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The Boivin antigen from Vibrio anguillarum was extracted with the use of 0.25 M trichloroacetic acid (TCA). Chemical analysis revealed that the endotoxin contained approximately 35.1% protein, 18.1% carbohydrate, 39.3% lipid, 1.8% glucosamine and evidence of ethanolamine. Sixteen different amino acids made up the protein. The aliphatic amino acid concentration was 41.6%. The acidic amino acid content was higher than the basic amino acid content (25.8% vs. 14.4%) and glutamic and aspartic acid were the predominant amino acids. The carbohydrate fraction was composed of fucose (7.3%), arabinose (1.2%), deoxyglucose (tentative identification) (4.2%), galactose (0.8%), glucose (2.1%), and an unidentified sugar (2.5%).

Injection of the endotoxin into mice resulted in an LD_{50} value of 794 μg. When injected intramuscularly into juvenile chinook salmon, the endotoxin caused a zone of hemorrhaging at the site of

injection. Upon prolonged storage of the endotoxin, this activity was lost.

When incorporated into the salmonids diet or injected intraperitoneally, the endotoxin protected against vibriosis. A higher degree of protection was obtained by injection than by feeding of the endotoxin. No anti-V. anguillarum agglutinating antibodies were evident in the serum of juvenile chinook salmon fed the endotoxin or whole cell vaccine. Fish receiving the endotoxin by intraperitoneal injection produced anti-V. anguillarum agglutinating serum antibodies.

Certain Chemical and Immunological Properties of the Endotoxin from Vibrio anguillarum

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CERTAIN CHEMICAL AND IMMUNOLOGICAL PROPERTIES OF THE ENDOTOXIN FROM VIBRIO ANGUILLARUM

INTRODUCTION

The bacterium, Vibrio anguillarum, is a gram negative, slightly curved, motile rod with a definite sodium chloride requirement for growth. It has been associated with the "red disease" of eels and vibriosis in various species of fish. Of particular concern is the occurrence of vibriosis in species of Pacific salmon. Mortality rates up to 90% have been known to occur. Salmonids infected with V. anguillarum show diffuse hemorrhages on the body surfaces and in the musculature. The intestines may be inflamed and petechiae are sometimes present on the viscera.

Successful oral immunization of salmonids against vibriosis has been accomplished by this laboratory. The methods of vaccination have involved using lyophilized formalin-killed cells, lyophilized sonicated cells and formalin-killed cells (a wet cell paste). These various vaccines were incorporated into the salmonids diet.

In this laboratory, various aspects of the vaccination and immunity conferred by the vaccines against vibriosis are being studied. The role of the V. anguillarum endotoxin in this acquired immunity has not been previously studied. It was the intent of this investigation to see if the endotoxin was involved in the immunity conferred by the various vaccines. Also, analysis of the chemical

constituents of the endotoxin was done in order to compare it with the endotoxin from other water and cholera vibrios. In addition, certain effects of the endotoxin on the host (salmonid fish) was examined.

The particular isolate employed in this study, Vibrio anguillarum -LS-170, was from the Oregon State University, Department of Microbiology culture collection. The bacterium was isolated from salmon involved in an epizootic of vibriosis at the saltwater rearing facilities at Lint Slough. Juvenile chinook salmon (Oncorhynchus tschawytscha) from the Oregon State Game Commission Research Laboratory were used in the immunization experiments. These fish were immunized at the Oregon State University Fish Disease Laboratory and transported to the saltwater rearing facilities of the Oregon State Game Commission at Lint Slough, Waldport, Oregon for exposure to the natural source of infection. This study covered a time interval of approximately eight months.

LITERATURE REVIEW

Vibrio anguillarum

The organism, Vibrio anguillarum, is a gram negative, slightly curved rod, and motile by means of a single polar flagellum. It is a non-sporeformer, anaerogenic fermenter and has a definite sodium chloride requirement for growth and survival. The temperature at which optimal growth occurs is between 20° - 27° C. At 37° C, growth is inhibited and death ensues (Cisar and Fryer, 1969; Evelyn, 1971).

Vibrio anguillarum was originally associated with a condition called "red disease" of eels. Since then, it has been found to be a pathogen of other marine and freshwater fishes (Rucker, 1959; Anderson and Conroy, 1970). Of particular concern is the occurrence of vibriosis in most species of Pacific salmon (Rucker, Earp and Ordal, 1953; Cisar and Fryer, 1969; Evelyn, 1971). The gross pathology of salmonids infected with V. anguillarum usually involve erythema at the bases of fins and within the mouth and gills. Hemorrhaging is also evident in the musculature and at times in the intestines, viscera, and body surfaces. Mortalities reaching 90% from an outbreak of vibriosis in juvenile salmon have been reported.

Bacterial Endotoxins

Because of a myriad of effects, the bacterial endotoxins have fascinated many scientists. Generally, the endotoxins are from gram-negative bacteria and thought of as being a lipopolysaccharide (LPS) complex whose extraction may be accomplished by a phenol/water method. The LPS obtained is non- or weakly antigenic whereas material obtained from the trichloroacetic acid (TCA) method yields a lipopolysaccharide-protein complex which is highly antigenic. In addition to these methods, the use of urea/ammonium sulfate, EDTA in Tris-HCl followed by phenol extraction, or hypertonic NaCl and sodium citrate, and various combinations of these procedures can also be used. It is known that these endotoxins have the O-antigenic specificity associated with their polysaccharide component (Luderitz, Westphal, Staub and Nikaido, 1971; Simmons, 1971; Mukasa and Slade, 1972). They are also heat stable and in the appropriate animal produce a biphasic fever, an inflammatory reaction in the skin and abortion. Both the dermal and generalized Shwartzman reactions have been produced in rabbits using bacterial endotoxins. In addition, an adjuvant effect, hemorrhagic necrosis of transplantable tumors, and a lethal effect can be observed (Milner, Rudbach and Ribi, 1971).

Recently two books edited by Weinbaum, Kadis and Ajl (1971a,

1971b) present information regarding the chemistry, immunology, structure, genetics, biosynthesis, pharmacology, and pathology of bacterial endotoxins.

Vibrio Endotoxins

Linton and Shrivastava (1933), working with cholera vibrios and a water vibrio, were able to extract several carbohydrates by heating the vibrios in acetic acid until they coagulated. The supernatant was then subjected to alcoholic precipitation. They found that the water vibrio extract contained glucuronic acid, galactose, and arabinose. The cholera vibrios contained glucuronic acid and galactose or arabinose. Later studies (Linton, 1940) revealed that some strains contained only glucose as the sugar constituent in the hydrolysate, although non-amino nitrogen, amino nitrogen, and phosphorus were found, thus indicating a complex structure.

Dennis (1959), using Vibrio fetus, isolated a toxic lipopolysaccharide (LPS) by a phenol extraction method in which the cells were treated previously by shaking in saline for one hour to remove the flagellum. The surface antigen was removed by treating with 0.5 M potassium thiocyanate for 72 hours at 37° C. The LPS was toxic, pyrogenic, antigenic, and was absorbed to human "O" blood cells giving hemagglutination when tested against V. fetus whole cell rabbit antisera. In rabbits a dose of 400 μg/kg I.V. was lethal; if

the solution was heated at 80° C for five minutes, a dose of only 40 μg/kg was needed to produce death. In mice an I.V. dose of 2.0 mg was lethal.

Winter (1966), using V. fetus var. intestinalis, isolated an LPS by extracting acetone-dried cells with 0.25 N TCA at 4° C followed by 45% phenol at 65° C. The material was then dialyzed after which it was lyophilized. The LPS was further purified by dissolving it in distilled water (1.0 mg/ml) and then precipitated by adding an equal volume of 0.2 M phosphate buffer (pH 7.5). The precipitate was collected by centrifugation for four hours at 130,000 x g. The sample was then dissolved in distilled water and lyophilized for storage.

Chemical analysis yielded a total carbohydrate content of 53.0%; hexose, 43.0%; pentose, 0%; methylpentose, 4.0%; lipids, 28%; hexosamine, 2.7%; phosphorus, 2.1%; nitrogen, 0.75%; protein, 2.0%; and nucleic acid, 0.2% (Winter 1966).

