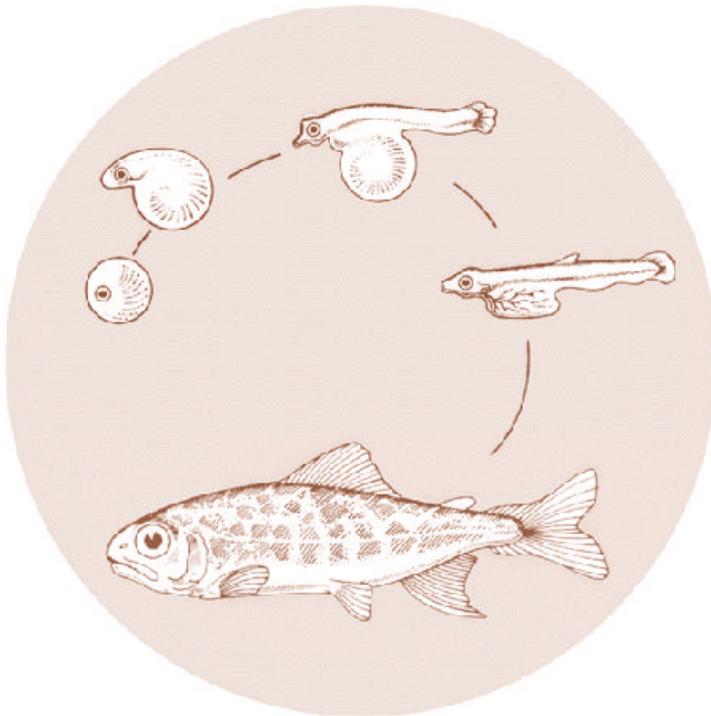


September 1985

EFFECTS OF VITAMIN NUTRITION ON THE IMMUNE RESPONSE OF HATCHERY-REARED SALMONIDS

Annual Report 1984



DOE/BP-18007-1



This report was funded by the Bonneville Power Administration (BPA), U.S. Department of Energy, as part of BPA's program to protect, mitigate, and enhance fish and wildlife affected by the development and operation of hydroelectric facilities on the Columbia River and its tributaries. The views of this report are the author's and do not necessarily represent the views of BPA.

This document should be cited as follows:

Leith, Dave, John Holmes - Abernathy Salmon Culture Technology Center, Effects Of Vitamin Nutrition On The Immune Response Of Hatchery-Reared Salmonids, Annual Report 1984, Report to Bonneville Power Administration, Contract No. 84BP18007, 1984BP16480, Project No. 198404500, 68 electronic pages (BPA Report DOE/BP-18007-1)

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ANNUAL REPORT

EFFECTS OF VITAMIN NUTRITION ON THE IMMUNE RESPONSE

OF

HATCHERY-REARED SALMONIDS

Project No. 84-45 A and B
Agreement No. DE-AI79-84BP18007

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EXECUTIVE SUMMARY

The Abernathy Salmon Culture Technology Center (A.S.C.T.C.) and the Department of Microbiology at Oregon State University, with funding from the Bonneville Power Administration, are presently conducting a study on the effects of vitamin nutrition on immunity and disease resistance in chinook salmon (Oncorhynchus tshawytscha). It is the long range goal of this project to define the dietary levels of pyridoxine, **folic** acid, pantothenic acid, riboflavin, and vitamin E required to assume maximum resistance to disease.

In the first year of this study we are 1) developing and optimizing a battery of immunological assays for the assessment of immunity in spring chinook salmon, 2) determining the effects of various pyridoxine levels on growth rate and food conversion, and exploring briefly, the means by which a purified diet may be modified to approximate the growth efficiency of a practical diet.

Results demonstrate that immunological functions of chinook salmon can be assessed quantitatively by standard immunological assays. Lymphocytes from both of the major lymphoid organs (spleen and anterior kidney) produce significant *in vitro* antibody responses to the antigen, **trinitrophenyl-lipopolysaccharide**. These cells also demonstrate significant mitogenic stimulation (proliferation) in response to bacterial lipopolysaccharide, phytohemagglutinin, and to a novel mitogen, Vibrio anguillarum extract. As an assessment of cell-mediated immunity, we have found that lymphocytes are capable of responding in a mixed lymphocyte reaction as demonstrated by increased incorporation of tritiated thymidine. Results also indicate that phagocytosis can be quantified by the uptake of radioiodinated Renibacterium

salmoninarum and that production of migration inhibition factor (MIF)-like activity can be induced in immunized animals. Polyclonal activation of chinook lymphocytes was elicited by both Vibrio anguillarum extract and E. coli lipopolysaccharide.

Groups of spring chinook **salmon** are currently being fed a practical, open formula diet (**Abernathy** Formula) containing five dietary levels of pyridoxine (15, 30, 60, 120, and 1500 mg/kg diet), The purpose of this feeding trial is to determine the crucial range in the dietary level of vitamin that will generate significant changes in the salmon's immune response and resistance to disease. Data collected on growth and feed efficiency in the first eighteen weeks show no significant difference between these formulations. Assays will be performed on these animals to determine if the *in vivo* and *in vitro* parameters of **humoral** immunity, cellular immunity, phagocytic activity, and disease resistance have been affected by these different formulations.

To provide a diet that can be replicated and have minimal compositional variability, a purified diet (Oregon Test Diet) is utilized in this study. In an exploratory study designed to improve the growth efficiency of the Oregon Test Diet, two variations were formulated. In one formulation, the diet was supplemented with six essential amino acids (L-arginine, L-histidine, L-lysine HCl, L-methionine, L-theonine, and L-tryptophan). In the second formulation glutamic acid and glutamic acid hydrochloride was substituted for the amino acid supplement described in the first formulation. After fourteen weeks of feeding, both diets approached the **growth** rate of the practical (Abernathy) diet and surpassed the practical diet in feed efficiency.

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INTRODUCTION

Infectious diseases are one of the most important problems affecting the efficiency and economics of **salmonid** culture. Total mortality experienced during the egg, fry, and fingerling stages of Pacific salmon (Onchorhynchus spp.) culture, for example, normally ranges from 15-20% (Hublo and Jones, 1970). About 50% of that mortality occurs during the fry and fingerling phases, and most of those deaths are probably attributable to infectious disease. This results in the loss of many millions of fish each year. Additionally, mortality can significantly exceed normal ranges in the event of epizootics resulting in even larger losses during artificial propagation.

Diseases can also produce debilitated fish or survivors which harbor latent infections. Low-grade or latent infections have the potential for significantly reducing the post-release survival of smolts from hatcheries. For example, Sanders (1979) and Banner et al. (1983) have found that coho salmon (O. kisutch) fingerlings, suffering a low-grade infection of bacterial kidney disease (R. salmoninarum), died at high rates after transfer to seawater. Also, Wedemeyer et al. (1976) reported similar effects when coho smolts had subclinical cases of furunculosis (Aeromonas salmonicida).

Considerable research has been done to better understand fish diseases and develop means for their prevention and treatment. That work has concentrated primarily upon identification of agents causing disorders, improving diagnostic methods, exploring chemotherapeutic treatments, and developing vaccines. Very little work, however, has been done to determine the relationship between the nutritional

state, immunocompetence, and disease resistance in fish (Bell et al., 1984; Blazer and Wolke, 1984; Hardy, 1979; Durve and Lovell, 1982).

Research with livestock, laboratory animals, and humans has provided a large body of information which demonstrates the importance of host nutrition in combating disease. Observations made by pathologists at hatcheries indicate this is also true for fish. For example, Wood (1974) reports case histories in which juvenile **coho** salmon fed either a dry diet or a moist pellet containing corn gluten meal suffered higher mortality from bacterial kidney disease than did fish receiving the standard moist pellet formula. **Wedemeyer** and Ross (1973) could not confirm that corn gluten meal increased kidney disease mortality, but fish fed the corn product exhibited a more severe, nonspecific, stress-response to the infection.

Many vitamins have significant roles in the functioning of immune systems in man and other animals. Several researchers and reviewers (Axelrod and Traketelelis, 1964; Beisel, 1982; Blalock et al., 1984; Cunningham-Rundles, 1982; Debes and Kirksey, 1979; Scott et al., 1976; **Wilgus**, 1977) have reported that deficiency of pyridoxine suppresses the immune response in a number of animals. The requirement is dependent, in part, on the amount of dietary protein (Scott et al., 1976). Hardy et al. (1979) found that increased pyridoxine in a high-protein diet rendered chinoolc salmon fingerlings more resistant to **Vibrio anguillarum**, but additional pyridoxine in a low-protein feed did not have the same effect. Cell mediated responses have been demonstrated to be dramatically affected by reduced pyridoxine, as evidenced by depressed mixed lymphocyte reactivity, prolonged allograft survival, and reduced skin hypersensitivity (Axelrod and

Traketellis, 1964). **Numbers** of blood lymphocytes are dramatically reduced, as well as the weight and size of the spleen and thymus during pyridoxine deficiency (Debes and Kirksey, 1979). Antibody formation is decreased as determined by bacterial agglutination and plaque forming cell responses in rats, swine, chickens and humans fed diets deficient in pantothenic acid (Beisel, 1982; Nelson, 1978; Panda and Combs, 1963; Scott et al., 1976), however, that vitamin apparently has little effect on cell mediated immunity. Insufficient amounts of dietary riboflavin have also caused decreased antibody responses to S. pullorum chicks (Beisel, 1982). Deficiencies of **folic** acid lead to reduced host resistance to salmonella and impaired **humoral** and cellular immune function in both man and experimental animals (Beisel, 1982; Cunningham-Rundles, 1982; Scott et al., 1976; Siddons, 1978). Impaired functioning of phagocytes and reduced response to phytohemagglutinin have also been observed in vitamin B₁₂ deficiency states. Inadequate biotin intake in rats causes a reduced hemagglutinating antibody response to diphtheria toxoid, and reduced development of splenic plaque-forming cells after inoculation with sheep red blood cells (Beisel, 1982;). The amount of ascorbic acid in the feed has reportedly influenced disease susceptibility in several animals (Chatterjee, 1978). Durve and Lovell (1982) found that channel catfish (Ictalurus punctatus) were more resistant to the bacterium Edwardseilla tarda when fed elevated doses of vitamin C and the effect was more pronounced at lower water temperatures. On the other hand, Bell et al. (1984) had equivocal results when studying the effects of dietary ascorbate on the development of bacterial kidney disease in sockeye salmon (*O. nerka*). The role(s) performed by this vitamin are still unclear and controversial, however indications are that it may play an important

role in the normal functioning of phagocytic cells (Beisel, 1982; Cunningham-Rundles, 1982). Two fat soluble vitamins, A and E, have recognized effects on immune systems of other animals. Vitamin A probably influences resistance to infections through its role in maintaining the integrity of the epithelial and mucosal membranes as well as affecting **humoral** and cell mediated immune responses (Beisel, 1982; Pandal and Combs, 1963). Vitamin E has been shown to improve the **humoral** immune responses of mice, chicks, turkeys, swine, sheep, and guinea pigs when challenged with either nonliving antigens, living bacteria, or live viruses (Beisel, 1982; Colnago et al., 1984; Ellis and Vorhies, 1976; Heinseling et al., 1974; **Nockels**, 1980). Blazer and **Wolke** (1984) found that rainbow trout (Salmo gairdneri) showed a significantly reduced **humoral** immune response and reduction of several non-specific resistance factors when fed diets deficient in Vitamin E. Since Vitamin E is an antioxidant which prevents autooxidation of lipids and/or protects the animal from toxic effects of oxidation products (Lee and Sinnhuber, 1972), the amount of lipid in the diet and its degree of rancidity could be important factors in the relationship between this vitamin and immunity.

Past research to define the quantitative vitamin needs of juvenile salmonids has concentrated primarily on determining requirements for maximum growth and most efficient feed utilization (Halver, 1972; National Academy of Sciences, 1973) without attempts to assess the immunocompetency of test animals. Work is needed to determine the quantities of key vitamins required to insure optimal functioning of immune systems and high resistance to common diseases.

EXPERIMENTAL DESIGN

The vitamins chosen for study were pyridoxine, pantothenic acid, riboflavin, **folic** acid, Vitamin E, and ascorbic acid. A practical fish feed formulation and a purified test diet are to be compounded to contain incremental amounts of the test vitamins. Each vitamin will be studied singly while dietary levels of all other vitamins are held constant, using the supplementation rates specified in the vitamin packages for each diet. The test diets are fed for twenty weeks to duplicate lots of spring chinook juveniles held indoors in circular tanks supplied with well water. During rearing, each entire lot of fish is weighed biweekly and dead fish are removed and recorded daily to: (1) determine growth rates, (2) calculate food conversion efficiencies, (3) provide population weights on which to base feeding rates, and (4) determine survival rates. Samples of test feeds and fish organs will be analyzed regularly to verify dietary concentrations and tissue stores of vitamins. Laboratory assays are designed to assess the degree of immune competence in fish from each diet group at regular intervals. The assays will be used to examine *in vivo* and *in vitro* parameters of **humoral** immunity, cellular immunity, phagocytic activity, and disease resistance. Data concerning growth, food conversion efficiencies, rearing mortality, times to death in disease challenges, and *in vitro* assays of immune responsiveness are tested by analyses of variance, regression techniques, and ranking to determine significance of differences (5% level of significance) and to calculate minimum amount of each vitamin required in the diet to insure maximum functioning of the immune system.

