Hospital, Corvallis, Ore.

Hemagglutination Inhibition

Sugars and peptones tested for the ability to inhibit hemagglutination were prepared to a final concentration of 0.02M (sugars) and 1 mg/ml (peptones) in PBS. The ova proteins were incubated with 10 µl of the sugar or peptone solution in the microtiter well for 30 min at 22 C before erythrocytes were added. After a further incubation period of 18 hr at 22 C, the hemagglutination titers were recorded.

The sugars were obtained from the following sources: D-mannose, fructose, deoxy-l-galactose, 2-deoxygalactose (Nutritional Biochemical Co., Cleveland, Ohio); L-rhamnose (Difco, Detroit, Mich.); D-galactose (Matheson, Coleman and Bell, Norwood, Ohio). Neopeptone, peptone and proteose peptone were purchased from Difco.

Chromatography Methods for Protein Purification

Two purification methods were used during the course of this study. Initially, the agglutinin was separated from other ova proteins by affinity chromatography on agarose. Subsequent studies were performed with material purified by gel filtration on Bio-Gel P-300.[6]

The affinity chromatography was performed as follows: agarose, previously washed three times in PBS, was mixed with five volumes of the ova homogenate. The mixture was incubated at 5 C for 16 hr, then centrifuged at 1600 x g for 5 min. After the supernate was removed, the agarose pellet was washed five times with PBS and then incubated with five volumes of a 0.2M L-rhamnose solution for 1 hr at 22 C. Consecutive second and third elutions were performed identically as described above. The eluates were dialyzed against four 1 liter volumes of PBS at 4 C and stored at - 20 C.

Gel filtration was performed with a column (2.5 x 35 cm) of Bio-Gel P-300. The P-300 beads were swelled in 0.1M phosphate buffer, pH 8.1, for 48 hr at 5 C, poured into the column, and the void volume was calibrated with Blue Dextran 2000[7] using descending flow. Previously dialyzed protein samples were applied in 1.0-2.5 ml volumes and washed through the column with the phosphate buffer.

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[6] Bio-Rad Labs, Richmond, California.

[7] Pharmacia Fine Chemicals, Piscataway, N.J.

Samples were collected with a drop-counting fraction collector and monitored for absorbance at 278 nm. The protein-containing fractions with hemagglutinin activity were pooled, passed through a 0.45 μm filter, and stored at - 20 C.

Carbohydrate Analysis

Quantitative and qualitative techniques were used to determine whether the agglutinin contained a carbohydrate moiety. The total carbohydrate content of the agglutinin was determined by the phenol-sulfuric acid test (Dubois et al., 1956). Glucose standards which contained 2.5-37.5 μg/ml were used to prepare a standard curve which related the amount of sugar to optical density.

Purified agglutinin was also analyzed on a gas chromatography apparatus[8] fitted with GE SE-30, Chromsorb W. Analysis was performed at 190 C for 1000 sec with helium as a carrier gas. The agglutinin was prepared for analysis as follows: a solution which contained 3.85 mg of purified chinook ova agglutinin was mixed with an equal volume of 4N HCl and placed in a boiling water bath for 4 hr. After cooling, the hydrolysate was titrated to the phenol red endpoint with 2N NaOH and lyophilized. The dried material was resuspended

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[8] F and M. Scientific, Avondale. Penn.

in Tri-Sil Z[9] and 1.0 µl injected onto the column. Arabinose and galactose served as markers.

Preparation of Rabbit Antisera

Antisera were prepared against chinook salmon ova homogenate and the purified salmonid agglutinin derived from chinook salmon ova. Adult New Zealand white rabbits were immunized with 5-10 mg of protein emulsified in Freund's Complete Adjuvant[10] by intrascapular injection and injections in both hind foot pads. Blood collected through incisions in the marginal ear veins at days 15, 19 and 65 was incubated at 22 C for 1 hr, 4 C for 18 hr, and centrifuged at 1000 x g for 15 min. The harvested serum was passed through a 0.45 µm filter and stored at -20 C. The subsequent booster immunizations (day 95 for anti-homogenate rabbit; day 122 for anti-agglutinin animal) were prepared in Freund's Incomplete Adjuvant[10] and administered intramuscularly.

Antigenic Comparison of Ova Proteins of Certain Salmonids

Immunodiffusion and immunoelectrophoresis were used to determine whether the agglutinin detected in chinook salmon ova was also

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[9]Pierce Chemical Co., Rockford, Ill.

[10]Difco Labs, Detroit, Mich.

present in ova from other salmonids. All ova used for these studies were homogenized and dialyzed as described above.

The immunodiffusion was performed as follows: a 1% agarose solution in barbital buffer (pH 8.6) was heated, cooled to 45 C, and dispensed into plastic Petri dishes (15 x 100 mm), 15 ml per dish. After the agarose cooled, a pattern with a central antiserum well and six surrounding antigen wells was cut. Centers of adjacent wells were 13 mm apart. Ova homogenates were placed in the outer wells and allowed to diffuse 6-8 hr prior to the addition of rabbit antiserum to the center well. The plates were incubated at 22 C until precipitin bands formed, usually 24-36 hr.

Immunoelectrophoresis was conducted in a manner similar to the electrophoresis procedure described above. After the ova homogenates were electrophoresed, a central trough was cut, filled with the appropriate rabbit antiserum, and the slides incubated in a moist chamber at 4 C until precipitin lines formed. The slides were then dried and stained as described.

Antigenic Comparison of Salmonid Agglutinin to Salmonid Immunoglobulin

Immunodiffusion was used to determine if the salmonid ova agglutinin was antigenically related to salmonid immunoglobulin. Rabbit anti-coho immunoglobulin was reacted against purified

agglutinin in an Ouchterlony double diffusion assay. In addition, rabbit anti-purified agglutinin was reacted against purified coho salmon and chinook salmon immunoglobulin prepared by Dr. E. W. Voss. Plates were incubated at 22 C until control precipitin bands appeared, usually 24-36 hr.

Bacterial Inhibition Studies

Experiments were conducted to determine whether ova proteins, particularly the agglutinin, affected the growth of certain bacteria. During the course of these studies it was shown that Vibrio anguillarum was strongly inhibited by the chinook salmon ova-derived agglutinin. For this reason, the V. anguillarum-chinook agglutinin system was used as a model for future experiments.

Sources of Bacteria

The bacterial fish pathogens used for these studies are listed in Table 2. With three exceptions, these organisms were maintained on BHI. The two myxobacteria, F. columnaris and C. psychrophila, were grown on Cytophaga media (Anacker and Ordal, 1959). The causative agent of bacterial kidney disease, considered by many to be a Corynebacterium sp., was propagated on Mueller-Hinton agar supplemented with 0.1% L-cysteine.

All the human-associated bacteria used in this study are listed

Table 2. Bacterial fish pathogens incubated with salmonid ova proteins.

Organism	Original Host Species	Source
Vibrio anguillarum type 1	Oncorhynchus kisutch	1
V. anguillarum type 2	O. kisutch	1
Aeromonas salmonicida (As-Sil-67)	O. kisutch	1
A. hydrophila (2035)	O. tshawytscha	1
A. hydrophila (K1)	Alosa sapidissima	1
Yersinia ruckeri	Salmo gairdneri	1
Pasteurella piscicida	Morone americana	2
Edwardsiella tarda	Ictalurus punctatus	2
Flexibacter columnaris (DD3)	O. tshawytscha	1
Cytophaga psychrophila	O. kisutch	1
Corynebacterium sp. (BKD)	O. kisutch	1

1 Dept. of Microbiology, Oregon State University, Corvallis, Ore.

2 G. L. Bullock, Eastern Fish Disease Lab., Kearneysville, W. Virg.

in Table 3. These organisms were grown on BHI medium and propagated at 37 C.

Effect of Salmonid Agglutinins on the Growth of Certain Bacteria

The following procedures were used to determine the effect of purified ova proteins on the growth of certain bacteria. Flexibacter columnaris and C. psychrophila were inoculated into Cytophaga broth and incubated at 18 C until turbid growth occurred. This required approximately 48 hr for F. columnaris and 72 hr for C. psychrophila. The bacteria then were treated as described below except they were cultured on Cytophaga agar for enumeration. All other organisms, except the causative agent of BKD, were inoculated into BHI broth and incubated at 22 or 37 C, depending on the species, until turbid broth cultures were obtained. The bacterial cells were washed three times with sterile PBS and resuspended to the original culture volume. Dilutions of the bacterial suspensions were prepared for enumeration by plate count (300-3000 cells/ml), mixed with an equal volume of the agglutinin (0.5 mg/ml) or substance to be tested, and then incubated at 22 C for the desired length of time. One hr incubation times were routinely used; however, experiments were performed to test different incubation periods. After incubation the mixtures were cultured in triplicate on BHI agar (0.1 ml/plate), incubated at 22 or

Table 3. Human-associated bacteria incubated with salmonid agglutinin derived from spring chinook salmon (Oncorhynchus tshawytscha) ova. [a]

Gram-positive bacteria	Gram-negative bacteria
Staphylococcus aureus	Klebsiella pneumoniae
S. epidermidis	Enterobacter aerogenes
Streptococcus pyogenes	Escherichia coli
S. faecalis	Salmonella typhi
S. lactis	S. paratyphi
Corynebacterium diphtheriae mitis	S. typhimurium
C. diphtheriae gravis	Shigella dysentariae
C. xerosa	S. sonnei
C. pseudodiphtheriticum	Vibrio cholerae
	V. parahemolyticus

[a] All bacteria were obtained from the culture collection of the Dept. of Microbiology, Oregon State University, Corvallis, Ore.

37 C, and the colonies enumerated after 24-48 hr to determine if inhibition had occurred.

The bacterial kidney disease organism was treated identically except that it was propagated on Mueller-Hinton agar supplemented with 0.1% L-cysteine and incubated at 18 C for 21 days. Since this bacterium could not be enumerated by the plate count method, the cultures were examined daily for growth and the time of appearance of control colonies compared to those which had been incubated with the agglutinin.

Control plates were used for each experiment so that plate counts of bacteria incubated with the agglutinin could be compared to plate counts of the same cultures incubated with PBS. In addition, after it was learned tha V. anguillarum was inhibited by the agglutinin, a culture of this bacterium was included as a positive 100% inhibited control when other species were tested. This assured that each agglutinin preparation expressed inhibiting activity.

Effect of the Chinook Ova Agglutinin Concentration on Bacterial Inhibition

The effect of agglutinin concentration on the percent reduction in viable bacteria was determined as follows: log phase V. anguillarum cells were grown (Gould, 1977), washed three times and resuspended in PBS. This suspension was diluted with PBS and

a 0.2 ml aliquot (which contained 600-6000 cells) was incubated with 0.2 ml of a known amount of chinook agglutinin in PBS for 1 hr at 22 C. A control suspension contained PBS, but no agglutinin. After the incubation period, the suspensions were cultured on BHI and colony counts made to determine the percent inhibition.

Effect of Incubation Time on Bacterial Inhibition by the Chinook Salmon Ova Agglutinin

The effect of incubation time on the percent reduction in number of V. anguillarum cells was tested in the following manner. Log phase V. anguillarum cells, prepared as described above, were incubated with 0.1 mg of chinook salmon ova agglutinin in 0.2 ml PBS for either 15, 30, 45, 60 or 90 min at 22 C, and then cultured on BHI for colony enumeration. Bacterial suspensions incubated with sterile PBS for identical time periods served as controls.