The endotoxin was lethal for white male mice weighing 13 to 15 grams when inoculated intraperitoneally. Death occurred within 48 hours and an LD_{50} of 350 μg was determined. An intravenous injection of 10 μg of endotoxin into rabbits weighing 3.5 and 3.3 kg produced a biphasic temperature response. Also a generalized Shwartzman reaction was produced when rabbits were given two 1,000 μg doses. The LPS was found to react with V. fetus whole

cell antisera and it could be adsorbed to sheep red blood cells to serve as an antigen for the passive hemagglutination reaction (Winter, 1966).

Sakaguchi et al. (1968) was able to extract an LPS from a K-minus strain of V. parahaemolyticus with 45% phenol containing 3% NaCl. The resulting crude extract (Fr. I) was further fractionated by centrifugation at 100,000 x g. The precipitated fraction (Fr. II) showed a high precipitin activity against "O" antisera. Lethality for mice was observed in Fraction II but not in Fraction I. Fraction II was hydrolyzed with 1% AcOH at 100° C for one or two hours. The non-dialyzable fraction of the hydrolyzed LPS obtained in a cellophane bag indicated much lower precipitin activity than that of the intact LPS. These observations appeared to be the result of the liberation of an acid-labile, immunologically active portion from the LPS.

Jenkin and Rowley (1959) working with Inaba and Ogawa strains of V. cholerae and water vibrios were able to isolate a toxic component from both, by use of various methods. An LPS of low toxicity was isolated by the phenol/water (45% w/v) method whereas a highly toxic protein (38% protein) was obtained by the TCA method. Further purification of the crude product by dissolving in 2.5 M urea and fractionation with ammonium sulfate produced a precipitate containing 92% protein and 8% carbohydrate. The toxicity was increased

twofold and could be destroyed by heating at 100°C for 10 minutes. Proteolytic enzymes (trysin, papain and pepsin) caused complete loss of toxicity while lyophilization caused a 50% loss.

Immune Responses

In Fish

The ability of teleosts to respond to an antigenic stimuli with a specific immune response has been well documentated (Ridgeway, Hodgins and Klontz, 1966; Klontz and Anderson, 1970; Sniezko, 1970). It was noted that the environmental temperature has a marked effect on the development of the immune response (Cushing, 1942; Muroga and Egusa, 1969). The lower the environmental temperature, the longer it took for the appearance of specific antibodies.

Endo (1961), during an epizootic of a disease of unknown etiology in hatchery fish, incorporated a multivalent vaccine into the hatchery diet. The vaccine consisted for four formalin killed fish pathogens; Vibrio piscium, Aeromonas salmonicida, Chondrococcus columaris and Haemophilus piscium. After administering the vaccine, a reduction in mortalities of approximately 60% was observed. Because no control group was included, the mortality figure was obtained by comparison with the previous epizootics. These epizootics had killed most of the hatchery population.

Hayashi et al. (1964) showed that the injection of formalin-killed cells into rainbow trout would protect against vibriosis. Some degree of protection against the disease by oral vaccination was shown.

Effective oral immunization of salmonids against vibriosis has been shown possible (Fryer, Nelson and Garrison, 1972). It is also possible to immunize salmonids with formalin-killed cells in Freund's incomplete adjuvant. Fryer et al., using sonicated lyophilized V. anguillarum cells incorporated into the salmonids' diet showed that increasing amounts of vaccine increased the degree of protection to a point where mortalities from vibriosis were about 10% as opposed to 98% in the non-vaccinated group.

Paterson (1972) has shown that an endotoxin extracted from Aeromonas salmonicida by the TCA method will elicit a specific antibody response. The endotoxin was absorbed to an aluminum hydroxide carrier and injected intraperitoneally into salmonids. It was found that a single dose of as low as 1.0 μg would cause a specific antibody response.

In Warm-Blooded Animals

Shirvastava, Singh and Ahuja (1948) using a phenol-extracted polysaccharide from V. cholerae were able to immunize mice against intraperitoneal infection with mucinized suspensions of

V. cholerae. This was accomplished by two subcutaneous doses of the polysaccharide given at weekly intervals.

Other papers (Bhatia et al., 1964; Kaur and Shirvastav, 1965; Watanabe and Verwey, 1965; Verwey et al., 1965) concerning V. cholerae have reported that a lipopolysaccharide can protect mice against infection by Inaba and Ogawa strains of cholera. The LPS isolated from one strain showed cross protection with the other strain.

Verwey et al. (1965) showed that the Inaba protective antigen differed from the Ogawa antigen by being damaged with the phenol extraction method. The Inaba antigen was also heat-labile. They hypothesized that the Inaba antigen was a complex of protein, carbohydrate, and lipid. The protein was thought to be the heat-labile, phenol-sensitive moiety necessary for full antigenicity.

Recently, Neoh and Rowley (1970) presented evidence that vibriocidal antibodies were directed against the polysaccharide determinants of the lipopolysaccharide. In addition, another vibriocidal antibody system against the protein closely associated with the LPS was present. Furthermore, the protein was antigenically distinct from the LPS but common to both Inaba and Ogawa strains of cholera. This indicated that the protein was the group antigen.

MATERIALS AND METHODS

Experimental Facilities

The facilities in the Department of Microbiology, Bioscience Building, Oregon State University; the Oregon State University Fish Disease Laboratory, Corvallis, Oregon and the saltwater rearing facilities of the Oregon State Game Commission at Lint Slough, Waldport, Oregon were used during the course of research.

Fish were held at the Fish Disease Laboratory in circular three foot diameter, fiberglass tanks with a capacity of 122 gallons. Well water free of pathogens at an ambient temperature of 12.2 °C was employed.

At Lint Slough the tanks used were circular three foot or five foot diameter fiberglass tanks with capacities of 100 and 250 gallons, respectively. The water supply was the brackish water from the slough.

Endotoxin Extraction

Two two-liter flasks containing one liter of tryptone 1.0%, yeast extract 0.5%, NaCl 0.25%, and glucose 0.25%, pH 7.1 (TYG broth) were each inoculated with 10 mls of a 24 hour TYG broth

culture[1] of Vibrio anguillarum. These flasks were then incubated at 26° C on a reciprocating shaker (linear displacement of 4 cm at a rate of 120 times per minute) for 24 hours. This was then inoculated into a 30 liter broth medium of TYG or Tryptic Soy Broth (Difco). Before each inoculation step, a wet mount and gram stain were made to examine for purity. The 30 liter broth medium had previously been prepared in a Fermacell Fermentor[2] to which five mls of an antifoaming agent[3] had been added. This was then sterilized for one hour at 121° C. The conditions of incubations were: temperature, 27° C; mixing at 200 rpm with an air flow rate of 2.0 CFM for a period of 24 hours. After a period of 12 hours a 500 ml solution of filter sterilized (0.45 μ) 20% glucose was added to the fermentor to replace the depleted carbon source. The cells were then harvested in a Sharples centrifuge.[4]

The extraction of the endotoxin was accomplished by use of trichloroacetic acid (TCA) (Williams and Chase, 1967), with slight modifications. The modified procedure consisted of washing the

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[1] In an effort to increase cell growth, the NaCl concentration of the 10 ml TYG broth was increased to 1.25% for the last three extractions.

[2] Fermacell Fermentor Model CF-50, New Brunswick Scientific Co., Inc.

[3] Medical Antifoam "C" Emulsion, Dow Corning Corp.

[4] Sharples Super-Centrifuge Type T 1 P, Sharples Corp.