MATERIALS AND METHODS

Facilities. The feeding and rearing phases of the experiments were performed at the Abernathy Salmon Culture Technology Center (SCTC), Longview, WA. Immunological assays were performed on chinook salmon obtained from Abernathy and **Marion** Forks Hatchery and raised at the Oregon State University Fish Disease Laboratory (OSUFDL), Corvallis, OR.

During rearing, the fish were held in **1200-liter**, steel, circular tanks furnished with constant temperature (12°C) well water. During optimization of the immunological assays, fish were housed in 460 liter circular fiberglass tanks supplied with 12°C well water. The compositions of the two water sources are given in Table 1.

The amount of water flowing into each **1200-liter** rearing tank was maintained at 12 liters per minute throughout the studies. The 460 liter tanks used to hold fish during disease challenges received flows of 19 liters per minute. Fish population densities (kg/inflow/min and kg/m^3 of space) never exceeded the guidelines of Banks et al. (1979).

Illumination of the experimental tanks was provided from fluorescent lights controlled by a photocell system to simulate the natural photoperiod at the latitude of the Abernathy SCTC.

Animals. Spring chinook salmon were used as test animals in all experiments. They were obtained as eyed eggs resulting from adult fish spawned at Carson National Fish Hatchery located about 15 miles north of Carson, WA on the Wind River. Eggs were transferred to the Abernathy Center, cushioned in moist burlap bags, supported in 40 cm x 40 cm x 25 cm egg baskets in order to prevent physical trauma. Upon arrival at the Abernathy S.C.T.C.,

Table 1. Composition of water supplies at Abernathy Salmon Culture Technology Center and Oregon State University, used in 1985

Measurement	Unit	Water supply	
		Fish rearing ^{1/}	Immunological Testing ^{2/}
Total gas saturation	% ^{3/}	100	100
N ₂ saturation	%	99	101-103
O ₂ saturation	%	89	91
pH	umhos	7.7	7.4 ^{4/}
Conductivity@ 25°C)	mg/l	244	--
Alkalinity, total(as CaCO ₃)	mg/l	76	--
Hardness, total	mg/l	90	--
Phosphate, ortho (as PO ₄)	mg/l	0.023	--
Solids, dissolved (@ 105°C)	mg/l	180	154
Solids, suspended (@ 105°C)	mg/l	1.2	< 1
Ammonia, total (as NH ₃ -N)	mg/l	<0.02	<.002
Nitrate (as NO ₃ -N)	mg/l	0.33	.97
Nitrite (as NO ₂ -N)	mg/l	<0.002	<.001
Carbon dioxide ²	mg/l	3.8	--
Chloride	mg/l	14	22
Cadmium	mg/l	<0.0003	--
Calcium	mg/l	18	17
Cobalt	mg/l	<0.13	<1.0
Copper	mg/l	<0.001	.0025
Iron	mg/l	0.17	.016
Lead	mg/l	0.005	.16
Magnesium	mg/l	11	8
Manganese	mg/l	0.10	.01
Molybdenum	mg/l	<0.16	<.025
Potassium	mg/l	2.8	1.5
Sodium	mg/l	15	8.8
Zinc	mg/l	0.009	.0015
Sulfate	mg/l	19	--
Turbidity	JTU	11	--

1/ Abernathy Salmon Culture Technologu Center well

2/ Oregon State University well

3/ per cent of barometric pressure

4/ data not available

the eggs were surface disinfected with an iodophor (Argentine, Argent Chemical Laboratories, Redmond, WA) according to the protocol recommended by Wood (1974). The eggs were then placed in incubator trays (Heath Techna Corp.) supplied with well water. After hatching and yolk absorption, the resulting fry were stocked in a 1200-liter, steel, circular tanks furnished with well water at 40 liters/minute. They were then fed ad libitum an Abernathy Diet (Fowler and Burrows, 1971) starter granule formulation (Table 2) until they reached an average size of about 4.5 grams or larger. Random distribution of the fish into each diet group and rearing tank was performed by the following procedure. Groups of ten fish were hand counted into separate baskets until a total of 300 fish per basket was reached. Each basket was then assigned to a diet (Each diet used in the study, and its replicate, was written on a separate piece of paper. All the pieces of paper were then placed into a container, mixed, and removed one at a time and assigned to a basket). Each basket was then assigned to a rearing tank using the same procedure.

Adult New Zealand white female rabbits and BALB/c female mice were maintained by the Laboratory Animal Resource Center on the O.S.U. campus in accordance with the "Guide for the Care and Use of Laboratory Animals" DHEW Publication No. 78.22.

Diets and Feeding. Pyridoxine was incorporated at five levels in a variation of the Abernathy Diet (Table 3). The base vitamin level in the Abernathy Diet was the 2.5 mg pyridoxine / Kg diet. Levels of 15, 30, 60, 120 and 1500 mg Pyridoxine/kg of Abernathy Diet were used. The complete diet was assayed after formulation to verify vitamin concentrations. This analysis determined the following (actual) vitamin concentrations for each of the five groups; 16, 26.7, 60.9, 127.5, and 905.3 mg / Kg diet. All

Table 2. Composition of Abernathy Diet starter granules used in the immunological study at Abernathy Salmon Culture Technology Center, 1985

Component	Percent
Herring meal, minimum protein 70%	58.00
Dried whey, minimum protein 12%	10.00
Blood flour, spray dried, minimum protein 80%	10.00
Fish solubles, condensed, minimum protein 30%	3.00
Wheat standard middlings, minimum protein 15%	2.72
Vitamin premix ¹	1.50
Choline chloride, 60% product	0.58
Ascorbic acid	0.10
Trace mineral premix ²	0.10
Lignin sulphonate binder	2.00
Tuna oil, stabilized with 0.04% BHA-BHT	<u>12.00</u>
	100.00

¹Mg/kg of diet unless otherwise indicated: riboflavin, 53; niacin, 220; folic acid, 12.7; thiamine, 43; biotin, 0.60; B-12, 0.06; vitamin K, 9; inositol, 132; d-pantothenic acid, 106; pyridoxine, 31; vitamin E, 503 IU; vitamin D₃, 441 IU; and vitamin A, 6614 IU.

²Mg/kg of diet: zinc, 75.0; manganese, 20.1; copper, 1.54; and iodine, 10.0. Mineral sources were zinc sulphate, manganese sulfate, copper sulphate, and potassium iodate.

Table 3. Composition of Abernathy Diet used as a vitamin test ration for spring chinook salmon (Oncorhynchus tshawytscha) fingerlings

Component	Percent
Herring meal, minimum protein 67.5%	40.57
Dried whey, minimum protein 12%	5.00
Wheat germ meal, minimum protein 23%	5.00
Wheat standard middlings, minimum protein 15%	26.65
Blood meal, spray dried, minimum protein 80%	10.00
Vitamin premix ¹	1.50
Ascorbic acid	0.10
Choline chloride, 60% product	0.58
Trace mineral premix ²	0.10
Herring oil, stabilized with 0.04% BHA-BHT (1:1)	<u>10.50</u>
	100.00

¹Mg/kg of diet unless otherwise indicated: riboflavin, 53; niacin, 220; folic acid, 12.7; thiamine, 43; biotin, 0.60; D-12, 0.06; vitamin K, 9; inositol, 132; d-pantothenic acid, 106; pyridoxine, 31; vitamin E, 503 IU; vitamin D₃, 441 IU; and vitamin A, 6614 IU.

²Mg/kg of diet: zinc, 75.0; manganese, 20.1; copper, 1.54; and iodine, 10.0. Mineral sources were zinc sulfate, manganese sulfate, copper sulfate, and potassium iodate.

vitamin assays were performed by **Hazelton** Laboratories America, Inc., Madison, Wisconsin.

Meal ingredients for the Abernathy Diet were ground through a hammer mill until they passed entirely through a 20 mesh screen. Components were blended in a paddle mixer and pelletized through a small compaction-type pellet mill without steam conditioning. Sufficient feed for 90 days was prepared at one time and stored at room temperature (22°C) until fed. The dry Oregon Test Diet (National Academy of Sciences, 1973) ingredients (Table 4) and water were blended in a dough mixer and then extruded through the plate of a laboratory grinder to form strings which were cut manually to produce the desired pellet length. The feed was then frozen (-25°C) in airtight containers until used. Fresh feed was prepared at 3 week intervals. Daily allotments of the purified diet were thawed at 4°C before feeding and held at that temperature between feedings. The particle sizes for types of diets were chosen according to the guidelines in Table 5. The approximate composition of completed feeds will be determined at the Abernathy SCTC during FY 1986 using methods described by Horwitz (1930).

Daily feed allotments for all feeds were based upon a uniform **weight** of dry food per unit weight of live fish computed by the methods of Buterbaugh and Willoughby (1967). Feeding was done by hand with frequencies ranging from hourly, when the fish were small, to two feedings per day for yearling fish.

Growth and Feed Efficiency. At biweekly intervals during the rearing phases of tests, each lot of fish was weighed to the nearest gram in a water-filled container. Dead fish were removed from tanks daily and data on their weight and numbers recorded.

Computation of food conversion of specific growth rates (Mahnken et al.,

Table 4. Composition of two modifications to the Oregon Test Diet formula (National Academy of Sciences, 1973). Immunological study, Abernathy Salmon Culture Technology Center, 1985.

Ingredients	<u>Diet Number</u>	
	1	2
Vitamin-free casein	40.9	40.9
Gelatin	7.2	7.2
Dextrin	15.6	15.6
Carboxy methyl cellulose	1.3	1.3
alpha-cellulose	13.229	13.299
alpha-tocopherol (250 IU/g)	0.264	0.264
choline chloride (99%)	0.707	0.707
Mineral mix ^a	4.0	4.0
Vitamin premix ^b	2.0	2.0
Herring oil	10.0	10.0
L-Arginine	0.95	-
L-Histidine	0.38	-
L-Lysine HCl	1.35	-
L-Methionine	1.06	-
L-Threonine	0.77	-
L-Tryptophan	0.26	-
L-Glutamic acid	-	3.42
L-Glutamic acid HCl	-	1.38

a, b are identical in composition to those described by the National Academy of Science (1973)

Final diet composition: 35% dry ingredients
65% water

Table 5. Guideline for dry feed particle sizes in relation to fish size used in immunological study involving spring chinook salmon (Oncorhynchus tshawytscha) fingerlings at Abernathy Salmon Culture Technology Center, 1985.

Feed Particle Size	Fish Size Range (grams)
2/64-inch (0.79 mm) granules	0.6 to 1.0
3/64-inch (1.19 mm) granules	1.0 to 2.5
4/64-inch (1.59 mm) granules	2.5 to 5.0
6/64-inch (2.38 mm) granules	5.0 to 6.0
6/64-inch (2.38 mm) pellets ²	6.0 to 10.0
8/64-inch (3.18 mm) pellets	10.0 to 25.0
12/64-inch (4.76 mm) pellets	25.0 to larger

¹Asymmetrical crumbles.

²Cylinders which have the same length as diameter.

1980), and gross feed efficiencies (Brett et al., 1969) as follows:

Specific growth rate, SGR:

$$\text{SGR} = \frac{\log, \text{ of weight at end} - \log, \text{ of weight at start}}{\text{days fed}} \times 100$$

Gross feed efficiency, GFE:

$$\text{GFE} = \frac{\text{total fish weight gain in grams}}{\text{food offered in grams}} \times 100$$

Culture media: Media components were purchased from Whittaker M. A. Bioproducts, Walkersville, MD, unless otherwise noted. These media were selected after testing two media and several added components. Data from pertinent tests are given in Appendix I. Mishell-Dutton holding medium (holding medium) consisted of 100 ug/ml gentamicin and 10% fetal calf serum in RPMI 1640 (Gibco). Mishell-Dutton modified RPMI (RPMI MDM) was used for tissue culture and consisted of RPMI supplemented with: non-essential amino acids, sodium pyruvate, L-glutamine, 10% fetal calf serum (hybridoma screened), 100 ug/ml gentamicin, 50 uM 2-mercaptoethanol (MCE, Cincinnati, OH), and the nucleosides, adenosine, uracil, cytosine, and guanine (10 ug/ml, Sigma, St. Louis, MO). The nutritional supplement was also prepared as previously described (Tittle and Rittenberg, 1978) and fed daily to the cultures as described below.

Cell Cultures: Sacrificed animals were transported to Oregon State University on ice, at which time the spleens and anterior kidneys

were removed and prepared for culture. A single cell suspension of each organ was obtained by aspiration through a 1 ml syringe after which holding medium was added. Organs from several fish were pooled when necessary to obtain the required number of cells for culture. The resulting cell suspension was incubated on ice to allow organ fragments to settle. The supernatant medium, containing a single cell suspension, was then washed two times in holding medium by centrifugation at 500 x g for 10 minutes at 4°C. The final washed cell pellet was resuspended in RPMI MDM. Lymphocytes were enumerated by the use of a hemocytometer or Coulter counter (Coulter Electronics, Hialeah, FL) adjusted for counting salmonid leukocytes. The cell suspension was then adjusted with RPMI MDM to a concentration of 1×10^7 cells/ml and held on ice until culture. Fifty μ l aliquots of the final cell suspension were added to the wells of a 96-well, flat-bottomed, tissue culture plate (Corning, Corning, NY) containing antigen or mitogen. Tissue culture plates were then incubated in plastic culture boxes (C.B.S. Scientific, Del Mar, CA) in an atmosphere of 10% CO₂ at 16°C. The cultures used for PFC assays were maintained by adding 10 μ l of nutritional supplement daily.