Microscopic Examination of Vibrio anguillarum Incubated with Chinook Agglutinin

A previous experiment quantitated the effect of incubation time on the reduction in colony counts of V. anguillarum. The purpose of this study was to learn if microscopically visible events could be correlated with the quantitative reduction in viable V. anguillarum cells with time.

One drop of washed V. anguillarum cells (600-6000 bacteria/ml) was incubated on a microscope slide with one drop of purified chinook salmon ova agglutinin (0.5 mg/ml). Adjacent slides which contained V. anguillarum cells incubated with either rabbit anti-V. anguillarum serum (Rohovec, 1976) or sterile PBS were examined concurrently. The slides were observed for agglutination or lysis of bacterial cells and for loss of motility.

Effect of Heat on the Chinook Salmon Ova Agglutinin

The following procedures were used to determine whether the bacterial inhibition property of the salmonid agglutinin was affected by certain heat treatments. Heated water baths set at 80 and 100 C were used. At each temperature, 0.4 ml volumes of purified chinook ova agglutinin (500 μg/ml) were held for specified lengths of time. Control volumes of sterile PBS were included at both temperatures. After the heat treatment, the material in each tube was assayed for V. anguillarum inhibition activity.

Effect of Lyophilization on the Bacterial Inhibition Activity

To determine whether purified chinook salmon ova agglutinin would retain bacterial inhibition activity after lyophilization, the following experiment was conducted. Dilutions of a purified agglutinin

preparation including 320, 32, 3.2, 1.6, 0.8 and 0.4 μg/ml were prepared. Each dilution was tested for bacterial inhibtion, and the percent reductions in bacteria numbers, relative to a PBS control, were determined. Another protion of the same agglutinin preparation containing 320 μg/ml was lyophilized and then stored at -20 C for 14 days. After the storage period, the material was resuspended to 0.5 ml in sterile PBS and identical dilutions as above were prepared and tested for bacterial inhibition activity. An analysis of the activity present before and after lyophilization compared the percent reductions in V. anguillarum colonies which had occurred.

Comparison of the Chinook Ova Agglutinin with a Plant Lectin

The plant lectin ricin, from Ricinus communis (the castor-oil plant), was chosen for comparison with the chinook ova agglutinin because this plant protein is well characterized and has a carbohydrate specificity for D-galactose (Gold and Belding, 1975).

The Effect of Ricin on Vibrio anguillarum

The first comparative test with ricin examined whether this lectin would inhibit the growth of V. anguillarum. The methods used were identical to those described above for the bacterial inhibition test except ricin, rather than the salmonid agglutinin was used.

Ricin[11] was tested at the following concentrations: 250, 500, 1250 and 2500 μg/ml. Controls which contained either sterile PBS or the salmonid agglutinin were included.

The Effect of Ricin on Bacterial Inhibition by the Salmonid Agglutinin

To determine whether ricin would compete with the salmonid agglutinin for binding sites on V. anguillarum cells, the following experiments were conducted. First, known amounts of ricin (250, 500, 1250 and 2500 μg/ml) were tested to determine if these concentrations would prevent the salmonid agglutinin from inhibiting V. anguillarum growth. Washed bacteria (0.2 ml with 600-6000 cells) were preincubated with 0.2 ml of the ricin solution for 1 hr at 22 C. The salmonid agglutinin (100 μg in 0.2 ml) was then added and the mixture was incubated for an additional hour at 22 C and cultured on BHI agar for colony enumeration.

A second experiment was performed to determine if a fixed amount of ricin would compete with varying amounts of the agglutinin. Paired culture tubes which contained washed V. anguillarum cells (600-6000 bacteria in 0.2 ml) preincubated with either 0.2 ml of PBS or ricin (50 μg in 0.2 ml) were prepared. Each pair then received one of the following amounts of purified salmonid agglutinin (0.2 ml/

[11] P. L. Biochemicals, Milwaukee, Wis.

tube): 100, 10, 1, 0.5 or 0.1 μg. These mixtures were incubated an additional hr at 22 C and cultured on BHI agar. A culture tube which contained 0.4 ml PBS and 0.2 ml of washed bacteria in PBS served as a control for this experiment.

The Effect of Intravenous Administered Agglutinin on the LD50 of *Vibrio anguillarum* for Coho Salmon

This experiment tested whether the administration of agglutinin to coho salmon would provide protection against subsequent challenge of the animals with *V. anguillarum*. The chinook ova agglutinin and an immunologically identical protein from coho salmon ova were tested.

For each experiment, 80 coho salmon (average weight, 20 g) received 125 μg (0.05 ml) of the purified chinook or coho protein by intravenous (i.v.) injection in the duct of Cuvier (Leed, Gjerde and Braekhan, 1975). These animals were placed in four 68 liter tanks, 20 fish per tank, and held at 18 C. An additional group of 80 salmon received 0.05 ml i.v. injections of sterile PBS and was divided equally among four tanks. After all the animals were injected, the aquaria were drained to 20 liters and the water supply shut off. A water-borne *V. anguillarum* challenge was administered as follows: each pair of tanks (a pair consisted of one tank of PBS-injected animals and one tank of agglutinin-injected animals) received either 0.2,

2.0, 20 or 200 ml of a log phase culture of V. anguillarum (approximately 10^9 cells/ml) per tank. After 15 min, water supply to the tanks was resumed. Fish were maintained at 18 C until deaths from V. anguillarum ceased; at this time the LD50 was computed (Reed and Meunch, 1938) for the control and experimental animals. All dead fish were necropsied and bacteriological cultures on BHI agar were prepared from kidney tissue. After incubation the plates were examined for typical V. anguillarum colonies and diagnosis confirmed by rapid slide agglutination tests with rabbit anti-V. anguillarum serum.

Amino Acid Analysis of Salmonid Ova Agglutinins

An amino acid analysis was conducted to further characterize and compare the immunologically identical ova agglutinins derived from chinook and coho salmon. The determination was performed on a Beckman Model 120B modified from a two-column system (Spackman, Stein and Moore, 1958) to a single 6 x 510 mm column system for use with samples which contained 10-50 nanomoles of each amino acid. Prior to the analyses, the proteins were hydrolyzed in evacuated sealed tubes in constant boiling 5.7N HCl at 110 C for 22 and 72 hr.

RESULTS

Examination of Unfertilized Chinook Salmon Ova for Maternal Antibody

Chinook salmon females which had high levels of circulating specific antibody did not pass a detectable amount of the classical salmonid immunoglobulin to their ova (Table 4). Ovarian fluids also lacked detectable antibody. When egg or ovarian fluid samples were reacted in Ouchterlony double diffusion with culture supernatants of the causative agent of BKD (Corynebacterium sp.), a heavy band of precipitation occurred. Further analysis of the egg material responsible for the precipitation showed it was a lipoprotein and not antibody. The lipoprotein reacted identically in gel against sodium dextran sulfate; this was a further indication of the non-antibody, nonspecific nature of the reaction obtained with this substance. It is likely the lipoprotein precipitated with a large sulfonated carbohydrate produced by the BKD organism and thus caused the nonspecific precipitation.

Characterization of Salmonid Ova Proteins

Electrophoresis of the chinook salmon ova homogenate indicated that four components were present in the nondialyzed preparation (Figure 1). When the homogenate was dialyzed against Tris (0.1M

Table 4. Examination of unfertilized chinook salmon (Oncorhynchus tshawytscha) ova for the presence of maternal antibody.

Fish no.	Serum titer[a]		Ovarian fluid titer[a]		Immunodiffusion assay for antibody[b]	
	Aeromonas salmonicida	Corynebacterium sp.	Aeromonas salmonicida	Corynebacterium sp.	Ovarian fluid	Ova
1	512	16	0	0	-	-
2	128	32	0	0	-	-
3	0	16	0	0	-	-
4	32	32	0	0	-	-
5	0	256	0	0	-	-
6	0	32	0	0	-	-
7	0	64	0	0	-	-
8	0	64	0	0	-	-
9	4096	32	0	0	-	-
10	0	128	0	0	-	-
11	0	128	0	0	-	-
12	0	64	0	0	-	-
13	0	128	0	0	-	-
14	128	128	0	0	-	-
15	64	512	0	0	-	-
16	0	128	0	0	-	-
17	32	64	0	0	-	-
18	0	32	0	0	-	-
19	256	128	0	0	-	-
20	0	256	0	0	-	-
21	0	32	0	0	-	-
22	0	64	0	0	-	-
23	0	128	0	0	-	-
24	0	128	0	0	-	-
25	0	64	0	0	-	-

[a] The titer is expressed as the reciprocal of the final dilution which showed visible agglutination.

[b] Ouchterlony double diffusion was used to react ovarian fluid and ova against rabbit anti-salmonid immunoglobulin serum. The (-) indicates no precipitin band formed between reactants.

Figure 1. Electrophoresis of chinook salmon ova fractions. The top well contained the PBS homogenate; the bottom well contained the supernatant from Tris-precipitated ova homogenate. Electrophoresis in agarose was conducted at 200 V until a fluorescein marker migrated to the anodal edge (left) of the slide. Ten microliters of the homogenate (31 mg/ml) and the Tris-precipitated material (10 mg/ml) were loaded into the respective wells.

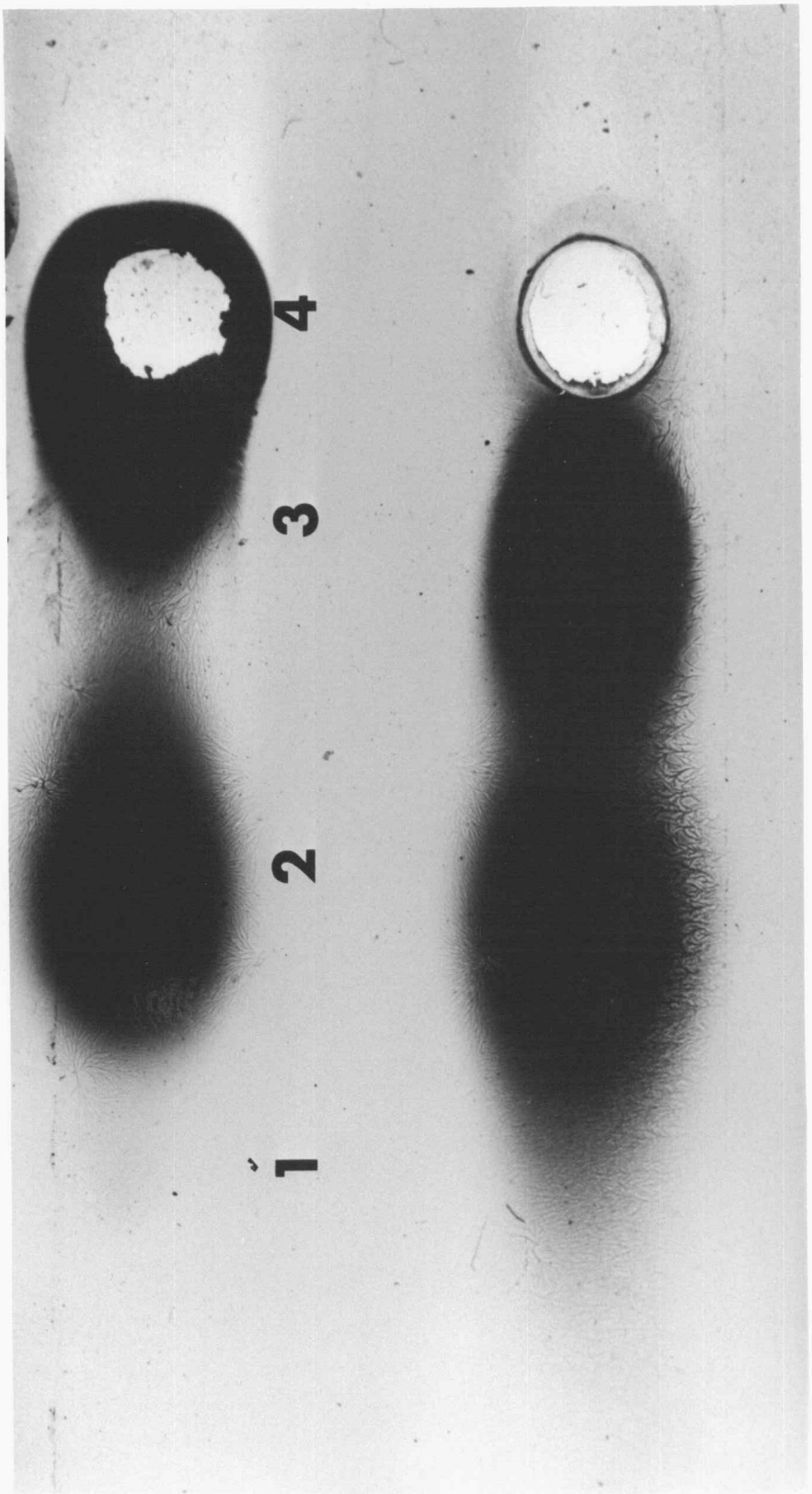

Figure 1

at 4 C for 24 hr), a pink or salmon-colored precipitate formed. The electroporetic pattern of the soluble material indicated that component four had precipitated in the Tris. Component one was present in relatively small quantities when compared to the other ova components stainable with Coomassie Blue.