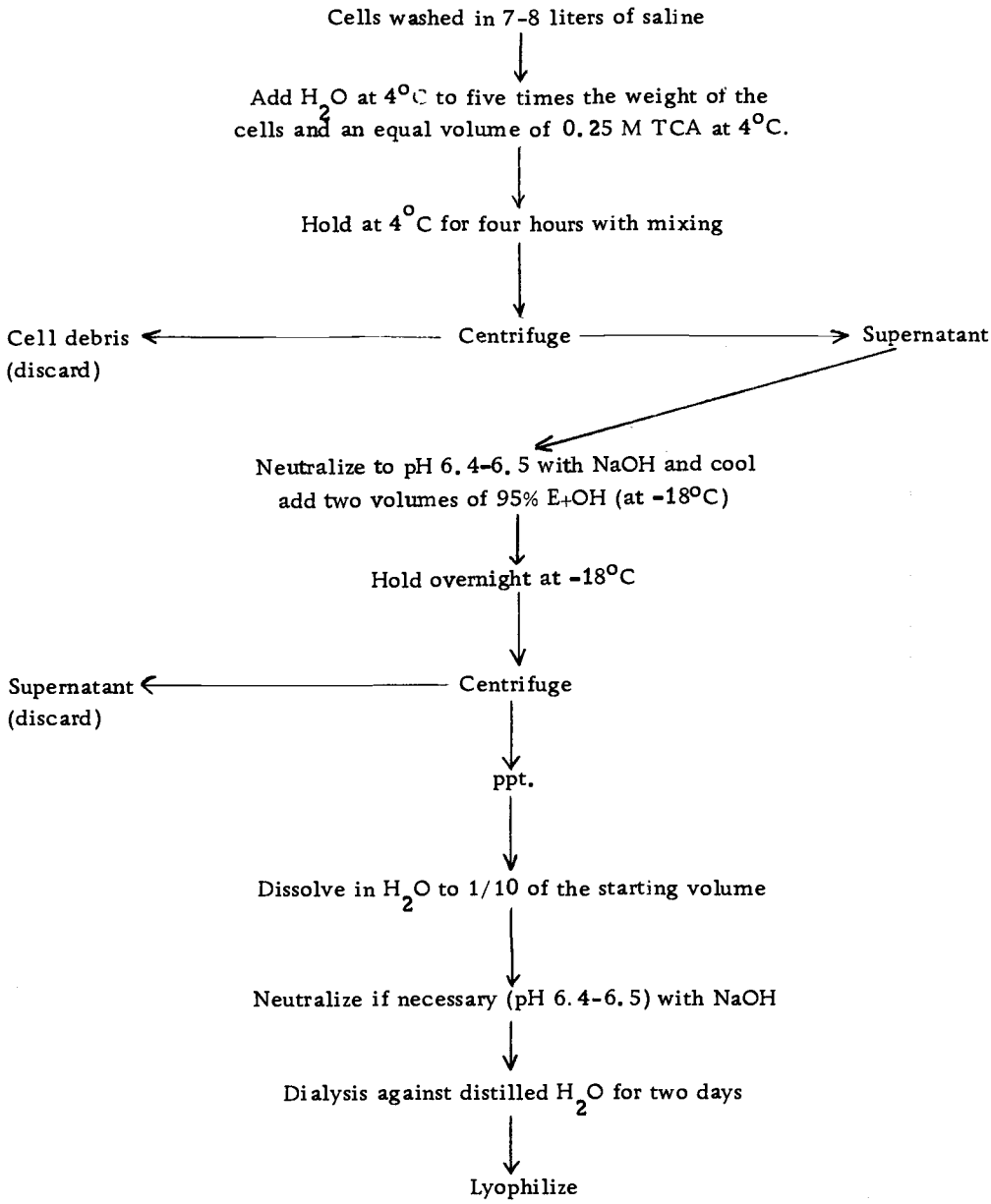

Figure 1. Flow chart for the extraction of the endotoxin from Vibrio anguillarum

harvested cells in seven to eight liters of cold 0.85% NaCl. The cells were then suspended in five times their weight of distilled water at 4° C. An equal volume of 0.25 M TCA at 4° C was then added and the solution maintained at 4° C for four hours with mixing. The mixture was then warmed to room temperature and centrifuged in a Sharples centrifuge. Neutralization of the supernatant to pH 6.4-6.5 was accomplished with NaOH. Precipitation of the complex was brought about by adding two volumes of 95% ethanol which had been cooled to -18° C. This was then held at -18° C overnight. The precipitate that sedimented overnight was harvested in the Sharples centrifuge and then dissolved in distilled water at one-tenth of the starting volume and neutralized (pH 6.4-6.5) with NaOH, if necessary. This was then dialyzed against 18 liters of distilled water for two days at 4° C. The final product was then lyophilized and stored in a dessicator in the cold.

Protein Purity

To determine protein purity of the endotoxin, polyacrylamide gel electrophoresis at pH 8.9 was employed. The V. anguillarum endotoxin extracted with 0.5 M and 0.25 M TCA were analyzed. Subsequent endotoxin extractions employed 0.25 M TCA.

An electrophoresis unit made especially for slab gel

electrophoresis was used.[5] Its design permitted the acrylamide slab to be continuously cooled during electrophoresis.

The original method of Davis (1964) was followed in preparing stock solutions, working solutions and gels. Acrylamide; N, N'-methylenbisacrylamide (BIS); N, N, N', N', -tetramethylenediamine (TEMED), and riboflavin were obtained from Eastman.[6] Tris (hydroxymethyl)aminomethane (Tris) was obtained from Eastman and Sigma.[7] Reagent grade ammonium persulfate was then from Matheson, Coleman and Bell.[8]

Endotoxin at a level of 200 μg in 20% sucrose was layered under the upper buffer. Electrophoresis was conducted with a constant potential of 200 volts across the electrodes of the unit (about 28 volts per cm of gel). The separation was stopped when the marker dye (bromphenol blue) approached within 3 mm of the bottom of the gel (about 2 hours). During electrophoresis the unit and gel slab were cooled with constantly flowing water from an ice bath. Gels were stained with Coomassie brilliant blue[9] in 12.5%

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[5]The unit was designed by Dr. A. W. Anderson, Department of Microbiology, Oregon State University, and made by the Physics Shop, Oregon State University.

[6]Eastman Organic Chemical, Division of Eastman Kodak Co., Rochester, New York.

[7]Sigma Chemical Co., St. Louis, Mo.

[8]Matheson, Coleman and Bell, Division of Matheson Co., Norwood, Ohio.

[9]Colab Laboratories, Inc., Chicago Heights, Illinois.

trichloroacetic acid (TCA) (Chrambach et al., 1967). After destaining in 10% TCA the gels were soaked in deionized distilled water and stored in 7.0% acetic acid.

Chemical Analysis

Lipid

Crude lipid content was determined by hydrolyzing the endotoxin in 1.0 N HCl for 15 minutes in a boiling water bath. This was immediately cooled to room temperature and neutralized to pH 7.0. Then 20 volumes of a chloroform-methanol (2:1) mixture was added, shaken and left standing for at least 20 minutes. The chloroform phase was then collected and evaporated by bubbling through nitrogen or air.

Amino Acids

Amino acid content was determined after 22 hours and 70 hours hydrolysis in constant boiling HCl. Samples were analyzed by a Spinco model 120 B Amino Acid Analyzer.

Carbohydrates

Analysis of sugars through gas-liquid chromatography was accomplished by their conversion to the alditol acetate derivative.

This was done by a modified method[10] of Albersheim et al. (1967). Prior to making the alditol acetate derivative, the samples were hydrolyzed in 0.25 N H_2SO_4 at 110° C for 2.5, 5, 18, 20, and 24 hours. Another sample was hydrolyzed at 125° C for 80 minutes. After hydrolysis, sulfates were removed by the addition of 1.5 g $BaCl_2 \cdot 2H_2O$ with stirring for five minutes. The pH was then adjusted to 5.0-5.5 with a saturated solution of $Ba(OH)_2$. This was centrifuged at 12,100 x g for 30 minutes and the supernatant reduced by adding sodium borohydride (0.15 g)[11] and left standing at room temperature for at least 30 minutes. The excess borohydride was decomposed by dropwise addition of glacial acetic acid until effervescence stopped. The mixture was evaporated to near dryness under reduced pressure and washed with five 10 ml aliquots of methanol each followed by evaporation to dryness with the rotary evaporator. This was then dried at 100° -110° C for 10 minutes. Acetylation of the alditols was accomplished by the addition of acetic anhydride (10 ml) and sodium acetate anhydride (0.5 g) with refluxing at 140° C for 25 minutes. The mixture was then evaporated to dryness on a rotary evaporator. The contents were then washed with three 5 ml

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[10] Personal communication with Dr. A. W. Anderson, Department of Microbiology, Oregon State University.