Mitogens and Antigens: A stock solution of lipopolysaccharide B from E. coli 055:B5 (Difco, Detroit, MI) was pasteurized for 30 minutes at 70°C in distilled water. Stock solutions of phytohemagglutinin P (PHA, Sigma), pokeweed mitogen (PWM, Sigma) and Concanavalin A Type III (Con A, Sigma) were made up in RPMI MDM and sterilized by filtration through a 0.45 μ m filter. Triphenylated-LPS (TNP-LPS) was prepared by the method of Jacobs and Morrison (1975). All mitogens and antigens were diluted into tissue culture medium at two times the final desired concentration.

Vibrio anguillarum extract. The Vibrio extract was prepared from V. anguillarum strain SL-174 which had been **formalin** killed and stored frozen. Fifty mls thawed packed cells were suspended in 10 volumes of 2% saline and placed in a boiling water bath for 2 hours. Cells were washed 3 times in 2% saline, centrifuged at 10,000 x g for 10 min at 4°C, resuspended in 95% ethanol, and incubated 48 hours at 37°C. The cells were then washed 2 times in acetone, centrifuging at 3,000 x g for 10 minutes and dried to a paste overnight at 37°C. The paste was ground to a fine powder with mortar and pestle and stored at 4°C. The soluble Vibrio extract used for these studies was prepared by boiling the powder in PBS at 10 mg/ml in a boiling water bath for 1 hour with frequent agitation. This suspension was then centrifuged at 1,000 x g to remove **particulates** and filter sterilized. Protein concentrations were determined by the method of Lowry et al. (1951).

Mitogen Assays. The ability of lymphocytes to undergo proliferation upon stimulation by various mitogens, was assessed by the uptake of tritiated thymidine. The radioactive counts / minute will increase as the amount of proliferation increases. This ability to proliferate then becomes a measure of lymphocytic activation. Briefly, fifty ul containing 5×10^5 cells were placed in individual wells of a 96-well flat bottom tissue culture plate with 50 ul of mitogen or culture medium. The plates were then incubated in gas boxes under 10% CO₂ at 17°C. Twenty four hours before harvest each well was pulsed with 1 uCi of tritiated thymidine (methyl- ³H, ICN Biomedicals, Irvine, CA) in 50 ul of RPMI MDM. Cells were harvested with distilled water onto glass fiber filters, with a Skatron cell harvester (Sterling, VA). The filters were then dried, placed in scintillation vials with a solubilizing agent (6g PPO, Sigma, 5 mg POPOP, Amersham, Arlington Heights, IL, in 1 liter toluene, after Etlinger et al., 1976), and counted on a Beckman liquid

scintillation counter (LS 8000). Data are reported as mean counts per minute (cpm) +/- standard error, of triplicate cultures, or as stimulation indices (SI) defined as experimental cpm/control cpm.

Mixed Lymphocyte Reaction (MLR). The ability of lymphocytes to recognize and respond to dissimilar antigens expressed on lymphocytes from other (heterologous) individuals is measured by a mixed lymphocyte assay. As in the mitogen assays, incorporation of tritiated thymidine is a direct measurement of the proliferation caused (in this case) by the stimulation of heterologous lymphocytes. These mixed lymphocyte reactions were performed utilizing the same media and culture conditions described for the mitogen assays. Briefly, fifty ul of a lymphocyte suspension (1×10^7 cells/ml) from individual fish were coincubated with fifty ul of lymphocytes from a heterologous source. Each combination of the mixed lymphocytes was cultured in triplicate, pulsed with tritiated thymidine 24 hours prior to harvesting, which was performed as described above. Cell proliferation was measured by the uptake of the tritiated thymidine.

Migration Inhibition Factor (MIF) Assays. The ability of the lymphocytes to express normal levels of important regulatory factors was determined by assaying for the production of migration inhibition factor. Antigen specific stimulation of lymphocytes causes the release of migration inhibition factor, which in turn, inhibits the migration of macrophages away from the antigen. The MIF assays used were a modification of the agarose microdroplet assay described by McCoy (1976). Fish anterior kidney cells were prepared as single cell suspensions in Lebowitz 15 medium (Cibco, Grand Island, NY) supplemented with 10% fetal calf serum and 100 ug/ml gentamicin (L-15 complete). Cells were washed twice and resuspended to a

concentration of $5 \times 10^5/12$ ul in a preparation of 0.27, Sea Prep agarose (ultra low temperature gelling agarose, FMC Corporation, Marine Colloids, Rockland, ME) in L-15 complete. These preparations were made with or without the test antigen. Two ul droplets of cells were placed in the center of sterile flat-bottom 96-well tissue culture plates (Corning) held on ice. The droplets were then allowed to solidify for 15-20 minutes. This incubation was then followed by an addition of 100 ul of 0.1% Sea Prep in L-15 complete, containing the same concentration of test antigen. This second layer of agarose was added gently so as not to dislodge the droplet. The plate was then placed in a sealed humidified box at room temperature for 48 hours. Migration was scored by inspection with an inverted microscope. Positive (+), or normal, migration occurred when the white blood cells (wbcs) appeared in a continuous ring exterior to the non-migrating red blood cells (rbcs) and pigmented kidney cells which were held in the agarose droplet. Moderate migration (+/-) was indicated when only scattered wbcs were found to be exterior to central rbcs and kidney cells. No migration (-, inhibition) was determined when no or few wbcs outside the droplet.

Radiolabelling of R. salmoninarum. One ml of a suspension of formalin-killed R. salmoninarum (one unit of 0.1). at 520 nm) was washed twice in phosphate buffered saline (PBS, pH 7.2) and resuspended in 250 ul of PBS. This solution was placed in a ice bath and 500 uCi of I^{125} (Amersham, Irvine, CA) in 5 ul was added while constantly stirring. Immediately following the I^{125} , 0.2 ml of 1% Chloramine T was added. After five minutes, 0.2 ml of 3.1% solution of sodium metabisulphite was added to stop the reaction. The labelled bacteria were then washed with PBS at 6,500 x g until the supernatant contained

less than 1% of the total cpm incorporated.

Phagocytosis Assay. Levels of phagocytic activity against R. salmoninarum were quantitatively measured by determining the uptake of radiolabelled bacterial cells by salmon leukocytes. Briefly 0.5 ml of a cellular suspension (3×10^6 cells/ml) in 5% FCS-L-15 was mixed with 0.05 ml of radioiodinated bacteria and diluted to a concentration of 2×10^6 cpm/ml. Phagocytosis was measured by removing non-phagocytized bacteria by two washes at 500 x g. The final pellet was counted and the % phagocytosis calculated as:

$$\frac{\text{cpm w/ leukocytes} - \text{cpm w/o leukocytes}}{\text{total cpm} - \text{cpm w/o leukocytes}} \times 100$$

Antibody-forming Cell Assay. Antibody production by lymphocytes in vitro was measured by the enumeration of lymphocytes producing plaques (cleared areas) in lawns of antigen coated red blood cells. Briefly, single cell suspensions of 1×10^7 cells/ml were prepared in RPMI MDN, as described above. Aliquots of 0.05 ml of the suspension were cultured with 0.05 ml of the appropriate dilution of antigen in RPMI MDN or in RPMI MDN alone. Cultures were fed 10 ul of cocktail daily until harvested. The contents of four microcultures were pooled for the antibody-forming cell (AFC) assay. Three such pools were tested per data point. Cells secreting anti-trinitrophenyl (TNP) antibodies were detected by a modification of the Cunningham plaque assay (Cunningham and Szenberg, 1968). 100 ul of the lymphocyte suspension, 25 ul of a 10% suspension of sheep red blood cells (SRBC) or TNP-SRBC (Rittenberg and Pratt, 1969) in modified barbital buffer (MBB), and 25 ul of steelhead serum, diluted in MBB, were mixed in individual wells of a 96-well microtiter plate (Linbro, McLean, VA). This mixture was deposited in a slide

chamber thus producing a thin lawn of cells, sealed and incubated for 1-3 hours at 16°C. Plaques were then enumerated under low power with the aid of a dissecting microscope.

RESULTS AND DISCUSSION

Growth and Feed Efficiency. The results indicate no significant difference in the specific growth rate and gross feed efficiency, among the five levels of pyridoxine (15, 30, 60, 120, and 1500 mg /Kg diet). Based on these results, the levels of pyridoxine above 15 mg /Kg do not appear to restrict the growth rate (Table 6).

The specific growth rate of the modified Oregon Test Diets (1.220 - 1.230), supplemented with essential amino acids or glutamic acid and glutamic hydrochloride, approached the specific growth rates found with the Abernathy diets (1.240 - 1.264). In comparing the gross feed efficiency, the Oregon Test Diets were higher (74.02 - 74.12) than values found with the Abernathy diets (64.88 - 66.05). Since this was an exploratory study, no statistical inference was made.

Mitogen Studies. Mitogen induced lymphocyte proliferations was produced by the four mitogens tested, lipopolysaccharide, phytohemagglutinin, concanavalin A, and pokeweed mitogen. In addition, we have discovered a new mitogen, an extract of Vibrio anguillarum, which is very effective for mitogenic stimulation and directly applicable to fish studies. Dose responses of all five mitogens for both anterior kidney and spleen are given in Figures 1-10, and the kinetics of response to lipopolysaccharide, phytohemagglutinin, and the Vibrio extract (Vibrio anguillarum extract) are given in Figures 9 and 10. The data presented are results from single experiments which are representative of the typical responses seen. However, these responses do tend to vary considerably between different fish or pools of fish, with regards to optimal dose for each mitogen and the level of

Table 6. Specific growth rate and gross feed efficiency of spring chinook salmon (Oncorhynchus tshawytscha) fingerlings fed different pyridoxine concentrations in the Abernathy Diet. Immunological study, Abernathy Salmon Culture Technology Center, 1985.

Diet	Specific Growth Rate ^A	Gross Feed Efficiency ^B
Abernathy Diet nominal mg Pyridoxine/Kg Diet ^D		
15	1.264±0.008 ^C	66.05±0.38
30	1.252±0.004	65.72±0.57
60	1.240±0.008	65.56±1.05
120	1.248±0.000	64.88±0.23
1500	1.252±0.020	65.58±2.03

^ASpecific Growth Rate = $\frac{\log, \text{ of the weight at end} - \log_e \text{ of weight at start}}{\text{days fed}} \times 100$

^BGross Feed Efficiency = $\frac{\text{total fish weight gain in grams}}{\text{total feed weight}} \times 100$

^Cvalues are $\bar{x} \pm SE, n=2$

^DTotal pyridoxine level, includes both basal level found in natural ingredients and supplemented pyridoxine

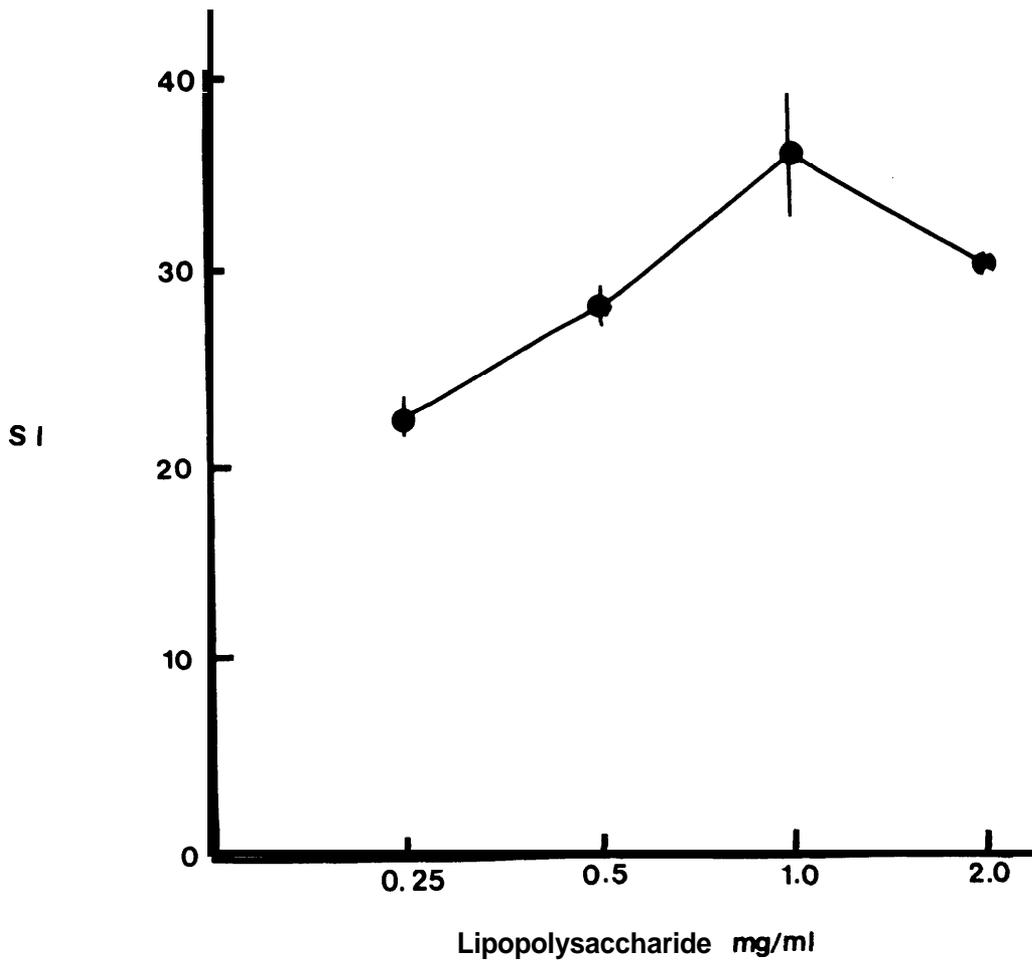


Figure 1. Dose response curve for the mitogenic stimulation of spring chinook salmon (*Oncorhynchus tshawytscha*) splenic lymphocytes with lipopolysaccharide (LPS). Mitogenic responses to lipopolysaccharide were examined at various doses of the mitogen. This assay was harvested on day 5 of incubation. SI refers to the stimulation index, counts per minute (cpm) incorporated in the presence of mitogen / cpm incorporated in the absence of mitogen. Experimental points represent the mean of triplicate cultures. Error bars represent one standard error.