Hemagglutination

The chinook salmon ova homogenate contained a component which specifically agglutinated rabbit and human type B erythrocytes (Table 5). This activity was stable in homogenates stored at - 20 C for 1 yr. The hemagglutinin was present in the Tris-soluble fraction, but not in the material which precipitated during Tris dialysis. This indicated that the hemagglutination activity was not due to electrophoresis component four, but rather to components one, two or three. Furthermore, this study showed that Tris dialysis was a useful step in purification of the hemagglutinin since it removed a major unwanted protein, component four.

Hemagglutination Inhibition

Hemagglutination inhibition assays were performed to characterize the carbohydrate specificity of the agglutinin. Results indicated that D-galactose and L-rhamnose were potent inhibitors whereas the other sugars tested did not reduce the hemagglutination

Table 5. Hemagglutination titer of chinook salmon ova homogenate and selected fractions with erythrocytes from certain species.[a]

Ova Fraction	Erythrocyte	Titer[b]
Whole homogenate	Rabbit	2560
Whole homogenate	Sheep	0
Whole homogenate	Human A	0
Whole homogenate	Human B	320
Whole homogenate	Human AB	160
Whole homogenate	Human O	0
Whole homogenate	PBS-control	0
Tris-precipitable[c]	Rabbit	0
Tris-soluble	Rabbit	1280

[a]Ova fractions were incubated with a 1% suspension of washed erythrocytes at 22 C.

[b]The titer, expressed as the reciprocal of the final dilution of the ova material which caused visible agglutination, was corrected for a 1:10 dilution of the ova homogenate in PBS.

[c]This material, soluble in PBS, precipitated when the ova homogenate was dialyzed against 0.1 M Tris at 4 C for 18 hr.

titer (Table 6). Since rabbit cells were consistently agglutinated at higher titers than were human type B erythrocytes, rabbit cells were used for all further tests that involved hemagglutination assays. All three peptones tested were effective inhibitors of hemagglutination (Table 7). Neopeptone was the strongest inhibitor of the three.

Purification of the Salmonid Agglutinin

These experiments examined two methods for purification of the salmonid ova agglutinin. Further characterization studies required a preparation of the agglutinin free of other proteins.

When ova homogenate, previously dialyzed against Tris, was adsorbed with agarose, the L-rhamnose eluate contained only one stainable protein (Figure 2A), and it corresponded to the electrophoretic band 3. Since this material contained hemagglutination activity for rabbit erythrocytes, and the other proteins did not agglutinate rabbit red cells, band 3 was identified as the salmonid ova agglutinin. Immunoelectrophoresis of the eluted protein produced only one precipitin band whereas a similar analysis of Tris-dialyzed ova homogenate showed two distinct and one lesser precipitin band (Figure 2B). The adsorbed supernate contained less hemagglutinating activity than the unadsorbed; this was a further indication that the agglutinin was specifically adsorbed by the agarose.

Gel filtration of Tris-dialyzed ova homogenate resolved two

Table 6. The effect of selected monosaccharides on the chinook salmon (Oncorhynchus tshawytscha) ova hemagglutination activity.[a]

Erythrocyte	Test Substance	Titer[b]
Rabbit	PBS-control	1280
Rabbit	D-galactose	40
Rabbit	L-rhamnose	0
Rabbit	Mannose	1280
Rabbit	Deoxy-1-galactose	1280
Rabbit	2-deoxygalactose	1280
Rabbit	Fructose	1280
Human Type B	PBS-control	400
Human type B	D-galactose	20
Human type B	L-rhamnose	400
Human type B	Mannose	400
Human type B	Deoxy-1-galactose	400
Human type B	2-deoxygalactose	400
Human type B	Fructose	400

[a]Ova homogenate was incubated with a final 0.02 M concentration of the sugar for 30 min at 22 C prior to addition of the 1% erythrocyte suspension.

[b]The titer, expressed as the reciprocal of the final dilution of the ova material which caused visible agglutination, was corrected for a 1:10 dilution of the ova homogenate in PBS.

Table 7. The effect of selected peptones on the chinook salmon (Oncorhynchus tshawytscha) ova hemagglutination activity.[a]

Erythrocyte	Substance Tested	Titer[b]
Rabbit	PBS-control	320
Rabbit	Neopeptone	0
Rabbit	Peptone	80
Rabbit	Proteose peptone	80

[a]Ova homogenate was incubated with a final 1.0 mg/ml concentration of the peptone for 30 min at 22 C prior to addition of the 1% erythrocyte suspension.

[b]The titer, expressed as the reciprocal of the final dilution of the ova material which caused visible agglutination, was corrected for a 1:10 dilution of the ova homogenate in PBS.

major protein peaks (Figure 3). Immunoelectrophoresis indicated that the first and largest peak contained the agglutinin while the second peak migrated as electrophoretic band 2. Gel filtration typically yielded 8-12 mg of purified agglutinin. Since the technique was relatively easy to perform and yielded mg quantities of the protein, it was used for all further purifications.

Ova homogenates from chinook, coho, kokanee, pink and chum salmon, steelhead and cutthroat trout all had identical elution profiles when passed through the P300 column. Proteins which were immunologically identical to the chinook ova agglutinin were purified from the other homogenates by this gel filtration method and used for further bacterial inhibition tests.

The purification procedure adopted as a result of these experiments consisted of the following:

1. Homogenization of the ova; centrifugation at 13,000 x g, 20 min.
2. Dialysis against Tris buffer; centrifugation at 1,000 x g, 10 min.
3. Dialysis against four changes of PBS.
4. Gel filtration on Bio-Rad P300.
5. Pass through 0.45 μm filter, store at -20 C.

Carbohydrate Analysis

The phenol-sulfuric acid assay indicated that purified chinook ova agglutinin preparations contained 0.8% carbohydrate. This figure

Figure 2. A. Electrophoresis of protein fraction eluted by 0.1M L-rhamnose from agarose previously incubated with a spring chinook salmon ova homogenate. Ten microliters of the eluted material (1.3 mg/ml) were electrophoresed in agarose at 200 V for 2 hr. Bottom well contained supernatant from a Tris-precipitated ova homogenate (10 mg/ml).
B. Immunoelectrophoresis of the L-rhamnose eluate (top) and Tris-precipitated ova homogenate (bottom). After electrophoresis, rabbit anti-ova homogenate serum was added to the trough and the slide incubated in a moist chamber for 24 hr.