[11] At this point 1.0 ml of the internal standard, myoinositol (10 mg/ml) was added.

aliquots of dichloromethane and centrifuged at 12,100 x g for 30 minutes. The supernatant solution was left standing at room temperature overnight for precipitate formation. If any precipitate formed, the sample was centrifuged at 12,100 x g for 30 minutes. The solution was then evaporated to 1.0 ml by bubbling through nitrogen and stored in a stoppered test tube.

The samples were analyzed on an F and M High Efficiency Gas Chromatograph Model 402 (F M Scientific Corp.) incorporating a high temperature flame ionization detector. The steel column employed was 1/8 inch by 4 ft. containing 100/120 mesh Gas-Chrom-P[12] coated with 0.2% diethylene glycol succinate,[13] 0.2% diethylene glycol adipate,[13] and 0.4% GE XF 1150.[12] The injector, column oven and flame temperature were 200° C, 180° C, and 205° C, respectively. The carrier gas helium and detector gases hydrogen and air had flow rates of 50, 25, and 200 mls per minute respectively.

Myoinositol (10 mg/ml) was used as the internal standard. For identification of the unknown peaks, known sugars were applied to the GLC and their times compared.

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[12] Applied Science Laboratories Inc., State College, Pa.

[13] F & M Scientific, Division of Hewlett - Packard, Avondale, Pa.

Amino Sugars

Amino sugars were determined after hydrolysis of the endotoxin in 6.0 N HCl at 110° C for 8, 12, 18, and 22 hrs. The samples were then cooled and neutralized to pH 6.8-7.3 with NaOH and centrifuged to remove particulate matter. The hydrolysates were then subjected to the method of A. Nowotny (1969) and read at 530 mμ after one hour. The amino sugar content is reported as glucosamine.

Effect of V. anguillarum Endotoxin in Mice

In an effort to determine the effect of V. anguillarum endotoxin in warm blooded animals, white male mice weighing 15-17 g were used. LD_{50} determinations were carried out by the I. P. injection of 0.1 ml of serial twofold dilutions of the endotoxin with six mice per dilution. These mice were observed for 3 days.

Effect of V. anguillarum Endotoxin in Fish

To investigate possible effects of V. anguillarum endotoxin on salmonids, juvenile chinook salmon were used. Prior to injection, fish were anesthetized in a solution of methyl pentynol (1 oz./gal.). Using 26 gauge needles, endotoxin at a level of 1000 μg in 0.1 ml saline was injected intraperitoneal (dorsal to the pelvic fins) and/or intramuscularly (on either side of the dorsal fins). Another group

of fish received 3000 μg of endotoxin in 0.1 ml saline intramuscularly. Control fish received 0.1 ml of saline I.P. or I.M. All groups were held at 64° F. The gross pathology of these fish were observed over a 48 hour period.

Serological Procedures

Serum Collection

Blood was obtained from juvenile chinook salmon by severing the caudal vein just posterior to the adipose fin. Individual blood samples were collected in small glass tubes. These tubes were incubated at room temperature for one hour to allow clotting, then overnight at 4° C for clot retraction. Separation of serum from blood cells was accomplished by centrifugation for 30 minutes at 1,000 RPM.

Microliter Procedure

The diluent, 50 μl of saline, was added to all wells of a microliter plate except the first row. Dilution of the serum was made in 0.1 or 0.2 ml pipets by prefilling the pipets with 0.08 ml of saline followed by the addition of the serum to the pipet by capillary action to bring the total pipet volume to 0.1 ml. The contents were then expelled into the first row of wells, allowed to take up their volume

of the serum dilution and mixed by rotating the dilutors. Dilutors were then placed in the next row of cells and the mixing repeated giving serial twofold dilutions. Then 50 µl of antigen was added to each well. The antigen had been prepared by using a 24 hour suspension of washed V. anguillarum cells. The adsorbancy of the suspension was measured at 525 mµ with a Spectronic 20. The adsorbancy was adjusted to 0.7-0.9. Before reading, the plates were gently mixed, covered and incubated for two hours at room temperature then 18-20 hours at 4° C.

After preparing a titration, the dilutors were blotted, rinsed twice in saline, twice in distilled water and heated to near incandescence before the next usage.

A positive agglutination was taken as a uniform dispersion of the antigen over the bottom of the well. A negative agglutination was taken as the formation of a button of antigen at the bottom of the well.

Immunization Experiment

Successful oral vaccination of salmonids against vibriosis by this laboratory have been accomplished. The vaccines consisted of formalin-killed whole or sonciated V. anguillarum cells, incorporated into the salmonids diet. In continuing studies of various vaccines against vibriosis, the endotoxin of V. anguillarum was

examined as to its involvement in this immunity. Immunization of salmonids was attempted by administration of the endotoxin through I. P. injection or incorporation into the salmonids diet.

Vaccine Preparation

An aluminum hydroxide adsorbent prepared according to the method of Rethy (1965) was used as a carrier for the endotoxin. The endotoxin was mixed at a concentration of 2000 μg per 7.8 mg of the $Al(OH)_3$ carrier. This was then injected I. P. (dorsal to the pelvic fins) into juvenile chinook salmon.

For the oral immunization of the salmon, the endotoxin was incorporated into Oregon Test Diet (OTD) (Lee, Roehm and Sinnhuber, 1967) at the appropriate concentrations.

Experimental Groups

Juvenile fall chinook salmon were divided into seven groups. Prior to injection, fish were anesthetized by a solution of methyl pentynol. The experimental groups were as follows:

Group 1. Fish weighing 8.0 g were used as controls. These fish were fed Oregon Moist Pellets (OMP) throughout the experiment.

Group 2. Fish weighing 3.0 g were used as controls. These fish were fed OMP throughout the experiment.

Group 3. Fish weighing 3.0 g were fed sonicated lyophilized cells (prepared by J. Rohovec of this laboratory) at a level of 2.0 mg/g of OTD for 28 days.

Group 4. Fish weighing 3.0 g were fed the endotoxin at a level of 20 μg/g of OTD for 28 days.

Group 5. This group consisted of salmon weighing 3.0 g. These fish received 0.1 ml of the $Al(OH)_3$ carrier containing 400 μg of endotoxin, I. P. once a week for three weeks. The endotoxin was also fed at a level of 100 μg/g of OTD for 28 days.

Group 6. These fish weighed 8.0 g. They received I.P., 0.1 ml of the $Al(OH)_3$ carrier containing 400 μg of endotoxin. The two injections were 16 days apart. The fish were fed plain OTD for 28 days.

Group 7. The fish weighed 3.0 g. They received I.P., 0.1 ml of the $Al(OH)_3$ carrier containing 800 μg of endotoxin. Infections were once a week for three weeks. This group also contained 100 fish which received only two injections. The fish were fed plain OTD for 28 days.

All groups were maintained at 12.2° C.

The actual level of OTD fed to the salmon was dependent upon their size. Groups 3, 4, 5, and 7 were fed OTD at level of 4.4%

of their body weight per day for 21 days. The next seven days the fish were fed as much OTD as they could ingest. Group six was fed OTD for 28 days at a level of 3.3% of their body weight per day. After the 28 day feeding of OTD, OMP was fed to all groups for 30 days. The groups were then transported to Lint Slough for a 30 day exposure to Vibrio anguillarum.

Assay of Fish Mortalities

The mortalities from the fish at Lint Slough were examined to determine if they died from vibrio. Material from the kidney was cultured onto furunculosis agar (Difco) and incubated at 30° C for 48-72 hours. A wet mount and gram stain was then made of suspected vibrio colonies.