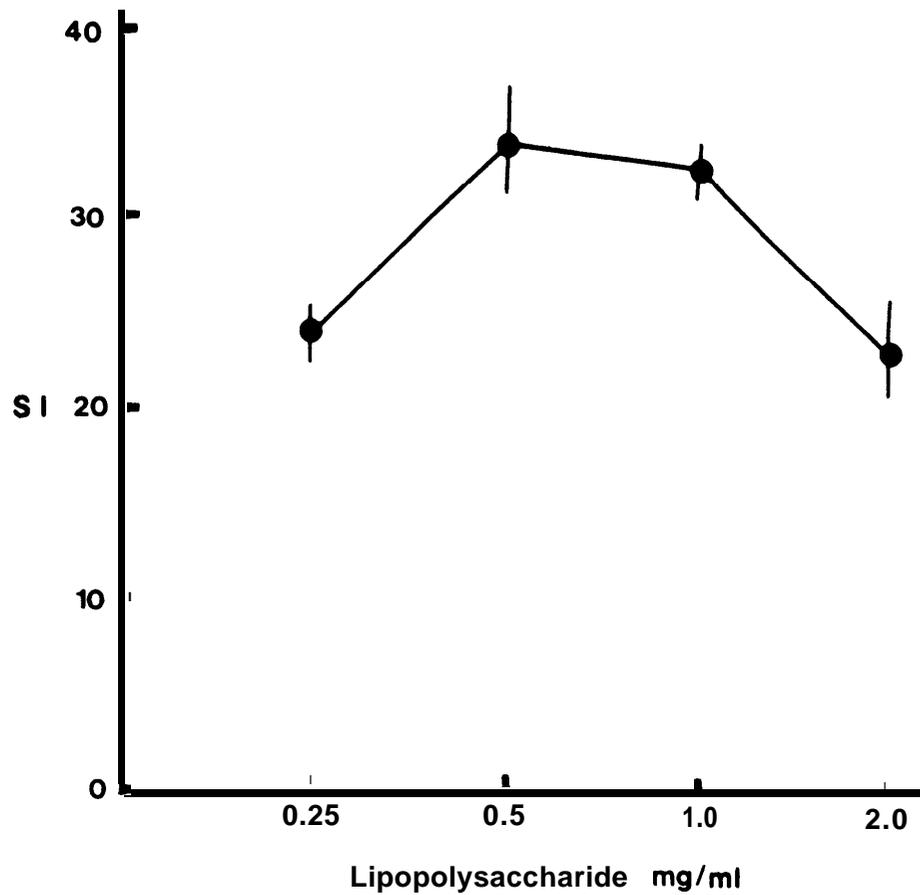


Figure 2. Dose response curve for the mitogenic stimulation of spring chinook salmon (*Oncorhynchus tshawytscha*) anterior kidney lymphocytes with lipopolysaccharide (LPS). Mitogenic responses to lipopolysaccharide were examined at various doses of the mitogen. This assay was harvested on day 5 of incubation. SI refers to the stimulation index, counts per minute (cpm) incorporated in the presence of mitogen / cpm incorporated in the absence of mitogen. Experimental points represent the mean of triplicate cultures. Error bars represent one standard error.

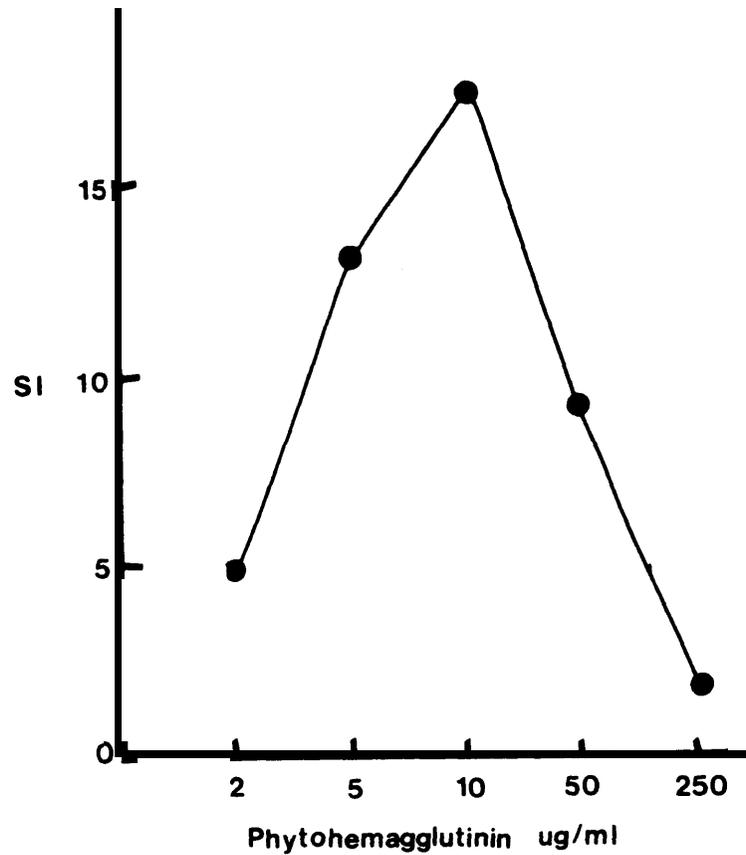


Figure 3. Dose response curve for the mitogenic stimulation of spring chinook salmon (*Oncorhynchus tshawytscha*) anterior kidney lymphocytes with phytohemagglutinin (PHA). Mitogenic responses to phytohemagglutinin were examined at various doses of the mitogen. This assay was harvested on day 5 of incubation. SI refers to the stimulation index, counts per minute (cpm) incorporated in the presence of mitogen / cpm incorporated in the absence of mitogen. Experimental points represent the mean of triplicate cultures. Error bars represent one standard error.

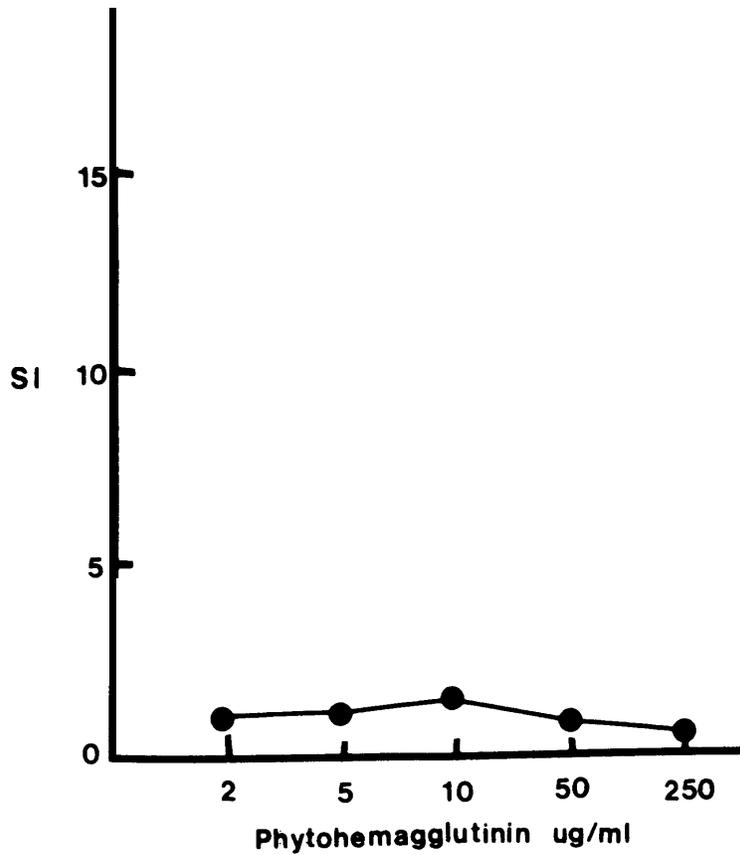


Figure 4. Dose response curve for the mitogenic stimulation of spring chinook salmon (*Oncorhynchus tshawytscha*) splenic lymphocytes with phytohemagglutinin (PHA). Mitogenic responses to phytohemagglutinin were examined at various doses of the mitogen. This assay was harvested on day 5 of incubation. ST refers to the stimulation index, counts per minute (cpm) incorporated in the presence of mitogen / cpm incorporated in the absence of mitogen. Experimental points represent the mean of triplicate cultures. Error bars represent one standard error.

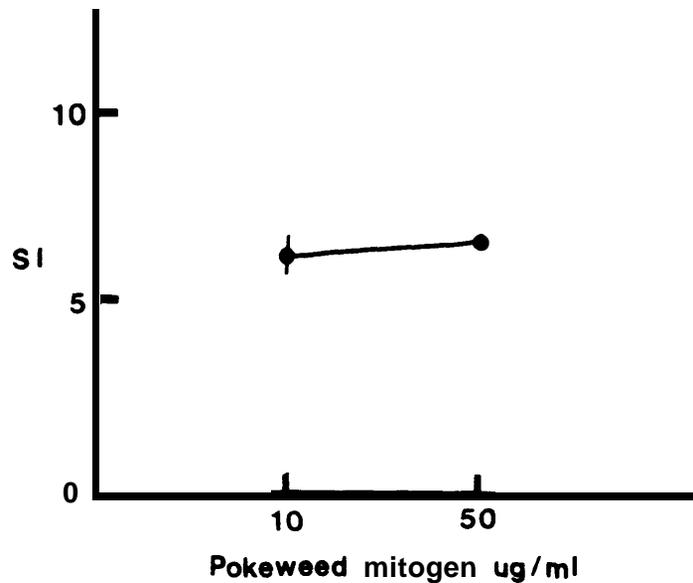


Figure 5. Dose response curve for the mitogenic stimulation of spring chinook salmon (*Oncorhynchus tshawytscha*) anterior kidney lymphocytes with pokeweed mitogen (PWM). Mitogenic responses to pokeweed mitogen were examined at various doses of the mitogen. This assay was harvested on day 5 of incubation. SI refers to the stimulation index, counts per minute (cpm) incorporated in the presence of mitogen / cpm incorporated in the absence of mitogen. Experimental points represent the mean of triplicate cultures. Error bars represent one standard error.

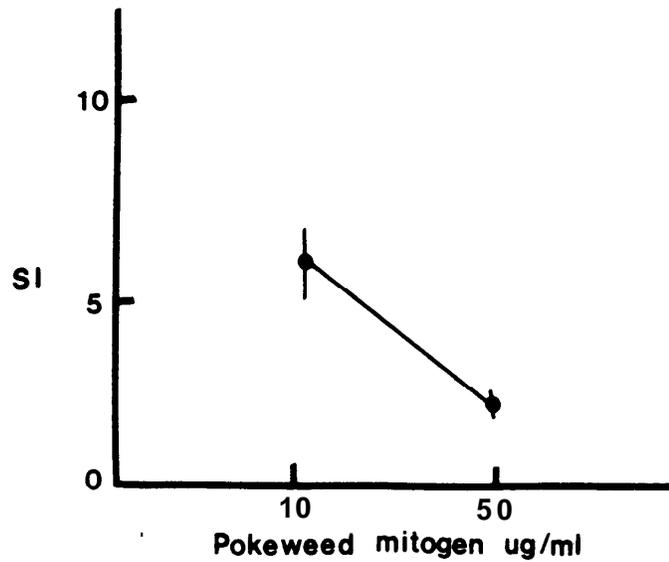


Figure 6. Dose response curve for the mitogenic stimulation of spring chinook salmon (*Oncorhynchus tshawytscha*) splenic lymphocytes with pokeweed mitogen (PWM). Mitogenic responses to pokeweed mitogen were examined at various doses of the mitogen. This assay was harvested on day 5 of incubation. SI refers to the stimulation index, counts per minute (cpm) incorporated in the presence of mitogen / cpm incorporated in the absence of mitogen. Experimental points represent the mean of triplicate cultures. Error bars represent one standard error.