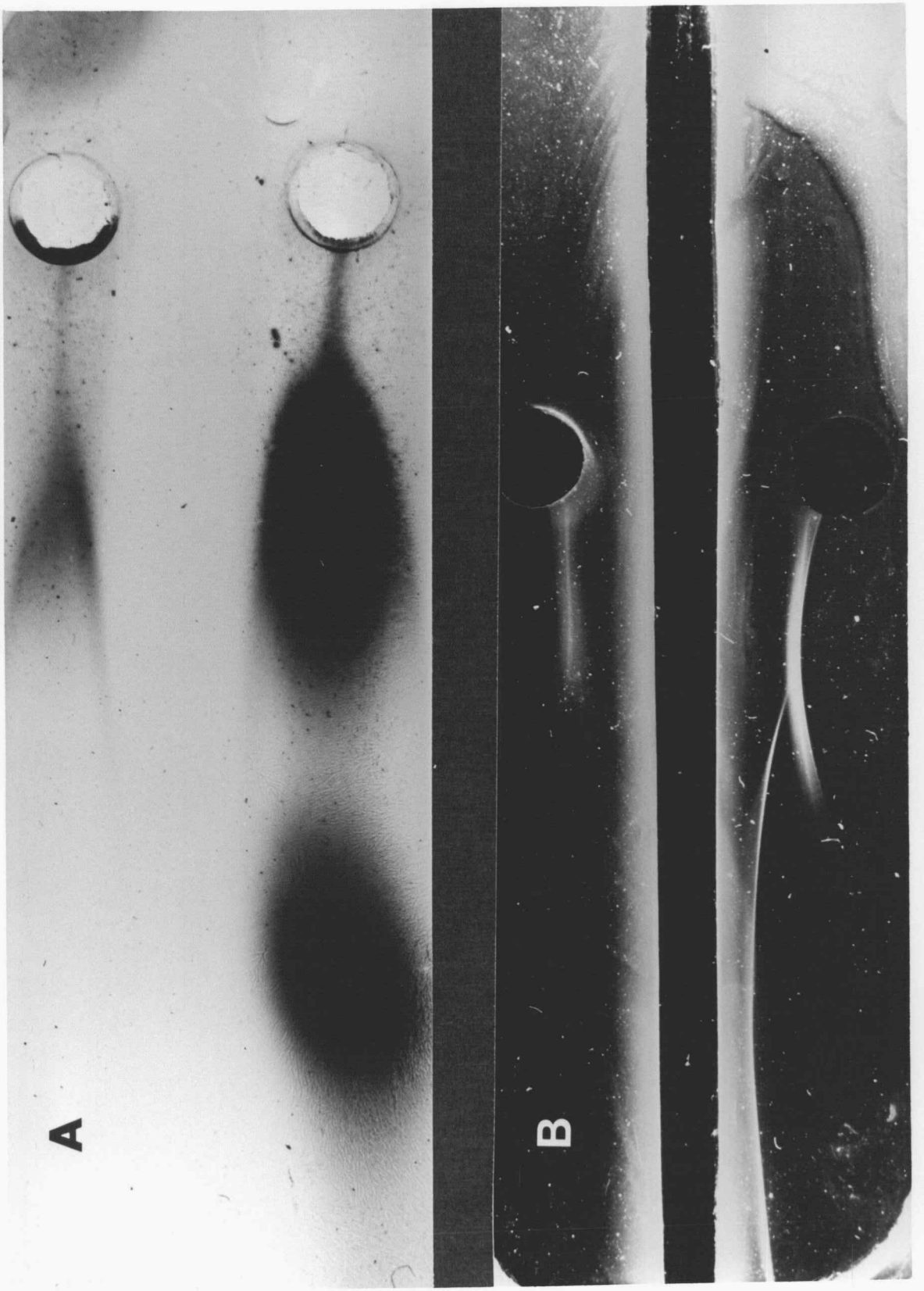

Figure 2

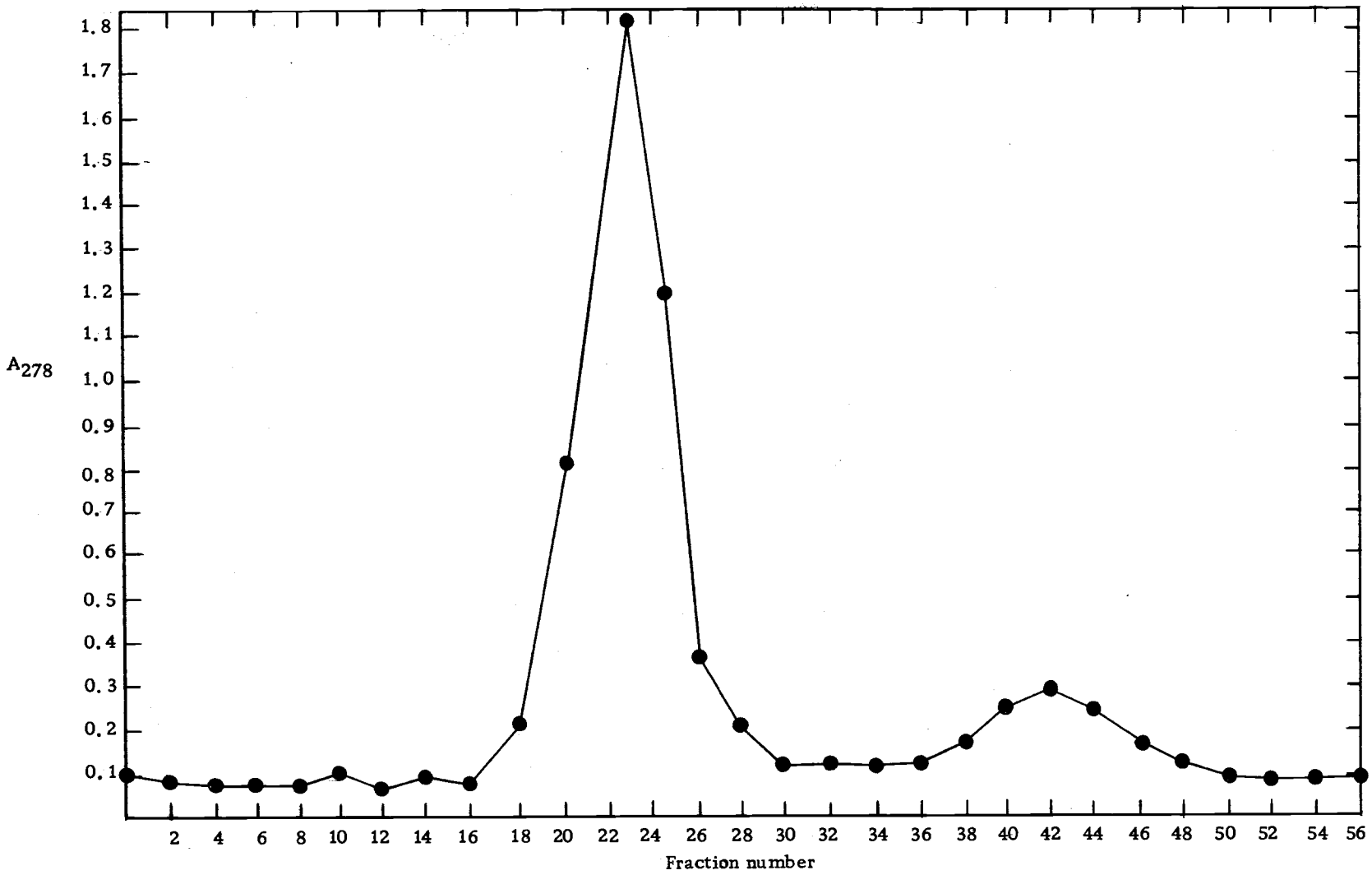

Figure 3. Bio-Rad P300 gel filtration of Tris-dialyzed chinook salmon (Oncorhynchus tshawytscha) ova homogenate. Ova homogenate, diluted with five volumes of PBS (pH 7. 2) and dialyzed against Tris (0. 1 M, pH 7. 8), was applied to a Bio-Rad P300 column (2. 5 x 35 cm) and eluted with phosphate buffer (0. 1 M, pH 7. 8).

represented an average of several determinations which ranged from 0.68% to 0.88%.

Gas chromatography was used in an attempt to characterize the carbohydrate moiety of the agglutinin; however, no hexose or pentose residues were detected when purified agglutinin was injected into the column. Arabinose and galactose standards were eluted at 161 and 419 seconds, respectively. Even a 2 µl agglutinin sample injected to increase the amount of agglutinin-associated carbohydrate failed to yield detectable hexose or pentose resiudes. This chromatographic analysis supported the conclusions derived from the spectrophotometric assay which indicated that carbohydrate was a very minor agglutinin component.

Antigenic Comparison of Ova Proteins of Certain Salmonids

The purpose of this experiment was twofold; first, to determine whether salmonids other than chinook salmon had a protein antigenically similar to the agglutinin within their ova; second, to determine if ova from these species shared other nonagglutinin antigens.

The rabbit antiserum prepared against the purified chinook ova agglutinin detected only one protein, and it was present in chinook, coho, steelhead, kokanee, Lahonten cutthroat trout, and pink and chum salmon ova (Figure 4A). When purified chinook ova agglutinin

Figure 4. Ouchterlony immunodiffusion in agarose. Ova homogenates from spring chinook, coho, pink, chum, kokanee and fall chinook salmon, steelhead and Lahonten cutthroat trout and spring chinook salmon were placed in wells 1-9 respectively. The BA well contained rabbit anti-spring chinook ova homogenate serum; BB contained rabbit anti-spring chinook ova lectin serum.

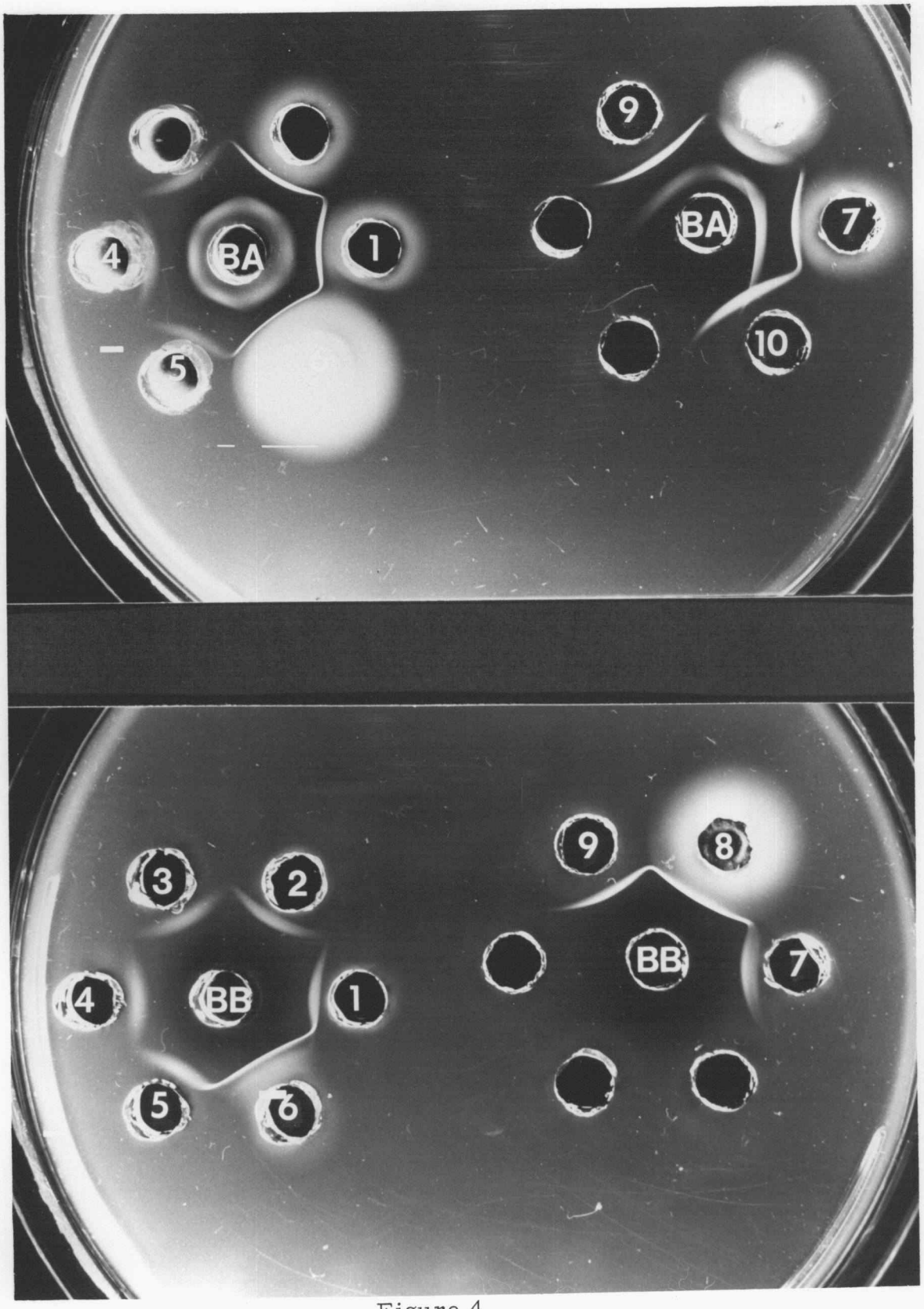

Figure 4

was reacted with this antiserum, a band of identity formed with the protein detected in the other species. These results indicated that the chinook agglutinin had an immunologically identical counterpart in the other salmonid ova.

Rabbit antiserum against Tris-dialyzed chinook ova homogenate detected other similarities among the different ova; all salmonids tested also had a nonagglutinin protein in common (Figure 4B). This protein diffused in gel faster than the agglutinin and formed the precipitin band nearer to the center antiserum well.

Certain quantitative differences existed between these specific proteins as found in the different species. Even though all ova were homogenized, diluted and dialyzed in the same manner, chinook, coho and steelhead ova contained more protein and formed stronger bands with the antiserum. Pink and chum salmon contained the least amount of these proteins; kokanee salmon and Lahonten cutthroat trout were intermediate.

Antigenic Comparison of Chinook Ova Agglutinin to Salmonid Immunoglobulin

The purpose of this experiment was to determine if the agglutinin was identical or similar to salmonid immunoglobulin. No precipitin band formed when rabbit anti-salmonid immunoglobulin was reacted with purified agglutinin or when rabbit anti-agglutinin was reacted with purified salmonid immunoglobulin. In addition,

when rabbit anti-salmonid immunoglobulin was reacted with Tris-dialyzed ova homogenate, no precipitin bands were formed. These results indicated that no detectable levels of classical salmonid immunoglobulin were present within the salmonid ova.