RESULTS

Endotoxin Extraction

The extraction of the Boivin antigen from V. anguillarum cells was accomplished by using 0.25 M TCA (Williams and Chase, 1967). The yields of endotoxin from five extractions are listed in Table 1. There was a high and two low yields with two yields occurring in the mid range. From a total wet cell weight of 2,529 grams, only 6.54 grams of lyophilized endotoxin was obtained.

Table 1. Yields of lyophilized Vibrio anguillarum endotoxin extracted with 0.25 M trichloroacetic acid.

Extraction number	Wet cell weight (g)	Weight of endotoxin (g)	% Yield
1	400	1.0	0.25
2	409	1.16	0.28
3[a]	510	2.24	0.44
4[a]	570	0.88	0.15
5[a]	640	1.19	0.19

[a]Used a 10 ml pre-seed broth culture (TYG) with 1.25% NaCl.

Protein Purity

To determine the protein purity of the V. anguillarum endotoxin, polyacrylamide gel electrophoresis at pH 8.9 was employed. A

comparison of the endotoxin extracted by 0.5 M TCA and 0.25 M TCA was made. Upon electrophoresis, the 0.5 M TCA extracted showed four protein bands. One band of protein was evident from the 0.25 M TCA extracted endotoxin (Figure 2). By the method employed, it was observed that the 0.25 M TCA extracted endotoxin exhibited a purer protein content. Thus, 0.25 M TCA was employed in subsequent extractions. The lyophilized endotoxin was stored in the cold and used in further studies. The endotoxin from each extraction was tested for purity by electrophoresis. All extractions showed one band of protein except number 2, which had two bands, a heavy and light band of protein (Figure 3). The polyacrylamide gel electrophoresis was done as an estimate of the protein purity of the endotoxin. For an accurate assessment involving the use of gel electrophoresis, different experimental conditions need to be employed. Buffers of differing ionic strength and pH's will affect resolution of the sample. Thus, many more runs would need to be done in order for a clearer picture of the protein purity of the endotoxin to appear.

Chemical Analysis

Chemical analysis was done in order to identify the constituents of the V. anguillarum endotoxin. From the analysis, it was found that the endotoxin contained approximately 1.0% moisture,

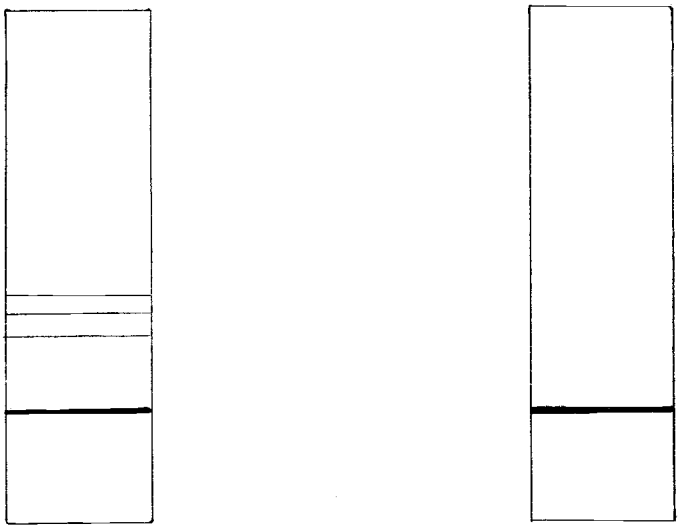


Figure 2. Polyacrylamide gel electrophoresis at pH 8.9: protein patterns of A) 0.5 M TCA extracted endotoxin and B) 0.25 M TCA extracted endotoxin.

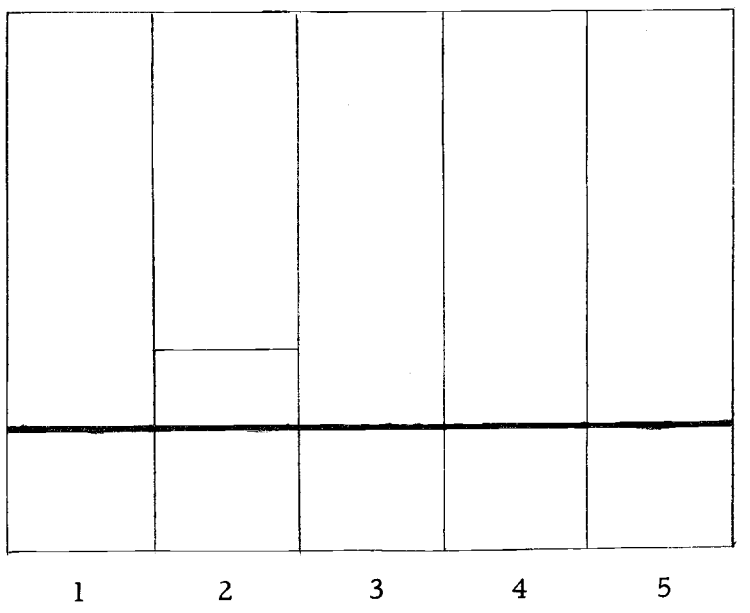


Figure 3. Polyacrylamide gel electrophoresis at pH 8.9: protein patterns of the endotoxin from separate extractions using 0.25 M TCA.

35.1% protein, 39.3% lipid, 18.1% carbohydrate, 1.8% glucosamine and evidence of ethanolamine. This indicated a complex macromolecule. The values obtained are based on the dry weight of the endotoxin.

Moisture

After drying the endotoxin in vacuo over Silica gel crystals at room temperature for three days, a moisture content of 1.0% was determined. This was the average value from three separate determinations (2.0%, 1.0, and 0%).

Protein

Analysis of the protein fraction of the endotoxin was accomplished by the Spinco model 120 B Amino Acid Analyzer. The amino acids present in this protein are summarized in Table 2. Besides the 16 different amino acids, the presence of an amino sugar and ethanolamine were indicated. From the amino acid content, a protein concentration of 35.1% was calculated. The aliphatic amino acids occurred at a level of 41.6%. The two acidic amino acids, aspartic and glutamic acid, occurred in a higher amount (25.8%) than the basic amino acids, lysine, histidine and arginine (14.4%). Tryptophane was destroyed by the hydrolysis method employed.

Table 2. Amino acids[a] present in protein[b] bound Vibrio anguillarum endotoxin.

Amino Acids	No. residues/10^5 g[c] 22 hrs.	70 hrs.	Average values (%)
lysine	62.4	66.4	8.0
histidine	9.1	10.5	1.3
arginine	32.3	35.0	5.1
aspartic acid	128.7	130.8	14.4
threonine	51.9	46.0	5.3[d]
serine	57.5	42.5	5.4[d]
glutamic acid	90.1	92.8	11.4
proline	19.6	21.4	1.9
glycine	96.1	102.6	5.5
alanine	109.0	111.8	7.6
valine	55.4	61.9	5.6
methionine	18.9	19.5	2.4
isoleucine	39.3	43.1	4.5
leucine	69.2	71.7	7.7
tyrosine	42.4	30.4	7.6[d]
phenylalanine	45.2	45.1	6.4

[a]An amino sugar and ethanolamine were detected.

[b]Tryptophane is destroyed by this method.

[c]The accuracy of these values is approximately 5.0%.

[d]Extrapolated to zero hydrolysis time.

Sugars

Sugars were converted to their alditol acetate derivative then analyzed by gas-liquid chromatography. Due to the differing rates of libe ration and destruction when subjected to acid hydrolysis, sugar content was determined after various hydrolysis times. The endotoxin was hydrolyzed in 0.25 N H_2SO_4 at 110° C for 2.5, 5, 18, 20, and 24 hours. Hydrolysis at 125° C for 80 minutes was also done.

Application of the samples to the GLC revealed seven peaks. A typical chromatogram is represented by Figure 4. The peaks were identified by comparing their times of retention with those of known sugars (Table 3).

Table 3. The identification of the unknown peaks revealed by gas-liquid chromatography. A comparison of the retention times with known sugars.[a]

	Unknowns Time (sec.)		Known Time (sec.)		
Peak	Run 1	Run 2	Run 1	Run 2	Sugars
A	357	377	366	380	Fucose
B	577	b	573	b	Arabinose
C	831	914	881	974	Deoxyglucose
D	b	1633	b	1660	Galactose
E	1171	2270	1154	2168	Glucose

[a]In addition, ribose, rhamnose, xylose and mannose were run to aid in the identification of the unknown peaks.