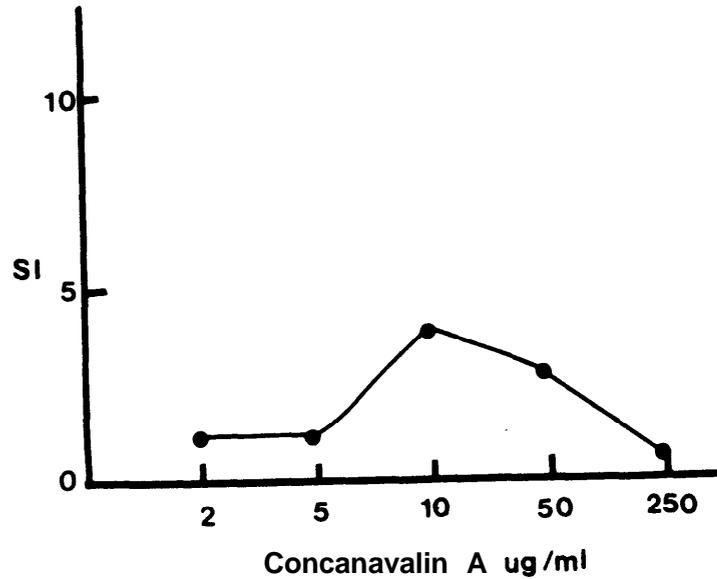


Figure 7. Dose response curve for the mitogenic stimulation of spring chinook salmon (*Oncorhynchus tshawytschs*) anterior kidney lymphocytes with concanavalin A (Con A). Mitogenic responses to concanavalin A were examined at various doses of the mitogen. This assay was harvested on day 5 of incubation. SI refers to the stimulation index, counts per minute (cpm) incorporated in the presence of mitogen / cpm incorporated in the absence of mitogen. Experimental points represent the mean of triplicate cultures. Error bars represent one standard error.

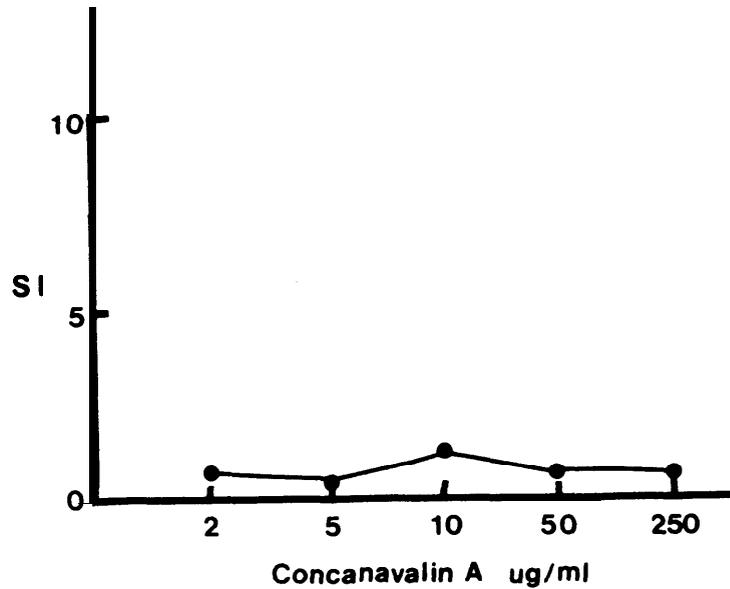


Figure 8. Dose response curve for the mitogenic stimulation of spring chinook salmon (*Oncorhynchus tshawytscha*) splenic lymphocytes with concanavalin A (Con A). Mitogenic responses to concanavalin A were examined at various doses of the mitogen. This assay was harvested on day 5 of incubation. SI refers to the stimulation index, counts per minute (cpm) incorporated in the presence of mitogen / cpm incorporated in the absence of mitogen. Experimental points represent the mean of triplicate cultures. Error bars represent one standard error.

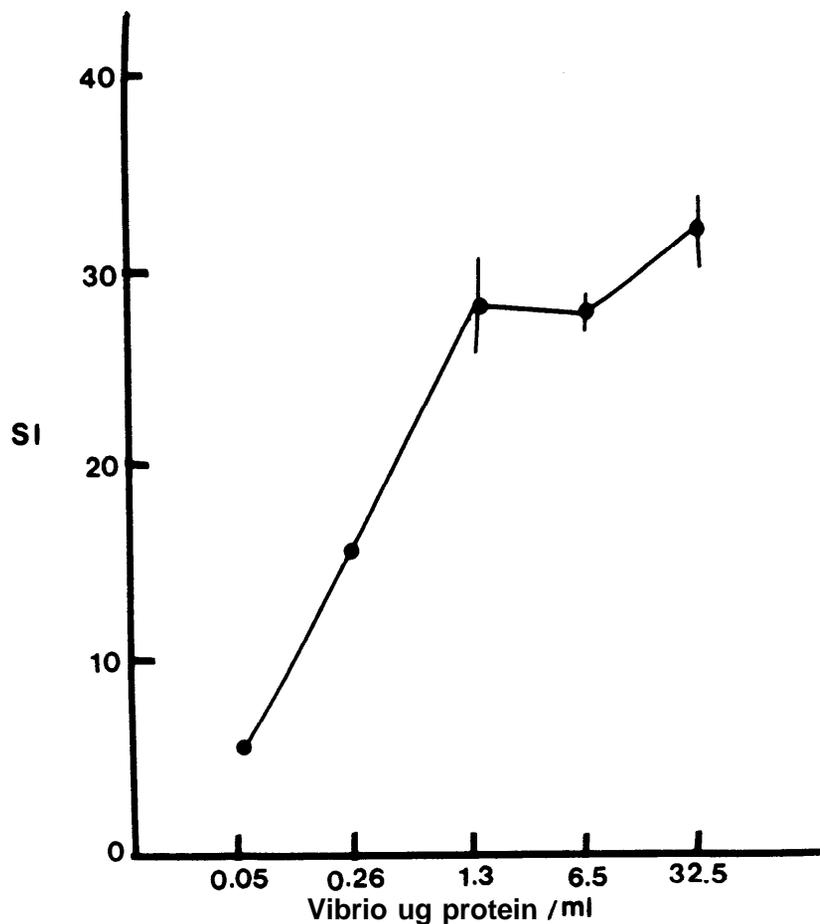


Figure 9. Dose response curve for the mitogenic stimulation of spring chinook salmon (*Oncorhynchus tshawytschaj* anterior kidney lymphocytes with *Vibrio anguillarun* extract (*Vibrio*). Mitogenic responses to *Vibrio* extract were examined at various doses of the mitogen. This assay was harvested on day 5 of incubation. SI refers to the stimulation index, counts per minute (cpm) incorporated in the presence of mitogen / cpm incorporated in the absence of mitogen. Experimental points represent the mean of triplicate cultures. Error bars represent one standard error.

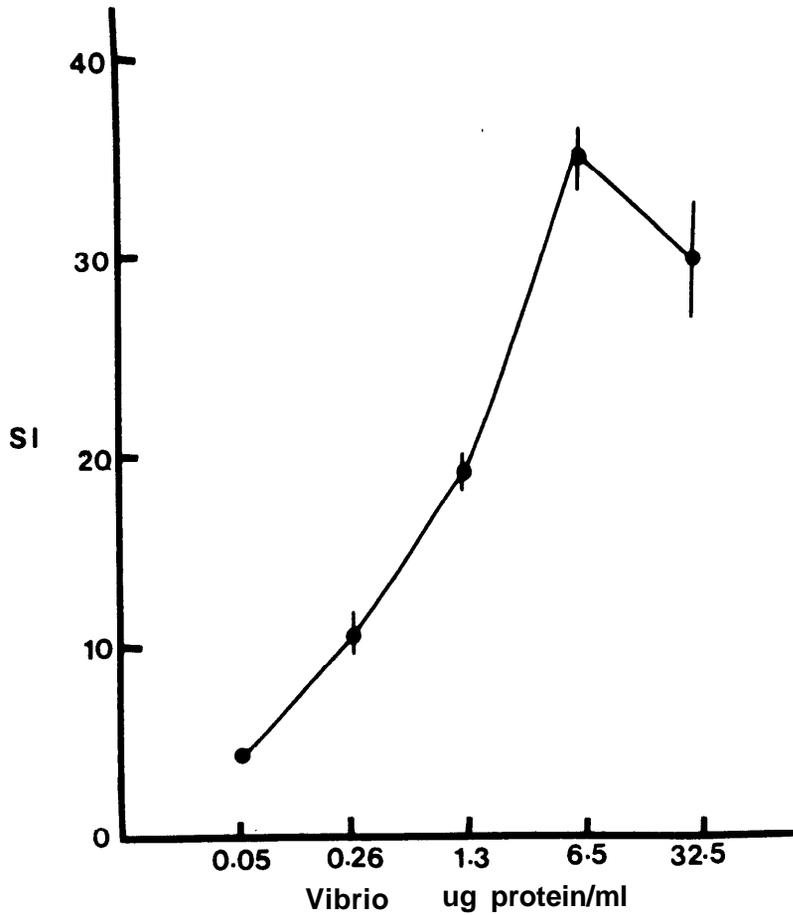


Figure 10. Dose response curve for the mitogenic stimulation of spring chinook salmon (*Oncorhynchus tshawytscha*) splenic lymphocytes with *Vibrio anguillarum* extract (*Vibrio*). Mitogenic responses to *Vibrio* extract were examined at various doses of the mitogen. This assay was harvested on day 5 of incubation. SI refers to the stimulation index, counts per minute (cpm) incorporated in the presence of mitogen / cpm incorporated in the absence of mitogen. Experimental points represent the mean of triplicate cultures. Error bars represent one standard error.

stimulation. For this reason, dose responses will be conducted on all fish fed the vitamin diets..

Lipopolysaccharide, a typical mammalian B cell mitogen, was consistently able to induce high stimulation indices in lymphocytes isolated from the spleen or anterior kidney (Figures 1 and 2). Both lymphoid populations demonstrated stimulation indices of 20-30. The peak stimulatory dose was found to be approximately 0.25-1.0 mg/ml.

In contrast, phytohemagglutinin, a T cell mitogen, was found to be capable of inducing stimulation indices of 15-20 in the anterior kidney only (Figure 3); no stimulation was found in the spleen (Figure 4). Occasionally, phytohemagglutinin stimulation can be seen in the spleen, **particulary** if fish serum is used (data not shown). These data may suggest that there are distinct phytohemagglutinin responsive sub-populations of lymphocytes in the anterior kidney but not in the spleen.

Pokeweed mitogen, a mammalian B and T cell mitogen was found to produce stimulation indices of 5 to 10 in both anterior kidney and spleen (Figures 5 and 6).

Concanavalin A, another T cell mitogen, produced the lowest stimulation index which was approximately 3.0 in the anterior kidney (Figure 7); no stimulation was seen in the spleen (Figure 3). It is of considerable interest that both of the T cell mitogens possess the ability to elicit responses from the anterior kidney only, while lipopolysaccharide and pokeweed mitogen stimulate responses in both anterior kidney and spleen. This may suggest that either there are few, if any, T cell like lymphocytes in the spleen, or that T cell responses in the spleen are heavily regulated or suppressed in response to mitogens.

The mitogenesis of the Vibrio anguillarum extract is of

particular interest because it is derived from a fish pathogen. This material has been found to produce similar or much higher mitogenic responses than the other mitogens (Figures 9 and 10). It also stimulated a non-specific anti-TNP plaque-forming responses (see the polyclonal activation section). This high degree of stimulation was found in both anterior kidney and spleen.

The kinetics of the mitogenic responses of the three most stimulatory agents (Vibrio anguillarum extract, lipopolysaccharide, phytohemagglutinin) was determined at their **respective** optimal doses (Figures 11 and 12). This analysis revealed distinctive differences between Vibrio anguillarum extract and lipopolysaccharide stimulation. Vibrio anguillarum extract appears to stimulate an early peak response (day 4) in both anterior kidney and spleen, whereas lipopolysaccharide demonstrates a rather protracted peak response over the seven days of culture, and distinct peak response at 6 days of culture for the splenic lymphocytes. Thus it appears that Vibrio anguillarum extract is similar to lipopolysaccharide in its ability to **stimulate** both anterior kidney and splenic lymphocytes, the difference in kinetics may indicate a different mechanism of stimulating lymphocytes or a preference for stimulating distinctly different lymphoid populations.

Phytohemagglutinin responses were inconclusive as to the resolution of a peak response day; however, the absence of any mitogenic responses with splenic lymphocytes was consistently observed at all times.