Effect of the Chinook Salmon Ova Agglutinin on the Growth of Certain Bacteria

Purified chinook salmon ova agglutinin inhibited the growth of certain bacterial fish pathogens (Table 8). Only gram-negative bacteria were inhibited, but not all gram-negative organisms were sensitive. A one-way analysis of variance supported the hypothesis that this inhibition was significant at $\alpha = .01$. Inhibition data that were not significant at $\alpha = .01$ were recorded as 0% inhibition. Since V. anguillarum was especially sensitive to this protein, it was used as a model organism for the inhibition assay. Control studies showed that the inhibition of V. anguillarum was a specific property of the agglutinin because when bovine serum albumin (1 mg/ml) or the other ova protein purified by P300 gel filtration were incubated with this bacterium, no inhibition of growth occurred.

Human-associated bacteria were not inhibited by purified chinook salmon ova agglutinin (Table 9). Gram reaction of the organism made no difference with these species. The minor inhibition recorded for certain of these bacteria was judged not significant ($\alpha = .01$).

Table 8. The effect of the spring chinook salmon (Oncorhynchus tshawytscha) ova agglutinin on the growth of certain bacterial fish pathogens.[a]

Test Organism	% Growth Inhibition[b]
Gram negative	
Vibrio anguillarum type 1	100
V. anguillarum type 2	100
Pasteurella piscicida	100
Yersinia ruckeri	0
Edwardsiella tarda	0
Aeromonas hydrophila	100
A. salmonicida	0
Flexibacter columnaris	76
Cytophaga psychrophila	0
Gram positive	
Corynebacterium sp.	0

[a] Log phase cells of test organisms were washed three times in PBS, incubated with purified agglutinin (500 μg/ml) for 1 hr at 22 C, and plated on appropriate media for enumeration of survivors.

[b] Growth inhibition is expressed as percent relative to control cells incubated with PBS. A one-way analysis of variance supported the hypothesis that the inhibition observed was significant at $\alpha = .01$.

Table 9. The effect of the spring chinook salmon (Oncorhynchus tshawytscha) ova agglutinin on the growth of certain bacteria associated with humans.[a]

Test Organism	% Growth Inhibition[b]
Gram positive	
Staphylococcus aureus	0
S. epidermidis	8
Streptococcus pyogenes	0
S. faecalis	0
S. lactis	0
Corynebacterium diphtheriae mitis	0
C. diphtheriae gravis	0
C. xerosa	10
C. pseudodiphtheriticum	7
Gram negative	
Enterobacter aerogenes	0
Klebsiella pneumoniae	0
Escherichia coli	0
Salmonella typhi	0
S. typhimurium	7
S. paratyphi	0
Shigella dysentariae	0
S. sonnei	9
Vibrio cholerae	0
V. parahemolyticus	0

[a]Log phase cells of test organisms were washed three times in PBS, incubated with purified chinook salmon ova agglutinin (500 μg/ml) for 1 hr at 22 C, and plated on appropriate media for enumeration of survivors.

[b]Growth inhibition is expressed as percent relative to control cells incubated with PBS. A one-way analysis of variance indicated that the observed inhibition was not significant at $\alpha = .01$.

Effect of Chinook Agglutinin-like Proteins Derived from Other Salmonids on the Growth of Certain Bacteria

The discovery that the chinook ova agglutinin inhibited the growth of certain fish pathogens prompted this study to determine whether immunologically identical proteins derived from other salmonid ova had similar bactericidal properties.

None of the other salmonid ova proteins were bactericidal for V. anguillarum, which was extremely sensitive to the chinook ova agglutinin (Table 10). The ova of some species did possess significant inhibitory activity against the myxobacteria tested. These results indicated that the agglutinin isolated from spring chinook salmon ova had unique and specific bactericidal properties not shared by other salmonids, and that the bactericidal site on the agglutinin molecule was distinct from the antigenic site detected by the rabbit antiserum.

Comparison of Bactericidal Properties of Fall and Spring Chinook Salmon (Oncorhynchus tshawytscha) Ova Agglutinins

Immunologically identical proteins derived from fall and spring strains of chinook salmon did not have identical bactericidal properties (Table 11). In fact, the fall chinook ova protein inhibited the growth of one species of myxobacteria, but none of the other bacterial fish pathogens tested. The spring chinook ova agglutinin, in contrast, inhibited a range of Gram negative pathogens as well as the

Table 10. The effects of chinook salmon ova agglutinin-like proteins derived from other salmonid ova on the growth of certain bacterial fish pathogens. [a]

Species of Bacteria	% Bacterial Inhibition by Selected Salmonids[b]					
	Oncorhynchus kisutch	*Salmo gairdneri*	*S. clarki*	*O. nerka*	*O. gorbuscha*	*O. keta*
Vibrio anguillarum I	0	0	0	0	0	0
V. anguillarum II	0	0	0	0	0	0
Yersinia ruckeri	0	0	0	0	0	0
Aeromonas hydrophila	0	0	0	0	0	0
A. salmonicida	0	0	0	0	0	0
Edwardsiella tarda	0	0	0	0	0	0
Pasteurella piscicida	0	0	0	0	0	0
Flexibacter columnaris	89	100	0	97	0	0
Cytophaga psychrophila	0	100	0	100	0	0

[a] Log phase cells of test organisms were washed three times in PBS, incubated with purified agglutinin-like protein (175 µg/ml) for 1 hr at 22 C, and plated on appropriate media for enumeration of survivors.

[b] Growth inhibition is expressed as percent relative to control cells incubated with PBS.

Table 11. Comparison of bactericidal properties of fall and spring chinook salmon (Oncorhynchus tshawytscha) ova agglutinins.[a]

Species of Bacteria	Percent Bacterial Inhibition by Each Salmonid[b]	
	Spring Chinook	Fall Chinook
Vibrio anguillarum I	100	0
V. anguillarum II	100	0
Yersinia ruckeri	0	0
Aeromonas hydrophila	100	0
A. salmonicida	0	0
Edwardsiella tarda	0	0
Pasteurella piscicida	100	0
Flexibacter columnaris	76	84
Cytophaga pyschrophila	0	0

[a] Log phase bacterial cells were washed three times in PBS, incubated with purified agglutinin (175 μg/ml) for 1 hr at 22 C, and plated on appropriate media for enumeration of surviving bacteria.

[b] Growth inhibition is expressed as percent relative to colony counts of control cells incubated with PBS.

myxobacterium.

The results of this experiment showed that the ova agglutinin derived from spring chinook salmon had bactericidal properties not even shared by a fall strain of the same salmonid species. Whether this difference was the result of specific induction of the spring chinook protein or merely a basic difference between the two salmonid strains was not determined.

Effect of Chinook Ova Agglutinin Concentration on Bacterial Inhibition

The concentration of purified agglutinin in the incubation mixture was directly related to the percent reduction of viable V. anguillarum cells (Figure 5A). Agglutinin concentrations as low as 0.5 μg/ml reduced the number of viable cells by greater than 75%. Less than 1.0 μg/ml was required to inhibit growth by 100%. The low concentration of agglutinin required for complete inhibition showed that V. anguillarum was very sensitive to this protein. These results also indicated that the agglutinin concentration (500 μg/ml) used to test other bacteria was adequate to detect sensitive species. The level of ova proteins from the other salmonids (175 μg/ml) used to test for bactericidal activity was also well above the minimum lethal dose.

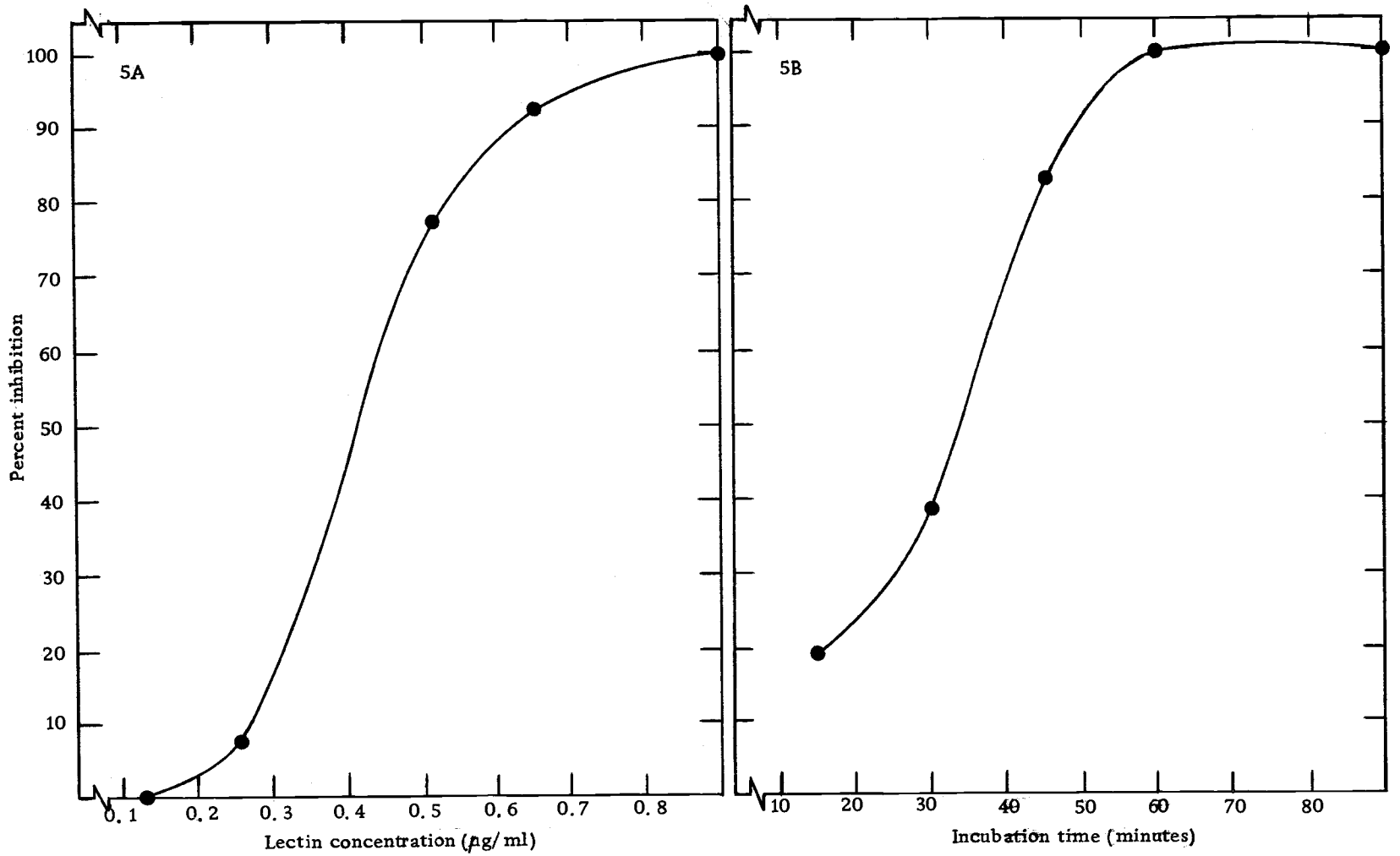

Figure 5. A. Dose-response curve. Certain concentrations of the spring chinook ova lectin were incubated with Vibrio anguillarum cells for 1 hr at 22 C, and cultured to measure the amount of bacterial growth inhibition that occurred. B. Effect of incubation time (purified lectin with V. anguillarum) on bacterial inhibition. All incubation occurred at 22 C.