[b]A second determination was not done.

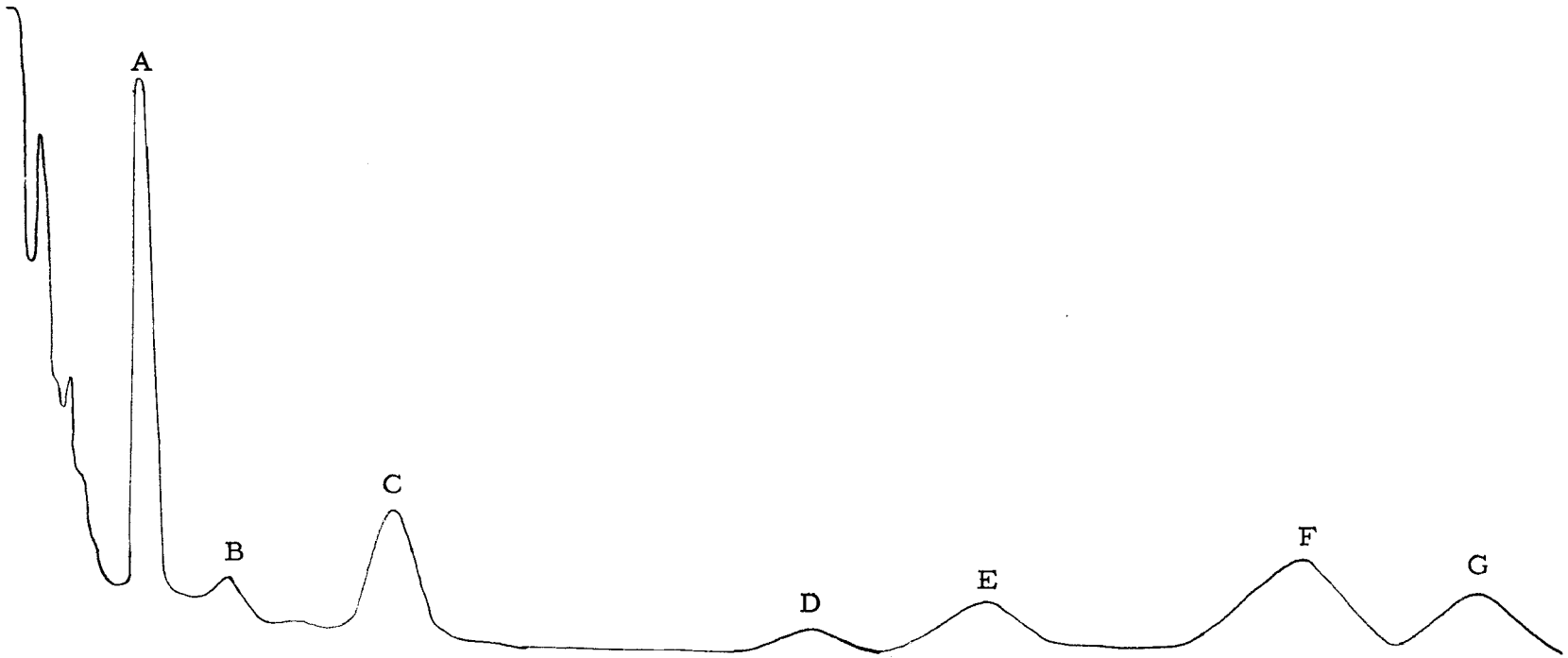


Figure 4. Gas-liquid chromatographic analysis of the sugars from the hydrolyzed endotoxin of Vibrio anguillarum. The peaks were identified as A. fucose B. arabinose C. deoxy-glucose* D. galactose E. glucose F. myoinositol (internal standard) G. unidentifiable sugar.

*Tentative identification.

The identification of the unknown sugars was accomplished from the data presented in Table 3. The unknown peaks were identified as A) fucose, B) arabinose, C) deoxyglucose, D) galactose, E) glucose, and G) unidentifiable sugar (Figure 4). The peak identified as deoxyglucose could be a different sugar. But, with the sugars employed in the identification of the unknown peaks, peak C was concluded to be deoxyglucose. During analysis, it was found that the presence of peak G could only be detected on a relatively new column. If an old column was used, there was no evidence of peak G. Thus, peak G was being eluted from the column in the myoinositol peak (peak F). This may indicate a structure close to myoinositol.

The approximate sugar concentration was determined by calculating the area of the triangle under the peaks (Table 4). The concentration of myoinositol (10 mg) was used as the baseline for calculations of sugar concentration. Fucose content is 7.3%; arabinose, 1.2%; deoxyglucose (tentative identification), 4.2%; galactose, 0.8%; glucose, 2.1%; and the unidentifiable sugar 2.5%. The accuracy of these values is dependent upon the type of sugar, some sugars show a greater variation than others. The concentration of the sugars (grams) may differ from 5 to 10%.

Table 4. Determination[a] of the approximate sugar content of the Vibrio anguillarum endotoxin by gas-liquid chromatography after hydrolysis in 0.25 N H_2SO_4 (110° C).

Sugars	Percentage of the sugars at various hydrolysis times					
	80 min.[b]	2.5 hr.	5 hr.	18 hr.	20 hr.	24 hr.
Fucose	5.8	4.9	5.1	6.6	5.9	7.3[c]
Arabinose	1.1	0.7	1.2[c]	1.2	1.2	0.9
Deoxyglucose[d]	4.2[c]	3.3	3.2	1.1	0.8	1.3
Galactose	0.6	0.4	0.6	0.6	0.8[c]	0.7
Glucose	1.8	1.8	1.6	2.1[c]	1.8	1.8
Unidentifiable	2.5[c]	1.7	1.7	1.7	1.4	2.1

[a]These values are the average from two determinations.

[b]Hydrolyzed at 125° C.

[c]These values are used in calculating the total carbohydrate content and the maximum value of each individual sugar.

[d]Tentative identification.

Lipid

Crude lipid content was determined after hydrolysis in 1.0 N HCl for 15 minutes. Three separate determinations, yielded values of 38, 38, and 42% lipid. The average value is 39.3% with a possible minimum error of 3.0%. The lipid content may be high in that carbohydrates and proteins may still be present.

Amino Sugar

The amino sugar content is expressed as glucosamine. In order to obtain free amino sugars, the endotoxin was hydrolyzed in 6.0 N HCl for 8, 12, 18, and 22 hours at 110° C. Using D-glucosamine-HCl as a standard, the amino sugar content was estimated to be 1.8%. This value was calculated from the data obtained with the eight hour hydrolysate. Two different concentrations of the eight hour hydrolysate were used. The determinations were done in duplicate, yielding values of 1.9, 2.0, 1.6, and 1.7% glucosamine. The 12, 18, and 22 hour hydrolysates yielded values of 1.7, 1.1, and 1.1% glucosamine, respectively.

A summary of the chemical constiutents of the V. anguillarum endotoxin revealed by the various analytical methods is presented in Table 5.

Table 5. Summary of the chemical constituents of the Vibrio anguillarum endotoxin extracted by 0.25 M trichloroacetic acid.

Component	Amount[a] (%)
Protein[b]	35.1
Lipids (crude)	39.3
Sugars (total)	18.1
Fucose	7.3
Arabinose	1.2
Deoxyglucose[c]	4.2
Galactose	2.1
Unidentifiable sugar	2.5
Glucosamine	1.8
Ethanolamine	present

[a]These are approximate values.

[b]Refer to Table 2 for the amino acids present in the protein fraction.

[c]Tentative identification.

Effect of V. anguillarum Endotoxin in vivo

To examine the toxicity of the V. anguillarum endotoxin, mice were employed. In addition, the effect of the endotoxin on the natural host (salmonid fish) was examined. Juvenile chinook salmon were used for this phase of the investigation.