Mixed Lymphocyte Reactions (MLR). It has been found that chinook salmon can exhibit a strong mixed lymphocyte reaction when cells from two or more fish are mixed (Figure 13). As can be seen from this representative figure, some degree of variability in

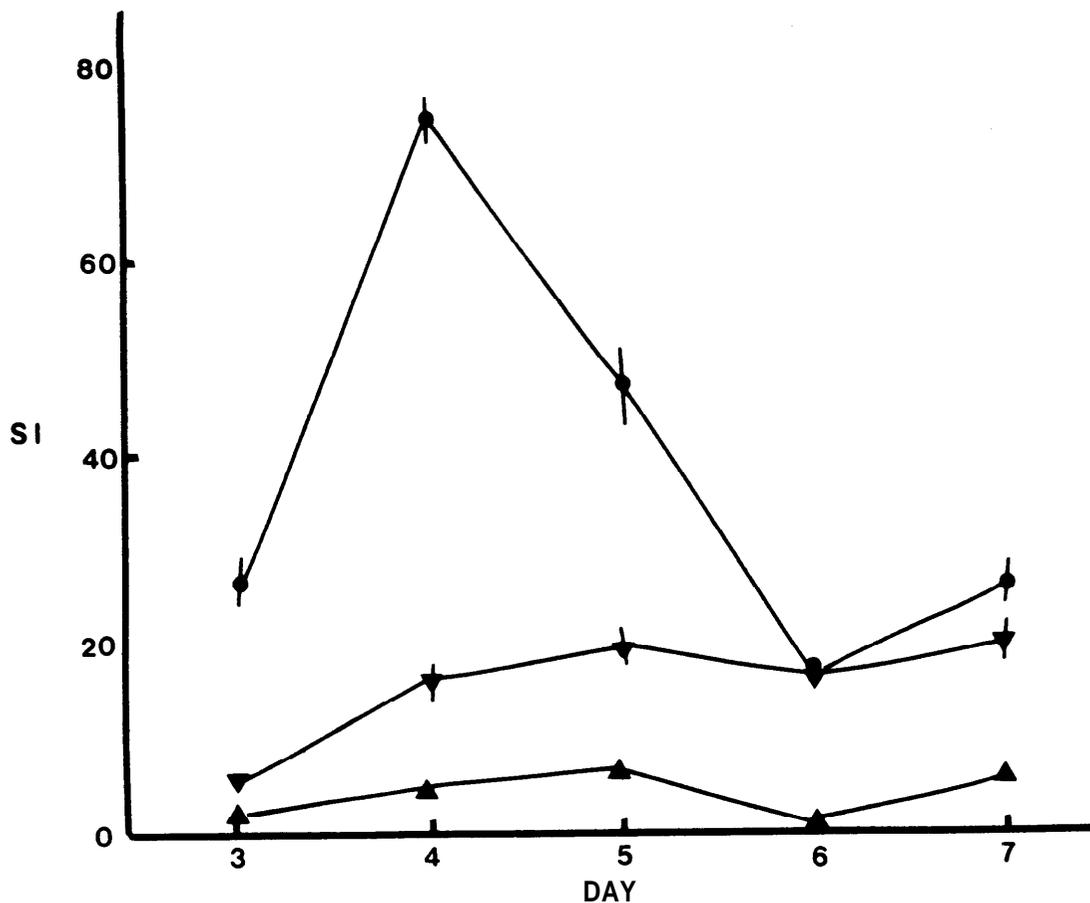


Figure 11. Kinetics (days 3-7) for the mitogenic stimulation of spring chinook salmon (*Oncorhynchus tshawytscha*) anterior kidney lymphocytes with *Vibrio*, lipopolysaccharide, and phytohemagglutinin. *Vibrio* (●), lipopolysaccharide (▼), and phytohemagglutinin (▲) were cultured at their optimal doses: 6.5 μg protein/ml, 1 mg/ml, and 10 μg /ml respectively. SI refers to the stimulation index, or counts per minute (cpm) incorporated in the presence of mitogen / cpm incorporated in the absence of mitogen. Experimental points represent the mean of triplicate cultures. Error bars represent one standard error.

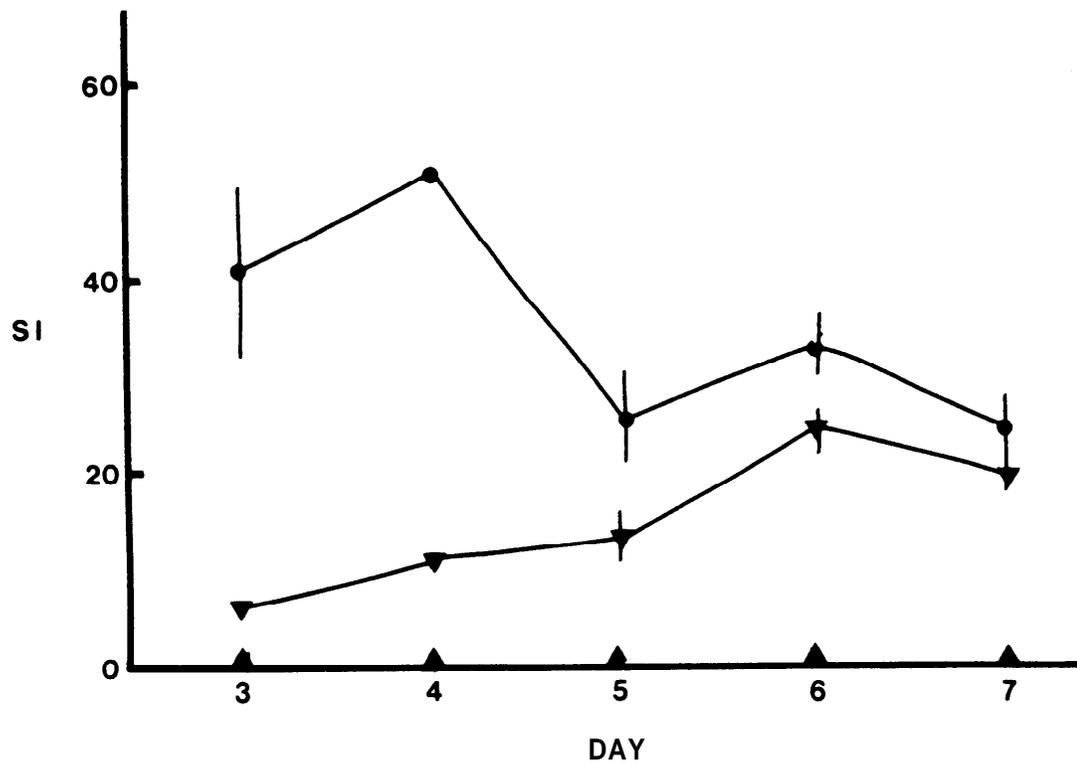


Figure 12. Kinetics (days 3-7) for the mitogenic stimulation of spring chinook salmon (*Oncorhynchus tshawytscha*) splenic lymphocytes with *Vibrio*, lipopolysaccharide, and phytohemagglutinin. *Vibrio* (●), lipopolysaccharide (▼), and phytohemagglutinin (▲) were cultured at their optimal doses; 6.5 ug protein/ml, 1 mg/ml, and 10 ug/ml respectively. SI refers to the stimulation index, or counts per minute (cpm) incorporated in the presence of mitogen / cpm incorporated in the absence of mitogen. Experimental points represent the mean of triplicate cultures. Error bars represent one standard error.

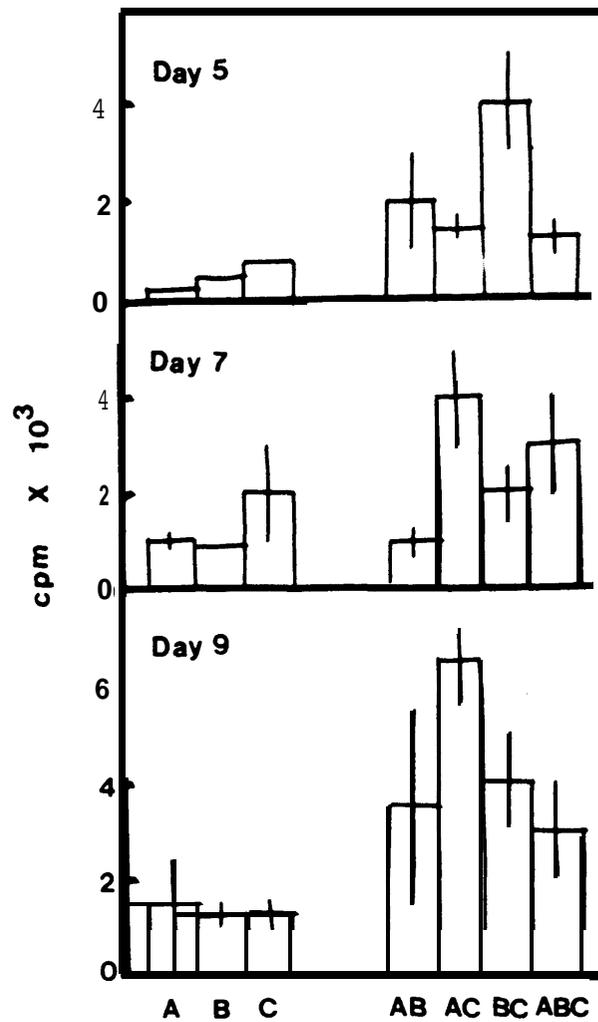


Figure 13. Nixed lymphocyte reaction (MLR) of spring chinook salmon (*Oncorhynchus tshawytscha*) anterior kidney lymphocytes. Cells from individual animals (A, B, C) were incubated alone or in combination (AB, AC, EC, AX). Individual cultures consisted of 50 ul of each individual cell type (1×10^7 cells/ml). Three way mixed lymphocyte reactions were composed of 33 ul of each individual cell type. CPM refers to counts per minute of radioactive thymidine incorporated into the lymphocytes. Each bar represents an average of triplicate cultures. The error bars represent one standard error.

stimulation can be seen. The **occasional** weak response or lack of a response, is most probably due to the partial **histocompatibility** of certain individuals. As can be seen in the figure, there appears to be a general increase in the cpm over time, the **greater** stimulation occurring at day 9. To counter the variability of this MLR, we will standardize this assay by incorporating stimulatory lymphocytes from a xenogenic source (i.e. rainbow trout), rather than an allogeneic one (other chinook cells). This should provide a constant stimulus to the chinook cells, independent of the individual tested. To prevent incorporation of tritiated thymidine by xenogenic cells, trout lymphocytes will be irradiated with 1,000 rads from a Co⁶⁰ irradiator prior to coculture. Preliminary evidence demonstrates that this amount of irradiation is sufficient to prevent tritiated thymidine uptake.

Migration Inhibition Factor (MIF) Analysis. Previous studies (McKinney et al., 1976) have demonstrated that cell mediated immunity in fish can be assessed by the analysis of the inhibition of macrophage migration. This assay measures the ability of "T"-like cells to elaborate a factor in response to antigen specific stimulation. This factor then induced the inhibition of macrophage migration.

Initial studies employing the defined antigen, TNP-BSA, revealed that TNP-BSA immunized fish were sensitive to TNP-BSA at concentrations of 1 to 100 ug/ml in culture (Table 7). The lowest concentration of TNP-BSA (0.01 ug/ml) was unable to stimulate sufficient elaboration of MIF to inhibit this response. Cells from naive or non-immune animals demonstrated no macrophage inhibition at any of the tested concentrations of antigen. Thus, within the chinook system, we should be able to evaluate

Table 7. Migration inhibition factor responses of immune and non-immune spring chinook salmon (*Oncorhynchus tshawytscha*) anterior kidney lymphocytes.^a

	TNP-Bovine Serum Albumin ug/ml											
	0			0.01			1			100		
	1	2	3	1	2	3	1	2	3	1	2	3
Injected fish ^b	+ ^c	+	+	+	+	+/-	/	-	-	+/-	-	-
Naive fish	+	+	+	+	+	+	/	+	+	+	+	+

^a Anterior kidney cells ($5 \times 10^5/12$) were cultured in 0.2% agarose in complete L-15 medium. Cultures were maintained in 96 well tissue culture plates, held in a humidified chamber at room temperature for 48 hours.

^b Cells were obtained from fish which had been injected one week previously with 100 ug TNP-bovine serum albumin (TNP-BSA) in complete adjuvant (X.4)

^c Migration scored as follows:
 + extensive migration
 +/- moderate migration
 - no migration (inhibition)
 / not scorable

the effects of vitamin nutrition on the ability of lymphocytes to **produce** an antigen specific cellular immune function.

Also, since macrophages play an important role in the progress of bacterial kidney disease (Fryer and Sanders, 1981; Young and Chapman, 1978), this assay may provide informative in vitro correlates to the in vivo studies relating resistance to BKD to vitamin nutrition.

Lymphocytes from R. salmoninarum injected or non-injected salmon will be assessed for their ability to respond to in vitro challenges of R. salmoninarum by the use of the MIF assay.

Phagocytosis Studies. The data in figure 14 demonstrate that the kidney and spleen possess varying degrees of phagocytic activity to R. salmoninarum. Leukocytes from either the anterior or posterior kidney produced the greatest amount of activity (approximately 23% within one hour), while the spleen produced considerably less (approximately 6%). Also, it should be noted that the spleen demonstrated no increased uptake of labelled KDB during the time period examined. This observation has led to the design of future studies to examine phagocytic activity at earlier time points than twenty minutes. It is felt that the close examination of the kinetics of phagocytosis will allow for the differentiation between non-specific and specific uptake of bacteria within the spleen. Aeromonas salmonicida cells will also be labelled to examine the phagocytic response to this challenge agent.

MIF and phagocytosis assays allow for the examination of two distinct phases of the cell mediated immune response. The MIF assay examines the initial antigen specific lymphocytic response to antigen and, thus, represents the inductive phase of the macrophage's response.

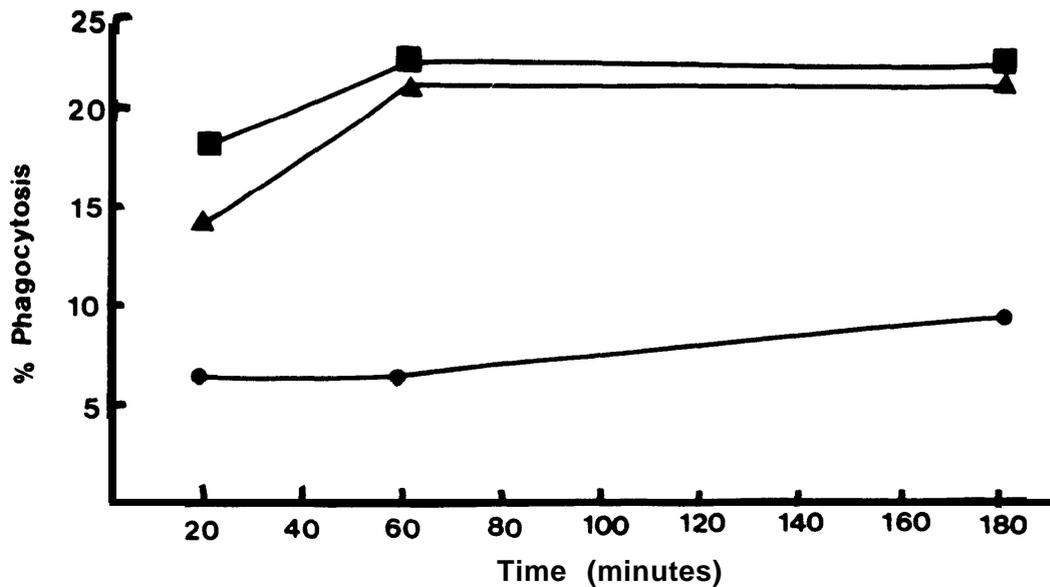


Figure 14. Phagocytosis of I¹²⁵ labelled R. salmoninarum.