Effect of Incubation Time on Bacterial Inhibition

The length of time V. anguillarum cells were incubated with purified chinook ova agglutinin was directly related to the percent reduction in colony counts which occurred (Figure 5B). A 60 min incubation period resulted in 100% inhibition of bacterial growth. When the 60 min incubation mixture was cultured on BHI and the plates maintained for 96 hr, no bacterial growth occurred. This indicated that the inhibition was likely bactericidal in nature.

Microscopic Examination of Vibrio anguillarum Incubated with Chinook Agglutinin

Vibrio anguillarum cells incubated with purified chinook ova agglutinin quickly lost their motility and became stationary; after 20 min, approximately 50% of the cells were no longer motile. In comparison, cells incubated with PBS retained their motility throughout the observation period. The agglutinin-treated cells were not lysed or clumped by the chinook protein; they merely became stationary. Control cells incubated with rabbit anti-V. anguillarum serum were agglutinated very early in the observation period; within 5 min, most cells were clumped.

Effect of Heat on the Chinook Ova Agglutinin

Purified chinook salmon ova agglutinin was extremely resistant

to heat treatment (Table 12). Neither 80 C for 6 hr nor 100 C for 60 min destroyed the bacterial inhibition activity. Dilutions prepared from a heated sample (80 C-6 hr) and tested for bacterial inhibition contained the same degree of activity as a nonheated preparation; 0.5 μg/ml of each preparation reduced colony counts approximately 60%.

Effect of Lyophilization on the Bacterial Inhibition Activity

This study tested whether purified spring chinook ova agglutinin retained bacterial inhibition activity after it was lyophilized. Results indicated that lyophilization did not diminish the amount of V. anguillarum inhibition activity present in the purified agglutinin preparation (Table 13). The results showed a slight increase in inhibitory activity after lyophilization; this likely represented an inaccuracy in reconstitution of the dried powder in PBS. The results of this study indicated that the agglutinin could be stored in a lyophilized form without loss of bacterial inhibition activity.

Effect of Ricin on Vibrio anguillarum

This study was conducted to determine if the chinook salmon ova agglutinin and a plant-derived lectin with similar carbohydrate specificity affected V. anguillarum in the same manner. The experiment indicated that the plant lectin, ricin, was not bactericidal for

Table 12. The effect of heat on bacterial inhibition activity of the chinook salmon (Oncorhynchus tshawytscha) ova agglutinin.[a]

Heat Treatment	% Inhibition of *Vibrio anguillarum*[b]
80 C, 1 hr	100
80 C, 2 hr	100
80 C, 3 hr	100
80 C, 4 hr	100
80 C, 5 hr	100
80 C, 6 hr	100
100 C, 20 min	100
100 C, 45 min	100
100 C, 60 min	100

[a]Purified agglutinin (500 μg/ml) was heated for the specified time, cooled and incubated with washed log phase bacteria suspended in PBS (approximately 600 cells/ml). After 1 hr incubation at 22 C, solutions were plated on Brain Heart Infusion agar (Difco).

[b]Inhibition is expressed relative to control tubes of PBS heated, cooled and incubated with the bacteria as described above.

Table 13. The effect of lyophilization on the Vibrio anguillarum inhibition activity of the chinook salmon (Oncorhynchus tshawytscha) ova agglutinin.[a]

Agglutinin Concentration (μg/ml)	Percent Inhibition	
	Before Lyophilization	After Lyophilization
320	100	100
32	100	100
3.2	99	100
1.6	94	100
0.8	42	48
0.4	18	22

[a]Purified salmonid agglutinin from chinook salmon ova was lyophilized and the dried protein stored at -20 C for 14 days prior to assay for bacterial inhibition activity.

V. anguillarum and, even when tested for high concentrations, failed to inhibit growth of the bacterium (Table 14). The chinook ova agglutinin, in contrast, inhibited growth of the pathogen by 100% when tested at a concentration of 500 μg/ml. These results showed that the bactericidal activity of the spring chinook ova agglutinin involved more than the mere recognition of a specific carbohydrate site on the bacterium.

Effect of Ricin on Bacterial Inhibition by the Chinook Ova Agglutinin

Since both chinook ova agglutinin and ricin specifically bound D-galactose, an experiment was performed to test whether ricin would compete for binding sites on V. anguillarum cells and reduce the apparent bactericidal activity of the spring chinook protein. The results of this experiment demonstrated that when present at greater than a 5:1 ricin to chinook ova agglutinin ratio, the plant lectin did compete with the salmonid-derived protein since less bactericidal activity occurred (Table 15). This study suggested that carbohydrate binding was an important factor in the bacterial inhi bition and that the salmonid protein likely had a greater affinity for V. anguillarum than did ricin.

Table 14. A comparison of the effects of the plant lectin, ricin, and the spring chinook salmon (Oncorhynchus tshawytscha) ova agglutinin on the growth of Vibrio anguillarum.[a]

Test Solution	Percent Growth Inhibition
PBS	0
Chinook agglutinin - 500 μg/ml	100
Ricin - 250 μg/ml	0
Ricin - 500 μg/ml	0
Ricin - 1250 μg/ml	0
Ricin - 2500 μg/ml	0

[a]Test solutions were incubated with washed V. anguillarum cells (approximately 1600 bacteria/ml) for 1 hr at 22 C. Mixtures were plated on Brain Heart Infusion agar (Difco) for enumeration of survivors.

Table 15. Inhibition of the spring chinook salmon (Oncorhynchus tshawytscha) ova agglutinin bactericidal activity by the plant lectin, ricin.[a]

Chinook Agglutinin (μg/ml)	Ricin (μg/ml)	Percent Growth Inhibition of Vibrio anguillarum
0	0	0
50	0	100
5	0	100
2.5	0	44
0	250	0
50	250	100
5	250	0
2.5	250	0

[a]Ricin, or PBS, was incubated with washed V. anguillarum cells (approximately 1500 bacteria/ml) for 1 hr at 22 C prior to the addition of the chinook salmon agglutinin. After the chinook agglutinin was added, an additional 1 hr incubation period followed. The mixtures then were plated on Brain Heart Infusion agar (Difco) for enumeration of the surviving bacteria.

The Effect of Intravenous Administered Agglutinin on the LD50 of Vibrio anguillarum for Coho Salmon

No protection was afforded juvenile coho salmon by i.v. injection of 125 μg of purified agglutinin. The LD50 values computed for experimental groups of fish were identical to control values. Both chinook agglutinin and an immunologically identical protein derived from coho ova were tested for protective effects; neither protein significantly affected the course of the V. anguillarum challenge.

This experiment indicated that purified agglutinin administered at this level by the i.v. route did not protect coho salmon from V. anguillarum. Different routes and amounts of the lectin were not tested.

Amino Acid Analysis of Salmonid Ova Agglutinins

This study showed that glutamine-glutamic acid and alanine were the most abundant amino acids in the salmonid ova agglutinins; the sulfur-containing amino acids (cysteine and methionine) were present in minor amounts (Table 16). No significant amounts of carbohydrate were detected during these determinations.

These results indicate that the chinook and coho salmon ova agglutinins were very similar proteins; differences in their amino acid compositions were minor and neither seemed to contain significant amounts of sugars.

Table 16. Amino acid composition of the hemagglutinin derived from the ova of two salmonid fishes.

	Coho Salmon				Chinook Salmon			
	Residues/1000		Corrected		Residues/1000		Corrected	
	22 hr	72 hr	average[a]	%	22 hr	72 hr	average[a]	%
Lys	63.6	59.9	61.7	6.2	64.5	63.7	64.1	6.4
His	30.8	28.9	29.9	3.0	28.8	27.1	27.9	2.8
Arg	45.1	42.6	43.8	4.4	45.0	45.9	45.4	4.5
Cysteic acid	8.4	7.2	8.4	0.8	9.6	8.8	9.6	1.0
AsX	85.3	79.2	82.3	8.2	85.9	81.8	83.8	8.4
Thr[b]	54.6	51.2	56.0	5.6	54.8	55.0	55.0	5.5
Ser[b]	47.1	38.3	51.8	5.2	50.7	40.9	55.0	5.5
GlX	103.5	108.1	105.8	10.6	102.5	104.0	103.2	10.3
Pro	49.4	58.4	58.4	5.8	58.0	58.6	58.3	5.8
Gly	46.9	49.8	48.5	4.9	44.3	47.4	45.8	4.6
Ala	136.9	141.2	139.0	13.9	133.6	137.8	135.7	13.6
Cys/2	--	--	tr	--	--	--	--	--
Val	81.4	89.3	89.3	8.9	79.4	86.4	86.4	8.6
Met[c]	13.3	8.4	13.3	1.3	14.6	6.9	14.6	1.5
Ile	58.4	62.9	62.9	6.3	60.0	65.0	65.0	6.5
Leu	99.2	100.7	99.9	10.0	97.5	99.2	98.3	9.8
Tyr	27.7	26.8	27.2	2.7	23.3	25.0	24.1	2.4
Phe	48.4	47.0	47.7	4.8	47.3	46.4	46.8	4.7
Trp[d]	--	--	--	--	--	--	--	--

[a] Corrected average values obtained from analysis of 22 and 72 hr hydrolysates.

[b] Corrected for losses which occur during hydrolysis.

[c] Determined as sulfoxide.

[d] Not determined.

DISCUSSION

This research investigated a previously little understood aspect of the salmonid immune system, namely possible mechanisms within the ova for protection of fry from infectious agents. Unfertilized chinook salmon ova were examined for the presence of classical salmonid antibody and a bactericidal hemagglutinin isolated from these ova was purified and characterized. The results reported here are the first record of bactericidal activity by a fish-derived lectin, and suggest a possible role for these ova proteins.

Classical salmonid immunoglobulin was not detected within the ova of chinook salmon. This is consistent with observations in other animals which indicated that IgM-like antibodies were not transmitted from mother to young (Malkinson, 1965; Franklin and Kunkel, 1958). The salmonid antibody is a 17S IgM-like macroglobulin (Cisar and Fryer, 1974) and thus not likely a protein that could be transmitted to the ova. Fidler, Clem and Small (1969) concluded that neither 7S nor 19S forms of nurse shark IgM were transmitted from the maternal serum to the young. Although 19S immunoglobulin was detected in the newborn shark serum, they predicted it was of fetal, and not maternal, origin.

The results reported in this investigation do not rule out the possibility that an antibody which is antigenically different from the

classical salmonid immunoglobulin is produced by the pre-spawning adult and transmitted to the developing ova. In fact, the chinook salmon ova agglutinin has certain characteristics of such a protein; it is present in maternal serum, has specific bactericidal activity, is a low molecular weight molecule, and is antigenically different from the classical immunoglobulin. However, it has not been shown to be inducible or to possess the classic Porter 2H-2L immunoglobulin structure; thus it cannot be considered an antibody.