In Mice

In initial experiments it was found that the endotoxin was lethal to mice (male, 12 to 17 g) when injected I. P. By the method of Meynell and Meynell (1970), using mice weighing 15-17 g an LD_{50} value of 794 μg was determined (Table 6). The true LD_{50} value is estimated to occur between 550 μg and 1,146 μg, this being the 95% confidential limit.

Table 6. LD_{50}[a] determination using male mice weighing 15 to 17 g. The Vibrio anguillarum endotoxin was injected I. P.

Dosage (μg)	Number of Animals	Number of Deaths	Number of Survivors
2,000	6	6	0
1,000	6	4	2
500	6	2	4
250	6	0	6
125	6	0	6

[a]The value at which 50% of the experimental animals will die.

LD_{50} = 794 μg.

In Juvenile Chinook Salmon

It was found that the endotoxin when injected I. P. (1,000 μg) into the salmon, did not produce any gross effect. Endotoxin injected I. M. at a level of 1,000 μg and 3,000 μg produced a zone of hemorrhaging at the site of injection (Figures 5 and 6). All fish were held at 64° F.

After about three months storage, the endotoxin lost its hemorrhagic activity. Because of this, a new batch of endotoxin was prepared and injected at a level of 2,000 μg I. M. into juvenile chinook salmon held at 64° F. The fish, examined 24 hours later showed hemorrhaging though to a lesser degree than previously observed.

Immunization of Salmon with *Vibrio anguillarum* Endotoxin

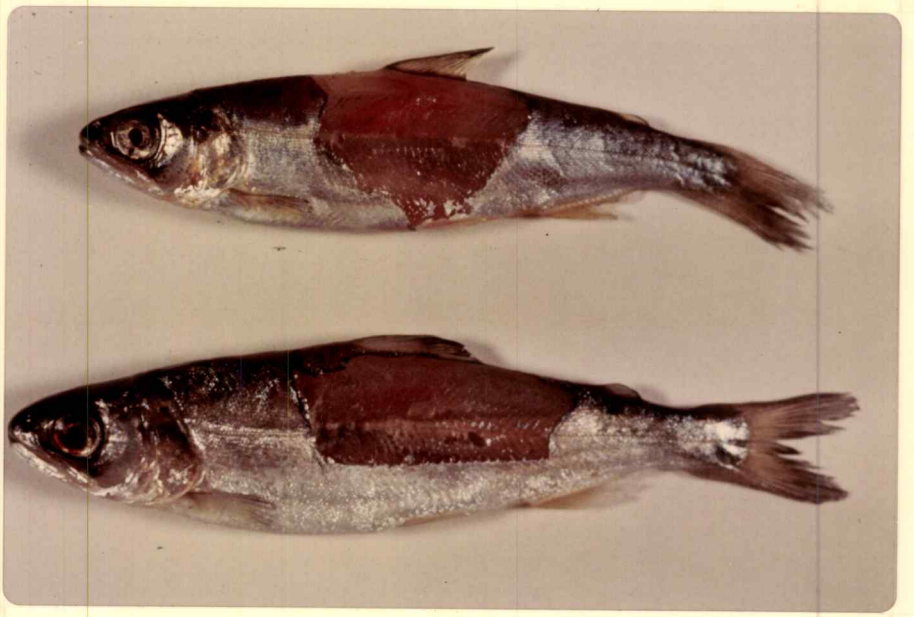
Experiments by this laboratory have shown that protection against vibriosis can be conferred by the use of formalin-killed whole cells or sonicated cells. Because of this success, it was desirable to investigate any role the endotoxin may play in the acquired immunity.

Antibody Production

A small number of fish from each group were bled in order to test for anti-vibrio agglutinating antibodies. The results are

Figure 5. Effect of the Vibrio anguillarum endotoxin injected intramuscularly into juvenile chinook salmon and observed 24 hours later.
Top fish - received 3, 000 μg of endotoxin (0. 1 ml),
Bottom fish - received 0. 1 ml of sterile saline.

Figure 6. Close-up of fish in Figure 5.
Top fish - Experimental
Bottom fish - Control.



summarized in Table 7. It was found that the I. P. injections of the endotoxin into chinook salmon held at 12.2° C, elicited agglutinating anti-vibrio serum antibodies. Fish which were fed the endotoxin or lyophilized sonicated cells produced no detectable agglutinating anti-vibrio serum antibodies. At the end of the exposure period to V. anguillarum at Lint Slough, groups previously showing no agglutinating antibodies, then had low levels of anti-vibrio antibodies. This is probably due to a sub-lethal infection with V. anguillarum.

Protection against Vibriosis

The V. anguillarum endotoxin was able to protect juvenile chinook salmon against vibriosis. The degree of protection conferred by the various methods of vaccinations are summarized in Table 8. The two control groups experienced losses due to vibrio at a 47.4% and 87.4% level. This is in marked contrast to all the vaccinated groups where the highest loss was 13.3%. The group fed the sonicated cells was included as a positive control (immunized) and showed a 6.9% loss. Salmon receiving the endotoxin by an intraperitoneal injection were protected to a much greater extent (approximately 1.0% dead) than fish fed the endotoxin (13.3% dead). The 13.3% fish loss experienced in the endotoxin fed group was probably due to an insufficient immunizing dose. There does not appear to be any appreciable difference in losses between the injected groups

Table 7. Anti-Vibrio anguillarum agglutinating antibody titers^{-1} of juvenile chinook salmon.

Experimental groups	mean weight of fish (g) at the start of the experiment	Agglutinating antibody titers^{-1} at time (weeks)		
		0	7[a]	12[b]
Control	3.0	0, 0, 0, 0, 0.	0, 0, 0, 0, 0.	10, 10, 10, 10, 20.
Control	8.0	0, 0, 0, 0, 0.	c	*
Positive control - fed 2.0 mg sonicated cells/g of OTD	3.0	0, 0, 0, 0, 0.	0, 0, 0, 0.	5, 5, 10, 20
Fed 20 µg endotoxin/g of OTD	3.0	0, 0, 0, 0, 0.	0, 0, 0, 0, 0.	10, 20, 20, 20
Injected 400 µg endotoxin plus fed 100 µg endotoxin/g of OTD	3.0	0, 0, 0, 0, 0.	80, 80, 80, 80, 640	20, 160, 160, 320
Injected 400 µg endotoxin	8.0	0, 0, 0, 0, 0.	20, 40, 40, 80	320, 1280, 10240.
Injected 800 µg endotoxin	3.0	0, 0, 0, 0, 0.	40, 80, 160, 320, 640.	160, 320, 640, 2560, 5120.

[a] One week before exposure to Vibrio anguillarum at Lint Slough.

[b] End of the exposure period at Lint Slough. Antibody titers were from the survivors.

[c] No serum samples were taken.

* All control fish were dead by this time.

Table 8. The degree of protection against vibriosis conferred upon juvenile chinook salmon by various vaccination methods.

Experimental groups[a]	mean weight of fish (g) at the start of the experiment	number of fish	total number of deaths	caused by vibrio number of deaths	caused by vibrio % deaths
Control	3.0	133	123	63	47.4
Control	8.0	95	95	83	87.4
Positive control - fed sonicated lyophilized cells at a level of 2.0 mg/g of OTD	3.0	130	11	9	6.9
Fed the endotoxin at a level of 20 μg/g of OTD	3.0	83	15	11	13.3
Injected the endotoxin at a level of 400 μg. Fed the endotoxin at a level of 100 μg/g of OTD	3.0	100	4	1	1.0
Injected the endotoxin at a level of 400 μg	8.0	151	11	2	1.3
Injected the endotoxin at a level of 800 μg	3.0	234	5	1	0.4

[a]These groups were exposed to Vibrio anguillarum infection for a maximum of 30 days at the saltwater-rearing facilities at Lint Slough, Waldport, Oregon.

(0.4% vs. 1.3% deaths), and the group fed and injected with the endotoxin (1.0% dead). There is the possibility that the endotoxin may be a better immunizing antigen than the sonicated cells. The endotoxin had been incorporated into the salmonid diet at a level which was 100 times less than that of the sonicated cells. And yet, these fish experienced a loss approximately double that of the sonicated cells (13.3% vs. 6.9% deaths). This may be due to the concentrated form of the endotoxin as opposed to the diluted state of the sonicated cells.