Phagocytosis was measured by incubation of the bacteria with normal leukocytes from the spleen (●), anterior kidney (▲), and posterior kidney (■) at 17°C. The vertical axis represents percent phagocytosis and the horizontal axis represents the period of incubation before harvest of the leukocytes.

The phagocytic assay, on the other hand, measures the **effector** phase of the macrophage's response. Examination of both of these phases will allow for the dissection of effects of vitamin nutrition on the **salmonid** cellular immune response.

Antibody-forming Cell (AFC) Assay. The antigen TNP-lipopolysaccharide has been found capable of inducing an in vitro antibody response as ascertained by the plaque forming assay. The spleen (Figure 15) demonstrated significant AFC responses to TNP-coated sheep red blood cells. At no time were AFC to sheep red blood cells alone detected (data not shown). Both figures indicate that approximately 20 ug/ml produces an optimal antibody response of 40-400 AFCs/ 10^6 total lymphocytes harvested. The optimal AFC response occurs in 7 to 9 days of culture.

This assay represents an extremely powerful tool for the analysis of the immune response. This is due to the fact that we are now capable of examining, quantitatively, the capability of lymphocytes to produce a specific antibody without the immunization of intact animals. Thus, investigators will not be required to immunize groups of fish and separate them into tanks until an in vivo response can be observed. Also, in utilizing this system, fish from various sources may be screened for their ability to produce a specific immune response without having to be subjected to stress of transport to other **holding facilities**.

Currently, this in vitro system is being used to determine if we can produce an in vitro antibody response to TNP-keyhole limpet hemocyanin (TNP-KLH). The carrier, KLH, is a protein and thus represents a probable **T cell dependent** antigen (Miller et al., 1985). Preliminary studies indicate that antibody responses can be generated

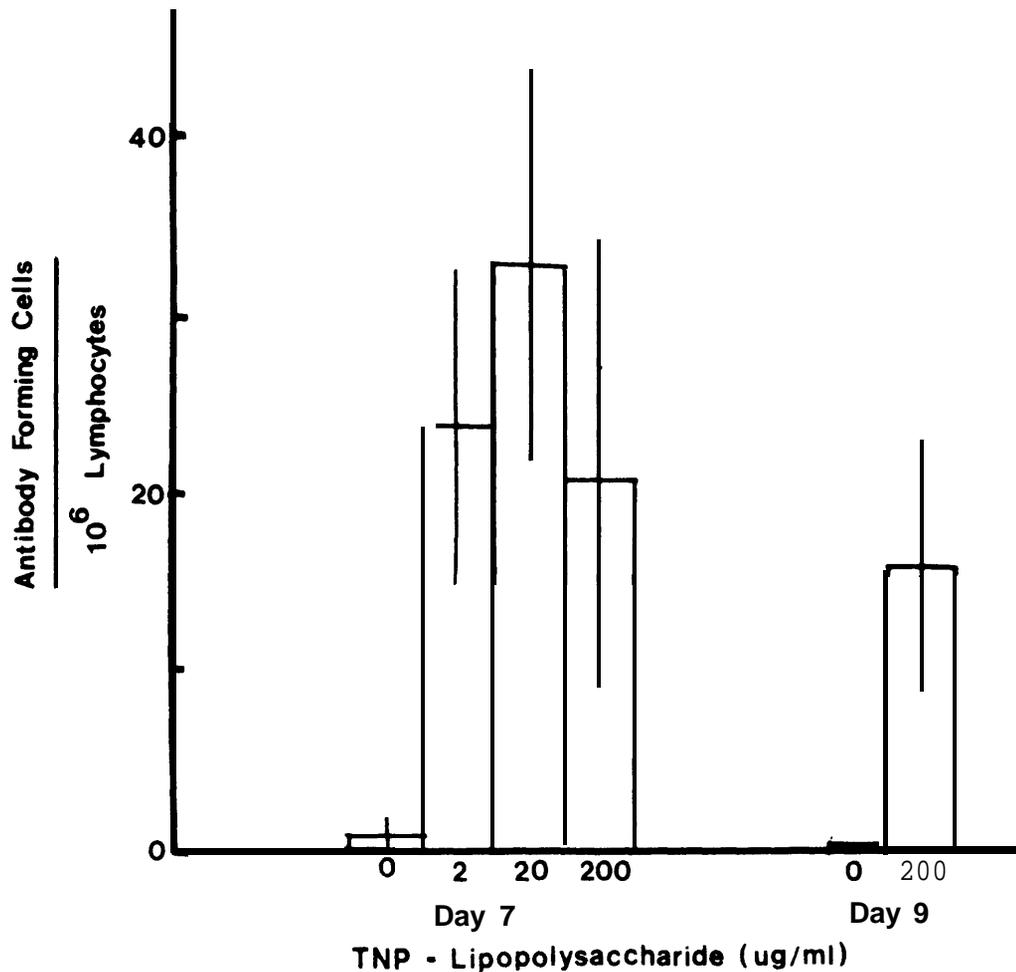


Figure 15. In vitro antibody forming responses of spring chinook salmon (*Oncorhynchus tshawytscha*) splenic lymphocytes. Splenic lymphocytes from normal non-immune salmon were cultured with or without the antigen TNP-lipopolysaccharide, Cultures were harvested after 7 or 9 days of culture. The cultured lymphocytes were dispersed in a field of TW-sheep red blood cells within Cunningham chambers. Plaques in the field of sheep red blood cells were enumerated after two to three hours incubation at 18^oC. Open bars represent average values for triplicate pools of cells. Error bars represent one standard error.

to this form of antigen also. The possession of a T-independent form of antigen (TNP-lipopolysaccharide) and a T-dependent form of antigen (TNP-KLH) will allow for the delineation of distinct effects of vitamin nutrition on B and T cell related functions in the generation of an immune response.

Recent evidence from our laboratory (Kaattari and Irwin, 1985) indicate that bacterial antigens such as that derived from Vibrio anguillarum cells can be coated onto sheep red blood cells and used for the detection of antigen specific responses to fish pathogens. Attempts are currently underway to determine if antibody producing cells can be detected that recognize R. salmoninarum and A. salmonicida antigens. Aside from the importance of analysing antibody responses to these pathogens, which are the disease challenge correlates for this project, such a system would allow investigators to screen various stocks of fish to determine if they vary in their abilities to respond to these two important pathogens.

Polyclonal Activation. Mitogenic materials were examined to determine if they might also be capable of stimulating spring chinook lymphocytes non-specifically to secrete antibodies. Such non-specific stimulation of antibody secretion was demonstrated by incubation with Vibrio extract (Figure 16). The figure demonstrates that when naive chinook lymphocytes are cultured with optimal concentration of Vibrio extract (6.5 ug/ml), an anti-TNP AFC response is elicited. Although of interest from an immunological perspective, it is felt that specific antibody induction in vitro will be of greater value in assessing the effects of dietary vitamin manipulations.

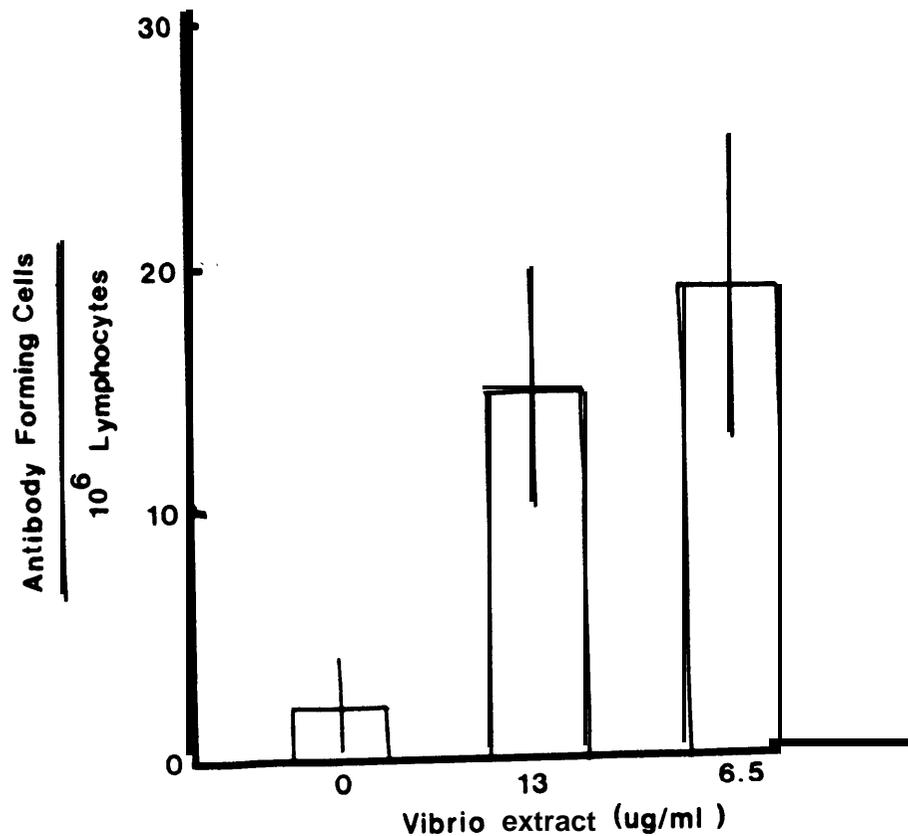


Figure 16. In vitro antibody forming responses by the polyclonal activation of spring chinook salmon (*Oncorhynchus tshawytscha*) splenic lymphocytes by *Vibrio* extract. Anterior kidney lymphocytes from normal non-immune salmon were cultured with or without the *Vibrio* extract and harvested on day 9. The cultured lymphocytes were dispersed in a field of TNP-sheep red blood cells within Cunningham chambers. Plaques in the field of sheep red blood cells were enumerated after two to three hours incubation at 18°C. Open bars represent average values for triplicate pools of cells. Error bars represent one standard error.

SUMMARY AND CONCLUSIONS

The primary goals in the first year of this project were to standardize various techniques for the assessment of immunocompetence. In addition, a range-finding study is now being performed to ascertain the approximate levels of B₆ in the diet which can lead to altered immunity or disease resistance.

The growth and feed efficiency studies reveal that the various test amounts of pyridoxine in the Abernathy Diet produce no significant difference in the growth rate or efficiency of the diets. The effect of pyridoxine on immunocompetency will be tested on this group of animals during the summer of 1985.

Another small diet study was conducted to determine how the Oregon Test Diet (OTD) might be altered to enhance its potential for growth and feed efficiency. This problem is of importance to this study, as well as other studies which require comparisons between practical and defined rations. In this study, two modifications of the OTD were produced, one of which contained a supplement of six essential amino acids, and a control modification which replaced the supplemented amino acids with glutamic acid and glutamic acid hydrochloride. Interestingly, both modifications resulted in similar specific growth rates and gross feed efficiency. The two Oregon Test Diets demonstrated feed efficiencies higher than that seen with the Abernathy diets. The growth rates obtained with these two diets approached the values attained with the Abernathy diet.

The standardization of the immunological assay systems has met with considerable success, in that all of the assays seem to function in the salmonid system. At this point, the most productive approach will be to ascertain which of the assays is the most sensitive in detecting changes in the various immunological parameters when vitamin levels are altered.

The assay systems which would be the simplest and most productive to use at this point would be: 1), the PFC assay, which measures the ability of lymphocytes to produce a specific antibody response to a defined antigen, 2) the mixed lymphocyte reaction, which measures a specific cell mediated response, and 3) the mitogen assays which appear to detect the responses of different lymphocytic subpoulations in both the anterior kidney and the spleen. The phagocytic assay may require further optimization to work efficiently with transported cells, such is not a concern with the three previously mentioned assay systems. The MIF assay, in its present form, would require the specific immunization of the fish before testing. The most convenient way to assess MIF production would be to analyse lymphocyte populations from fish challenged with R. salmoninarum or A. salmonicida. This assay may then provide an informative in vitro correlate to be studied during our disease challenge studies.