The results of the hemagglutination and carbohydrate specificity tests indicate that the chinook salmon ova agglutinin is similar to, and very likely, identical to the hemagglutinins reported from Salmo eriox, S. gairdneri, S. salar, S. trutta (Todd, 1971), and S. irideus (Anstee, Holt and Pardoe, 1973). The immunodiffusion analysis presented in this study also indicates that identical proteins occur in Oncorhynchus kisutch, O. nerka, O. gorbuscha, O. keta and S. clarki. The natural hemagglutinins isolated from rainbow trout serum were of two molecular species and both agglutinated rabbit erythrocytes (Hodgins, Weiser and Ridgway, 1967); the smaller 4.2S protein was likely similar to the chinook ova agglutinin. Hemagglutinins isolated from salmonids agglutinated either rabbit or human type B erythrocytes; for this reason they are distinct from the eel (Anguilla anguilla) anti-H agglutinin which recognizes human red cell H-antigen and reacts with L-fucose (Springer and Desai,

1971). Harisdangkul et al. (1972) purified a fructosan-specific protein from nurse shark serum; although this substance precipitated with addition of certain fructosans, it did not agglutinate human type A, B or O erythrocytes. These observations show it is quite distinct from the salmonid ova hemagglutinins. Lectins isolated from invertebrate sources are also quite different from the salmonid ova protein. The oyster (Crassostrea virginica) hemagglutinin is a large (33.4S) protein which agglutinates sheep red cells (Acton et al., 1969). Similarly, the horseshoe crab (Limulus polyphemus) hemolymph agglutinin is a larger protein (approximate molecular weight 400,000) which agglutinates human A, B and O cells by virtue of reaction with N-acetylneuraminic acid (Cohen, 1968). Other invertebrate-derived hemagglutinins also differ from the salmonid ova agglutinin in erythrocyte and carbohydrate specificity, size and heat stability (Gold and Belding, 1975).

The chinook ova lectin reaction with carbohydrate was highly specific; while D-galactose inhibited the agglutination of rabbit erythrocytes, 2-deoxygalactose did not. The anomer specificity of the reaction was not determined. Anstee, Holt and Pardoe (1973) reported that ova hemagglutinin derived from Clupea harengus and S. trutta also were specific for L-rhamnose and D-galactose and that hemagglutination was inhibited to a lesser extent by L-arabinose, D-fucose and L-fucose. Their results indicated that the α-anomeric

configuration was preferred and that the determinant groups at C_2 and C_4 of the sugars were essential for binding; groups at C_3 and C_5 were less important. The work reported here supports their conclusions. As noted above, a simple change of the C_2 hydroxyl group of D-galactose to the deoxy form was sufficient to eliminate recognition of the sugar by the salmonid ova agglutinin.

Gel filtration analysis of chinook ova homogenate previously dialyzed against Tris buffer showed that the unfertilized salmon egg contained relatively few proteins; these results were supported by the electrophoresis data. Two major protein peaks were resolved by gel filtration, and the larger of these contained the hemagglutination activity. The estimated molecular weight of 120,000 daltons indicates the chinook lectin is similar in size to the 4.2S natural hemagglutinin derived from rainbow trout serum (Hodgins, Weiser and Ridgway, 1967). This size also shows that the salmonid ova agglutinin is quite distinct from the classical 17S IgM-like salmon immunoglobulin.

When purified chinook salmon ova agglutinin was assayed for carbohydrate content, a very minor sugar moiety was detected. Previous studies with hemagglutinins derived from salmonid sera or ova did not include carbohydrate analyses, so no comparisons can be made. The carbohydrate content of other lectins ranges from a total lack of carbohydrate in concanavalin A (Agrawal and

Goldstein, 1965) to the 13% sugar content of the oyster hemagglutinin (Acton et al., 1969).

Immunodiffusion analysis of ova homogenates derived from seven species of salmonids showed that the unfertilized eggs from these fish contained very similar proteins. All species tested had a protein immunologically identical to the chinook salmon ova agglutinin. It seems likely that other salmonids will be found which contain this protein in their ova. Whether nonsalmonid fish have immunologically similar ova proteins is unknown. Atlantic herring (Clupea harengus) ova contain an agglutinin which recognizes human type B erythrocytes and is inhibited by D-galactose; however, this protein was not compared, by immunological methods, to a salmonid hemagglutinin (Anstee, Holt and Pardoe, 1973).

The discovery of the bactericidal properties of the spring chinook salmon ova agglutinin was of special interest. Although these proteins have been postulated to serve as "protectins" in young salmon, no previous studies reported bactericidal properties for fish-derived lectins. The specificity of the bactericidal activity was especially striking; only fish pathogens were affected, but not all were sensitive to the protein. While A. hydrophila was sharply inhibited, A. salmonicida was unaffected. Both motile and non-motile species were sensitive to the lectin. These observations suggest that the spring chinook ova agglutinin likely represents more

than a broad-spectrum anti-bacterial agent. Based upon the specificity of the bactericidal reaction, it is tempting to speculate that this activity is inducible, that is, produced in response to a previous contact with the pathogen, or an antigenically-related agent. This hypothesis could be tested by injection of a foreign bacterium into the adult salmon during the period of ova formation, with subsequent testing of the ova for specific bactericidal activity against that agent. Cushing (1952) suggested that hemagglutinin specificity noted in skipjack tuna sera may have resulted from immune responses to parasitic or other infections. No further work was done, however, to test this hypothesis with tuna.

The observation that immunologically identical proteins derived from other salmonid species had different and reduced bactericidal effects supported this hypothesis. These other species, unlike the spring chinook salmon, are not severely debilitated and therefore not as susceptible to bacterial invasion during the period of their ova formation. The spring chinook salmon returns from the ocean to freshwater 3-4 months prior to spawning and spends this period of time in a debilitated condition; losses at some hatcheries reach 50-70% of these adults, and these deaths are usually attributed to multiple bacterial infections.[12] It is during this period of time

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[12]Personal communication, R. A. Holt, Oregon Dept. Fish and Wildlife.

that egg development occurs. Ova development in the other species tested does not occur while the animal is in a debilitated state; the coho, pink and chum salmon and steelhead trout enter freshwater only shortly prior to spawning. Lahonten cutthroat trout and kokanee salmon are freshwater species and never enter the ocean. Because of this, they are not subjected to the physiological and osmotic stress of moving from a saltwater to a freshwater environment during their ova development. These considerations suggest that the spring chinook is more susceptible to pathogenic bacteria during the period of egg formation, and has a greater need to respond with protective mechanisms at this time.

The comparative study of spring chinook and fall chinook salmon supported this hypothesis. Although the fall chinook is taxonomically identical to the spring chinook, it has a different life cycle. The fall strain returns to freshwater only shortly before spawning; ova development occurs while the animal is in salt-water and relatively healthy. As the hypothesis would predict, the fall chinook, not being subjected to antigenic stimulation as the result of severe bacterial invasion during ova formation, would not produce bactericidal lectin. Indeed, the results showed that the ova protein derived from the fall chinook had a limited bactericidal activity.

As mentioned earlier, proof of this hypothesis must await further experimentation. The data discussed above only indicated

that the species (spring chinook salmon) most likely exposed to large numbers of pathogenic bacteria possessed an ova lectin with specific bactericidal properties. To test this hypothesis, one of the other salmonids should be exposed to a foreign bacterium during the period of ova development; if the hypothesis is sound, resulting eggs would be bactericidal for that specific agent.

The observation that 0.5 μg/ml of purified spring chinook ova agglutination reduced viable V. anguillarum cells by greater than 75% was of interest. It appears that salmon, though occupying a relatively low taxonomic position among vertebrates, are able to produce highly efficient anti-bacterial molecules. Cisar and Fryer (1974) found that the coho salmon immunoglobulin, at a concentration of 0.006 μg N/ml, agglutinated A. salmonicida cells. This is one of the most sensitive bacterial agglutination systems known.

After the effect of incubation time for bacterial inhibition was determined, questions concerning the mode of bactericidal action were raised; a 60 min incubation period resulted in 100% inhibition of V. anguillarum. Spectrophotometric examination of cells incubated with the lectin indicated that cell lysis had not occurred. Microscopic examination of these cells showed a loss of motility occurred among 50% of the cells after 20 min, but again no lysis was observed. The mode of action remains unknown; whatever the killing mechanism is, it is rapid, even when the agglutinin is present in low

concentrations.

The heat stability of the bactericidal property indicated that the lectin killing mechanism was likely not due to enzymatic activity. This degree of stability (100 C for 60 min) is similar to that of the Enterobacter cloacae enterotoxin which retained its toxigenic characteristics after heating at 100 C for 30 min (Klipstein and Engert, 1976).

The bactericidal activity was also stable after lyophilization. This showed that purified agglutinin could be safely stored as a dried powder. Anstee, Holt and Pardoe (1973) found that the type B-specific agglutinin isolated from S. trutta ova also was unaffected by lyophilization. Since the protein in S. trutta ova was very similar to the spring chinook ova agglutinin in several other respects, the lyophilization stability was expected.

The comparative studies with ricin and the spring chinook ova agglutinin posed some questions concerning the relation between carbohydrate binding and bactericidal activity. Although both lectins have specificity for D-galactose, ricin was not toxic for V. anguillarum. This indicated that the toxicity involved more than the mere recognition of a specific carbohydrate site on the bacterium. However, it seemed that ricin did compete for binding sites on the cells; when ricin was present at greater than a 5:1 ratio over the chinook protein, bactericidal activity was diminished. This suggested that

carbohydrate-binding might be prerequisite to the bactericidal activity.

More work is required to determine the role between carbohydrate-binding and bacterial inhibition. One experiment was performed in which D-galactose was incubated with the spring chinook ova agglutinin prior to the addition of V. anguillarum cells; the degree of bactericidal activity was unchanged under these conditions. It can be argued, however, that the agglutinin had a much greater affinity for the bacterial cells than for the monosaccharide, and thus the bactericidal reaction was energetically favored.

This study reports the first amino acid analysis of a salmonid-derived agglutinin. The salmon protein is distinct from other lectins that have been analyzed. The anti-H lectin derived from eel serum has significant amounts of cysteic acid and reduced levels of alanine (Springer and Desai, 1971). Lectins from the horseshoe crab and the oyster also have been purified and analyzed; each of these proteins has an amino acid composition that is different from the salmonid protein (Marchalonis and Edelman, 1968; Acton et al., 1969). The amino acid data indicate that the chinook and coho salmon ova agglutinins are nearly identical in composition; this confirms the immunodiffusion results which demonstrate their immunological identity. Since these proteins differ markedly in their bactericidal properties, it seems that the portion of the molecule responsible for toxicity is relatively small and consists of a minor fraction of the total amino

acids. This is similar to the situation which occurs with immunoglobulins.

The finding that i. v. -administered spring chinook ova agglutinin had no effect on the V. anguillarum LD50 for coho salmon does not rule out a possible protective role for the lectin in salmon fry. Administration of greater amounts of the protein by a different route may affect the course of the challenge. Knowledge concerning the location of the agglutinin in salmon fry would be very helpful for conducting this study. One alternative route of administration, intraperitoneal injection, may also prove fruitless for demonstration of a protective role; apparently very little agglutinin administered by this route is absorbed into the circulatory system.[13] Further in vivo studies of this nature will be needed to determine the biological role of this protein in salmon fry.

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[13]Personal communication, W. J. Groberg, Oregon State University.

SUMMARY AND CONCLUSIONS

1. Classical-type salmonid immunoglobulin was not detected within unfertilized spring chinook salmon ova. It is likely that 17S salmon antibodies are not transmitted from the maternal serum to the ova in sufficient quantities to provide protection to newly hatched fry.
2. A bactericidal lectin isolated from spring chinook salmon ova agglutinated human type B and rabbit erythorcytes. This hemagglutination was inhibited by D-galactose and L-rhamnose.
3. Since the lectin was selectively adsorbed to agarose, affinity chromatography on agarose could be used to purify the protein. However, gel filtration on Bio-Rad P300 was a more efficient means for purification of this ova lectin.
4. Purified spring chinook salmon ova lectin contained a minor (0.8%) carbohydrate moiety.
5. Coho, pink, chum, kokanee and fall chinook salmon, steelhead and Lahonten cutthroat trout ova contained a protein immunologically identical to the spring chinook ova lectin.
6. The spring chinook salmon ova lectin was bactericidal for two serotypes of V. anguillarum, P. piscicida, A. hydrophila and F. columnaris, but not for Y. ruckeri, A. salmonicida, E. tarda, C. psychrophila, or Corynebacterium sp. None of 19

species of human-associated bacteria tested were inhibited by this protein.