Thus, in addition to eliciting a specific antibody response, the endotoxin was capable of protecting against vibriosis.

DISCUSSION

It was found that the endotoxin could be extracted from Vibrio anguillarum by the use of trichloroacetic acid (TCA). The endotoxin was then examined for possible biological effects, its chemical constituents, and its role in the immunity against vibriosis.

The lethality of the endotoxin to mice and its non-toxicity in salmonids (I. P.) was expected. W. E. Paterson (1972) had injected the Boivin antigen from Aeromonas salmonicida (causative agent of furunculosis in fish) into fish (I. P. and I. M.) at levels up to 5,000 μg without observing any effects. Wedemeyer, Ross and Smith (1969) found that coho salmon and rainbow trout were highly resistant to the LPS from E. coli and A. salmonicida. They observed no effects when the LPS was given intravenously or subcutaneously up to levels of 80 μg/kg of body weight. Berezi, Bertok and Bereznai (1966) found that the LPS from E. coli had no effect in fish when injected I. P. In the mammalian species, areas of edema and erythema can be produced upon subcutaneous injection of the endotoxin, but the hemorrhaging in the salmonid was unexpected. Also unexpected was the loss of this hemorrhagic ability of the endotoxin after prolonged storage in the cold. This would seem to indicate the presence of a labile substance, which may be an endotoxin bound enzyme. There is the possibility that a TCA soluble, alcohol precipitated

enzyme is responsible for this action.

In light of the polyacrylamide gel electrophoresis results, the use of 0.25 M instead of 0.5 M TCA for endotoxin extraction was preferable. A purer protein content was shown by the 0.25 M TCA extracted endotoxin.

Analysis of the protein by the amino analyzer yielded 16 different amino acids and the presence of an amino sugar and ethanolamine. The calculated protein content of 35.1% is in close agreement with the two values (35% and 38%) reported by Jenkin and Rowley (1959) for their Boivin antigen from cholera vibrios. Neoh and Rowley (1970) have indicated a Boivin antigen from the Inaba strain of cholera containing 20.3% protein.

The protein obtained in this study showed an aliphatic amino acid content of 41.6%. The acidic amino acids, glutamic and aspartic acid, occurred at a higher level than the basic amino acids, lysine, histidine, and arginine (25.8% vs. 14.4%). Linton (1940) noted that cholera vibrios had a relatively high content of simple amino acids and a low content of basic amino acids. Jackson and Redmond (1970) found the major amino acids of their protein (V. cholerae-Inaba) to be glycine and methionine. Homma and Suzuki (1966) found that an endotoxin protein prepared from an autolysate of Pseudomonas aeruginosa contained 12.0% glutamic acid and 9.9% aspartic acid. Thus, it appears that there is some similarity

between various endotoxins.

The carbohydrate content (18.1%) of the V. anguillarum endotoxin is similar to that reported (18.7%) by Neoh and Rowley (1970) for their Boivin antigen (V. cholerae-Inaba). From the gas-liquid chromatography results in this work, fucose is the predominant sugar of the polysaccharide. Prior to this study, deoxyglucose had not been mentioned as a constituent of vibrio polysaccharides. There is the possibility that the peak identified as deoxyglucose is in fact another sugar. This is in view of the 50 to 60 second time difference between the unknown peak and the known peak. The GLC also showed the presence of an unidentifiable sugar which was eluted from the column after the internal standard, myoinositol. It is possible that the sugar could be a heptose or an unusual six-carbon sugar. The sugars identified in this study except for deoxyglucose, have been reported to occur in the polysaccharide fractions of other vibrios (Linton and Mirta, 1936; Shrivastava, 1965; Watanabe and Verwey, 1965; Jackson and Redmond, 1971). The presence of 2-keto-3-deoxyoctonic acid (KDO) and the dideoxyhexoses have not been reported to occur in vibrio polysaccharides. Though a heptose in V. fetus has been reported. Jackson and Redmond (1971) postulated that the vibrio LPS structure is fundamentally different from that of the Salmonella sp. where the KDO is responsible for linking the polysaccharide and Lipid A (a water insoluble, chloroform soluble

fraction resulting from the hydrolytic degradation of endotoxin). They also noted the presence of ethanolamine (about 3.0%) in their LPS and that it had a possible structural function.

From the results of the immunization of the juvenile chinook salmon, it is shown that the groups fed the endotoxin or whole cells had no detectable serum agglutinating antibodies. And yet, these groups were protected against infection. Due to its smaller size, it was thought that perhaps the endotoxin could penetrate the gut wall and elicit serum antibodies, but this was not the case. Since no humoral antibodies were present in the above mentioned groups, it appears that protection occurs at a cellular level or from secretory antibodies.

The endotoxin when injected, conferred a higher degree of protection than the group fed the endotoxin (approximately 1.0% vs. 13.3% deaths). This is due to the small amount of endotoxin per gram of food, which perhaps resulted in a non-uniform dosage. In the injected fish there was no appreciable difference in the losses experienced by the group receiving the 400 μg or 800 μg injections of the endotoxin (1.3% vs. 0.4% deaths) or the group receiving 400 μg by injection plus 100 μg of endotoxin per gram of food (1.0%).

The fact that the endotoxin caused specific antibody formation and protected against vibriosis, leads one to think that it is the endotoxin or some component of it which is the actual "protective"

antigen. That the fish anti-V. anguillarum endotoxin serum could agglutinate V. anguillarum cells indicates that the endotoxin may be synonymous with the somatic antigen (as with the Salmonella sp. and Shigella sp.) or involved in some critical site on the bacterium.

The prospect of using the endotoxin to immunize salmonids is very remote. The cost of extracting the endotoxin would be most prohibitive. In contrast, the cost of producing the formalin-killed cells is low. Plus, the formalin-killed cells produce a high degree of protection against vibriosis.

The results obtained in this study present the following stimulating problems worthy of investigation:

1) the determination of the V. anguillarum endotoxin structure,
2) determining if the protein of the endotoxin is involved with group specificity and the polysaccharide with strain specificity as with V. cholerae (Neoh and Rowley, 1970),
3) determining whether there is a difference between pathogenic and non-pathogenic V. anguillarum in their sugar constituents as with the Salmonella sp. (Roantree, 1971),
4) determining if a particular sugar of the endotoxin may serve as the determinant for the immunological specificity of the bacterium.

SUMMARY AND CONCLUSIONS

1. The use of 0.25 M trichloroacetic acid extracted the Boivin antigen complex.
2. Chemical analysis of the endotoxin revealed the approximate concentration of its constituents. The endotoxin consisted of 35.1% protein, 39.3% lipid, 18.1% carbohydrate, 1.8% glucosamine, and ethanolamine.
3. The protein fraction contained 16 different amino acids. The two acidic amino acids, glutamic and aspartic were the predominant amino acids.
4. The carbohydrate fraction contained fucose (7.3%), arabinose (1.2%), deoxyglucose (tentative identification) (4.2%), galactose (0.8%), glucose (2.1%), and an unidentifiable sugar (2.5%).
5. The endotoxin was lethal for white male mice weighing 15 to 17 g. The LD_{50} value was 794 μg with a 95% confidence limit of 550 μg to 1,146 μg.
6. The endotoxin causes hemorrhaging at the site of injection when injected I.M. into juvenile chinook salmon but caused no observed gross effect when injected I.P.
7. The hemorrhagic ability of the endotoxin is lost after prolonged storage.
8. Intraperitoneal injections of the V. anguillarum endotoxin into

juvenile chinook salmon caused the formation of humoral anti-V. anguillarum agglutinating antibodies. It was also found that a high degree of protection against vibriosis was obtained.

9. The feeding of the endotoxin or a whole cell vaccine to juvenile chinook salmon did not elicit the formation of detectable levels of humoral anti-V. anguillarum agglutinating antibodies. But these fish were protected against vibriosis.

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