7. Immunologically identical proteins from seven other salmonids had little, if any, bactericidal activity against the bacterial fish pathogens. Even the protein from fall chinook salmon ova had very limited bactericidal activity.
8. Less than 1.0 μg/ml of the purified spring chinook ova lectin caused complete inhibition of V. anguillarum growth. Incubation of the lectin with bacteria for 1 hr was sufficient for 100% growth inhibition. The mechanism of bactericidal activity was not cell lysis.
9. Bacterial inhibition activity of the lectin was not reduced by heating at 80 C for 6 hr, 100 C for 1 hr, or by lyophilization.
10. Ricin, a plant lectin with a carbohydrate specificity similar to that of the chinook lectin, was not toxic for V. anguillarum. However, this plant lectin did compete for binding sites on the bacterium.
11. Intravenous administration of 125 μg of purified spring chinook ova lectin to coho salmon did not protect the animals from V. anguillarum challenge.
12. Amino acid analysis of coho and chinook salmon-derived agglutinins showed that these proteins are very nearly identical; this supported the immunodiffusion results. Alanine, glutamine-glutamic acid and leucine were the most abundant amino acids detected.

BIBLIOGRAPHY

Acton, R. T., et al. 1969. Physical and chemical characterization of an oyster hemagglutinin. Journal of Biological Chemistry 244:4128-4135.

Agrawal, B. B. L. and I. J. Goldstein. 1965. Specific binding of concanavalin A to cross-linked dextran gels. Biochemistry Journal 96:23c-25c.

Amend, D. F. and F. C. Fender. 1976. Uptake of bovine serum albumin by rainbow trout from hyperosmotic solutions: a model for vaccinating fish. Science 192:793-794.

Anacker, R. L. and E. J. Ordal. 1959. Studies on the myxobacterium Chondrococcus columnaris. 1. Serological typing. Journal of Bacteriology 78:25-32.

Anstee, D. J., P. D. J. Holt and G. I. Pardoe. 1973. Agglutinins from fish ova defining blood groups B and P. Vox Sanguinis 25: 347-360.

Antipa, R. 1976. Field testing of injected Vibrio anguillarum bacterins in pen-reared Pacific salmon. Journal of the Fisheries Research Board of Canada 33:1291-1296.

Avtalion, R. R. 1969. Temperature effect on antibody production and immunological memory, in carp (Cyprinus carpio) immunized against bovine serum albumin (BSA). Immunology 17:927-931.

Bohlool, B. B. and E. L. Schmidt. 1974. Lectins: a possible basis for specificity in the Rhizobium-legume root nodule symbiosis. Science 185:269-271.

Brambell, F. W. R. 1970. The transmission of passive immunity from mother to young. Frontiers of Biology, volume 18. American Elsevier Publishing Company, New York. 385 p.

Buxton, A. 1952. On the transference of bacterial antibodies from the hen to the chick. Journal of General Microbiology 7:268-286.

Callow, J. A. 1975. Plant lectins. Current Advances in Plant Science 18:181-193.

Cisar, J. O. and J. L. Fryer. 1974. Characterization of anti-Aeromonas salmonicida antibodies from coho salmon. Infection and Immunity 9:236-243.

Cohen, E. 1968. Immunological observations of the agglutinins of Limulus polyphemus and Birgus latro. Transactions of the New York Academy of Science 30:427-443.

Conrath, T. B. 1972. Handbook of Microtiter Procedures. Dynatech Corporation, Cambridge. 475 p.

Corbel, M. J. 1975. The immune response in fish: a review. Journal of Fish Biology 7:539-563.

Cushing, J. E. 1952. Individual variation in the hemagglutinin content of yellowfin tuna and skipjack bloods. Journal of Immunology 68:543-547.

Dazzo, F. B. and W. J. Brill. 1977. Receptor site on clover and alfalfa roots for Rhizobium. Applied and Environmental Microbiology 33:132-136.

Dazzo, F. B. and D. H. Hubbell. 1975. Cross-reactive antigens and lectin as determinants of host specificity in the Rhizobium-clover association. Applied Microbiology 30:1017-1033.

Dubois, M., et al. 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28:350-356.

Fidler, J. E., L. W. Clem and P. A. Small. 1969. Immunoglobulin synthesis in neonatal nurse sharks (Ginglymostoma cirratum). Comparative Biochemistry and Physiology 31:365-371.

Finstad, C. L., et al. 1972. The evolution of the immune response. XIII. The characterization of purified erythrocyte agglutinins from two invertebrate species. Journal of Immunology 108: 1704-1710.

Franklin, E. C. and H. G. Kunkel. 1958. Comparative levels of high molecular weight (19S) gamma globulin in maternal and unbilical cord sera. Journal of Laboratory and Clinical Medicine 52:724-727.

Fryer, J. L., et al. 1976a. Vaccination for control of infectious diseases in Pacific salmon. Fish Pathology 10:155-164.

Fryer, J. L., et al. 1976b. Temperature, infectious diseases, and the immune response in salmonid fish. EPA report #600/3-76-021. Office of Research and Development, Duluth, Minnesota. 71 p.

Fryer, J. L., et al. 1977. Development of bacterins and vaccines for control of infectious diseases in fish. Oregon State University Sea Grant Publication no. ORE SU-T-77-012. 10 p.

Gold, E. R. and P. Balding. 1975. Receptor-specific proteins. Plant and animal lectins. Academic Press, New York. 440 p.

Gould, R. W. 1977. Development of a new vaccine delivery system for immunizing fish and investigation of the protective antigens in Vibrio anguillarum. Ph. D. thesis, Oregon State University, Corvallis, Oregon. 120 p.

Grey, H. M. 1969. Phylogeny of immunoglobulins. In: Advances in immunology 10. ed. by F. J. Dixon and H. G. Kunkel. Academic Press, New York. p. 51-104.

Groberg, W. J., E. W. Voss and J. L. Fryer. 1978. Metabolism of coho salmon Ig. II. Intraperitoneal absorption properties of coho salmon tetrameric Ig. (manuscript in preparation).

Gunnels, R. D., H. O. Hodgins and M. H. Schiewe. 1976. Failure of vaccines to protect salmon from vibriosis enzootic in Puget Sound, Washington. American Journal of Veterinary Research 37:737-740.

Harisdangkul, V., et al. 1972. A protein in normal nurse shark serum which reacts specifically with fructosans. I. Purification and immunochemical characterization. Journal of Immunology 108:1244-1258.

Hildemann, W. H. 1962. Immunogenetic studies of poikilothermic animals. American Naturalist 96:195-204.

Hodgins, H. O., R. S. Weiser and G. J. Ridgway. 1967. The nature of antibodies and the immune response in rainbow trout (Salmo gairdneri). Journal of Immunology 99:534-544.

Klipstein, F. A. and R. F. Engert. 1976. Partial purification and properties of Enterobacter cloacae heat-stable enterotoxin. Infection and Immunity 13:1307-1314.

Klontz, G. W. and D. P. Anderson. 1970. Oral immunization of salmonids: a review. In: Symposium on diseases of fishes and shellfishes, ed. S. F. Snieszko. American Fisheries Society Special Publication No. 5, p. 16-20.

Kuhns, W. J. and J. Chuba. 1968. Intrageneric blood group differences between ictalurids (freshwater catfishes). (Abstract). Federation Proceedings 27:491.

Leed, E., J. Gjerde and O. R. Braekhan. 1975. Simple and rapid technique for repeated blood sampling in rainbow trout (Salmo gairdneri). Journal of Fisheries Research Board of Canada 32:699-701.

Malkinson, M. 1965. The transmission of passive immunity to Escherichia coli from mother to young in the domestic fowl (Gallus domesticus). Immunology 9:311-317.

Marchalonis, J. J. 1969. Isolation and characterization of immunoglobulin-like proteins of the Australian lungfish (Neoceradotus forsteri). Australian Journal of Experimental Biology and Medical Science 47:405-419.

Marchalonis, J. J. and G. M. Edelman. 1968. Isolation and characterization of a hemagglutinin from Limulus polyphemus. Journal of Molecular Biology 32:453-465.

Murphy, F. A., et al. 1964. Gamma-globulins of bovine lacteal secretions. Archives of Biochemistry and Biophysics 108: 230-239.

O'Leary, P. J., J. O. Cisar and J. L. Fryer. 1978. The effect of temperature on agglutination activity of coho salmon (Oncorhynchus kisutch) antiserum. Journal of Fish Diseases 1:123-125.

Paterson, R., et al. 1962. Antibody production and transfer to egg yolk in chickens. Journal of Immunology 89:272-278.

Pauley, G. B., S. M. Krassner and R. A. Chapman. 1971. Bacterial clearance in the California sea hare, Aplysia california. Journal of Invertebrate Pathology 18:227-239.

Pistole, T. G. and R. M. Furman. 1976. Serum bactericidal activity in the horseshoe crab, Limulus polyphemus. Infection and Immunity 14:888-893.

Prokup, O., et al. 1974. Protectins: past, present problems, and perspectives. Annals of the New York Academy of Sciences 234:228-231.

Reed, L. J. and H. Meunch. 1938. A simple method of estimating fifty percent endpoints. American Journal of Hygiene 27:493-497.

Rohovec, J. S. 1974. Oral and parenteral immunization for the control of Vibrio anguillarum, the etiological agent of vibriosis in salmonid fish. Ph. D. thesis, Oregon State University, Corvallis, Oregon. 82 p.

Sharon, N. and H. Lis. 1972. Lectins: cell-agglutinating and sugar-specific proteins. Science 177:949-958.

Simpson, D. L., D. R. Thorne and H. H. Loh. 1977. Developmentally regulated lectin in neonatal rat brain. Nature 266:367-369.

Spackman, D. H., W. H. Stein and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Analytical Chemistry 30:1190-1206.

Springer, G. F. and P. R. Desai. 1971. Monosaccharides as specific precipitinogens of eel anti-human blood-group H(O) antibody. Biochemistry 10:3749-3761.

Tebbit, G. L. 1976. Viruses infecting salmonid fishes from Oregon. A. The occurrence and distribution of infectious pancreatic necrosis virus. B. The development of an attenuated strain of infectious hematopoietic necrosis virus (IHNV) for the immunization of salmonids. Ph. D. thesis, Oregon State University, Corvallis, Oregon. 81 p.

Teichberg, V. I., et al. 1975. A β-D-galactoside binding protein from electric organ tissue of Electrophorus electricus. Proceedings of the National Academy of Sciences 72:1383-1387.

Todd, G. M. 1971. Blood group antibodies in Salmonidae roe. Vox Sanguinis 21:451-460.

Voss, E. W., J. L. Fryer and G. M. Banowetz. 1978. Isolation, purification and partial characterization of a lectin from chinook salmon ova. Archives of Biochemistry and Biophysics 186:127-136.

Voss, E. W., W. J. Groberg and J. L. Fryer. 1978. Metabolism of coho salmon Ig. I. Catabolic rate of coho salmon tetrameric Ig in serum. (Manuscript in preparation.)

Uhr, J. W., M. S. Finkelstein and E. C. Franklin. 1962. Antibody response to bacteriophage ϕX174 in nonmammalian vertebrates. Proceedings of the Society for Experimental Biology and Medicine 111:13-15.