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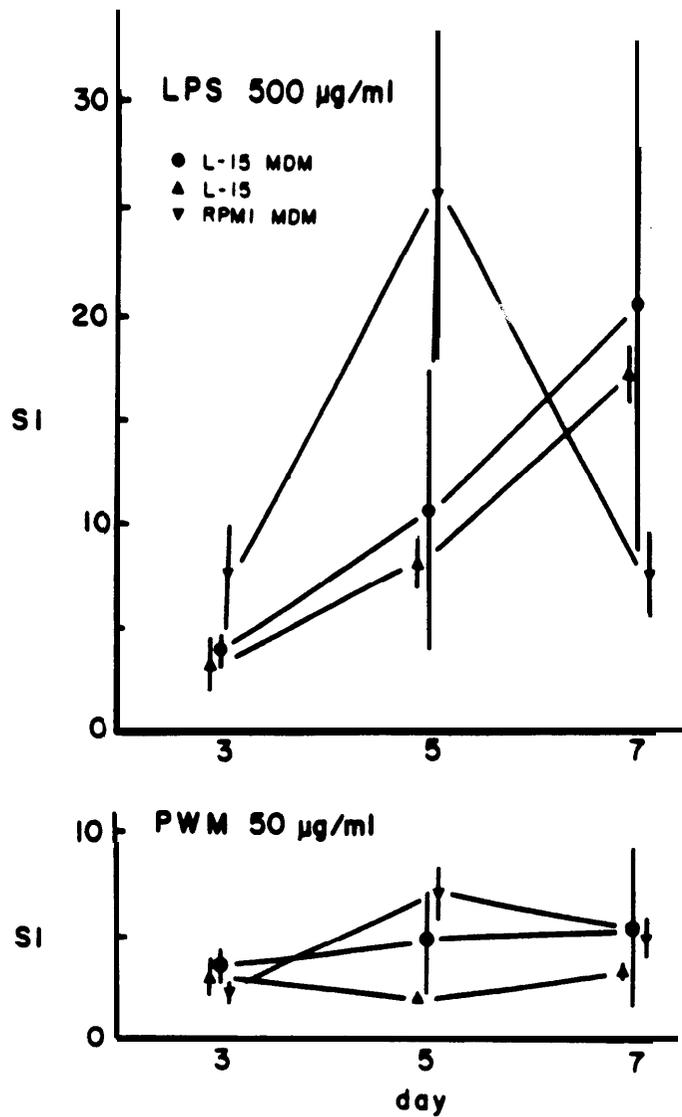


Figure A. Comparison between three medium preparations for efficacy in supporting the mitogenic responses of spring chinook (*Oncorhynchus tshawytscha*) lymphocytes. Three tissue culture media, L-15 MDM (●), L-15, with fetal calf serum and gentamicin (▲), and RPMI MDM (▼) were tested with the mitogens, lipopolysaccharide (500 µg/ml) and pokeweed mitogen (50 µg/ml). Tritiated thymidine incorporation was measured on days 3, 5, and 7 of culture. Data are reported as mean stimulation indices of triplicate cultures. Error bars represent one standard error.

APPENDIX B. Comparison between fetal calf serum and fall chinook salmon serum at various concentrations, as a tissue culture medium supplement for mitogen assays.

To determine the best serum type and **concentration** to maximize mitogenic stimulation, fetal calf and chinook salmon sera were tested. 5×10^5 anterior kidney cells were cultured in RPMI MDM supplemented with 5, 10, 15, or 20% fetal calf serum or salmon serum as described in Materials and Methods. Cells were pulsed with tritiated thymidine in medium with the appropriate type and concentration of serum, 24 hours before harvest, and uptake was measured as described.

Fetal calf serum was found to support both lipopolysaccharide and phytohemagglutinin mitogenic responses (Figures E1 and B2). For lipopolysaccharide stimulation, 10 and 20% fetal calf serum gave the best results although all were adequate. For phytohemagglutinin stimulation, 10 and 54 fetal calf serum gave the highest stimulations, although, again, all were adequate. Chinook salmon serum, on the other hand, did not support lipopolysaccharide stimulation at any concentration tested. salmon serum also suppressed phytohemagglutinin stimulation except at 5% and 25 $\mu\text{g}/\text{ml}$ phytohemagglutinin on day 6.

Based on these results, 10% fetal calf serum was selected to be used routinely in RPMI MDM in mitogen assays.

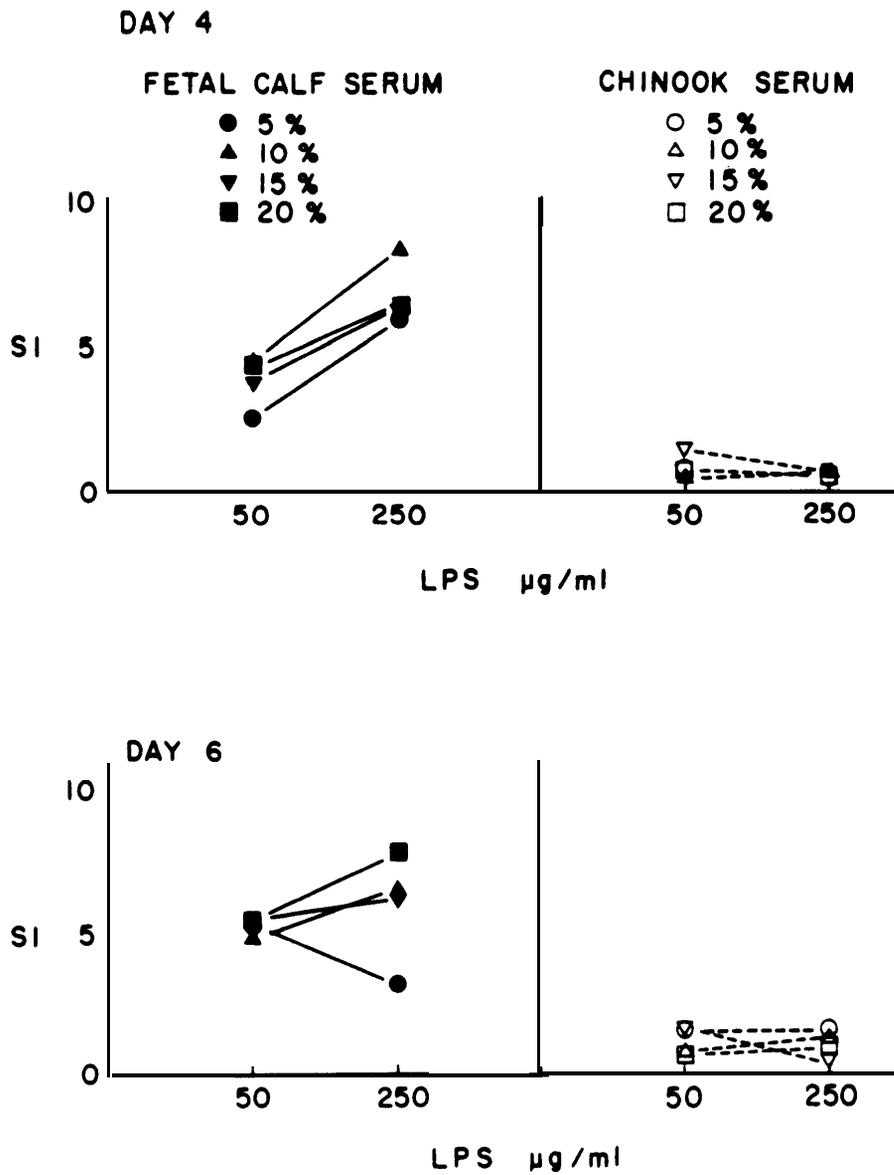


Figure B1. Lipopolysaccharide mitogenic stimulation of anterior kidney cells from spring chinook salmon (*Oncorhynchus tshawytscha*) in medium containing various concentrations of fetal calf or salmon serum. Mitogenic responses to lipopolysaccharide were compared in RPMI MDM with 5 (● C), 10 (AA), 15 (▼▼), or 20 (■ □)% fetal calf serum (closed symbols) or salmon serum (open symbols). Cells were harvested on days 4 and 6 of culture. Data are mean stimulation indices of triplicate cultures.

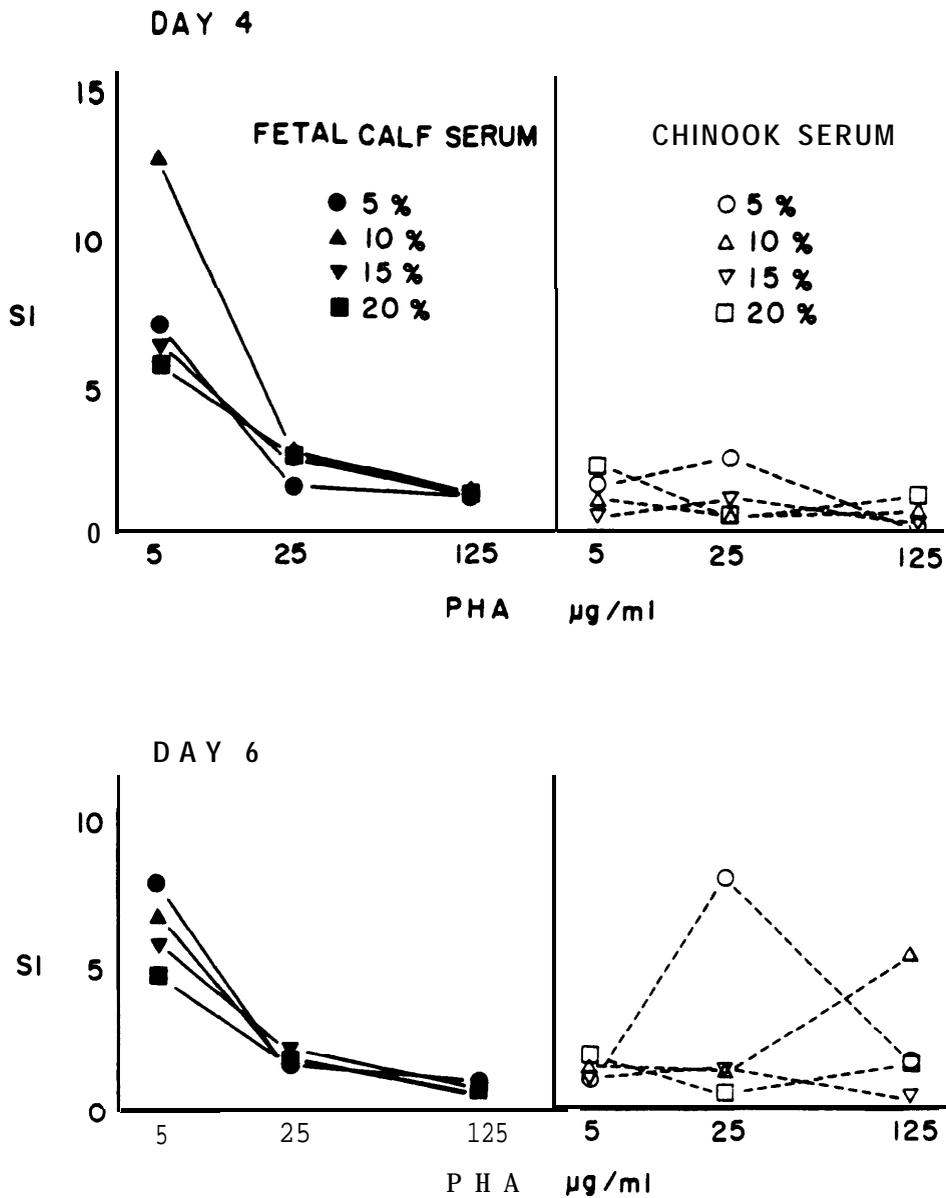


Figure B2. Phytohemagglutinin mitogenic stimulation of anterior kidney cells from spring chinook salmon (*Oncorhynchus tshawytschaw*) in medium with various concentrations of fetal calf or salmon serum. Mitogenic responses to phytohemagglutinin were compared in RPMI MDM with 5 (●), 10 (▲), 15 (▼), or 20 (■) % fetal calf serum (closed symbols) or salmon serum (open symbols). Cells were harvested on days 4 and 6 of culture. Data are mean stimulation indices of triplicate cultures.

APPENDIX C. Production and detection of lymphokines by phytohemagglutinin stimulated anterior kidney cells, a potential assay to assess cell-mediated immunity.

These studies were initiated to explore other possible assays to be used in assessing immunocompetence in fish. These results are quite preliminary and further studies are currently underway.

24 wells with 1×10^6 anterior kidney cells in each were incubated for various periods of time with either 10 $\mu\text{g/ml}$ phytohemagglutinin (phytohenagglutinin supernatant) or no mitogen (control supernatant) in RPMI MDM as described previously. After 13, 24, and 72 hours, culture supernatants were collected, centrifuged at 500 x g to remove cells, and stored at -20°C . These supernatants were then tested with fresh anterior kidney cells for their ability to enhance mitogenic stimulation, which would indicate the presence of elaborated lymphokines. Triplicate cultures of 5×10^5 anterior kidney cells were set up with either 1.25, 5, 20, or 80% (v/v) phytohenagglutinin-induced supernatant in fresh RPMI MDM, or the same percentages of control supernatants with and without the addition of 10 $\mu\text{g/ml}$ phytohemagglutinin, Tritiated thymidine uptake was measured on days 3 and 5 of culture.

Phytohemagglutinin supernatants at most concentrations and all three culture times (18, 24, and 72 hours) gave higher stimulation than control supernatants obtained at the same time even with phytohemagglutinin added at 10 $\mu\text{g/ml}$ (Figure C). These data suggest that a lymphokine for cellular proliferation may be present in the supernatant of phytohemagglutinin stimulated cells, which cannot be attributed to direct phytohenagglutinin stimulation. These differences are evident in both day 3 and 5 cultures although results

from only day 5 are given. We may be able to use this method to assess the ability of lymphocytes to produce the soluble factors required in cell mediated immunity.

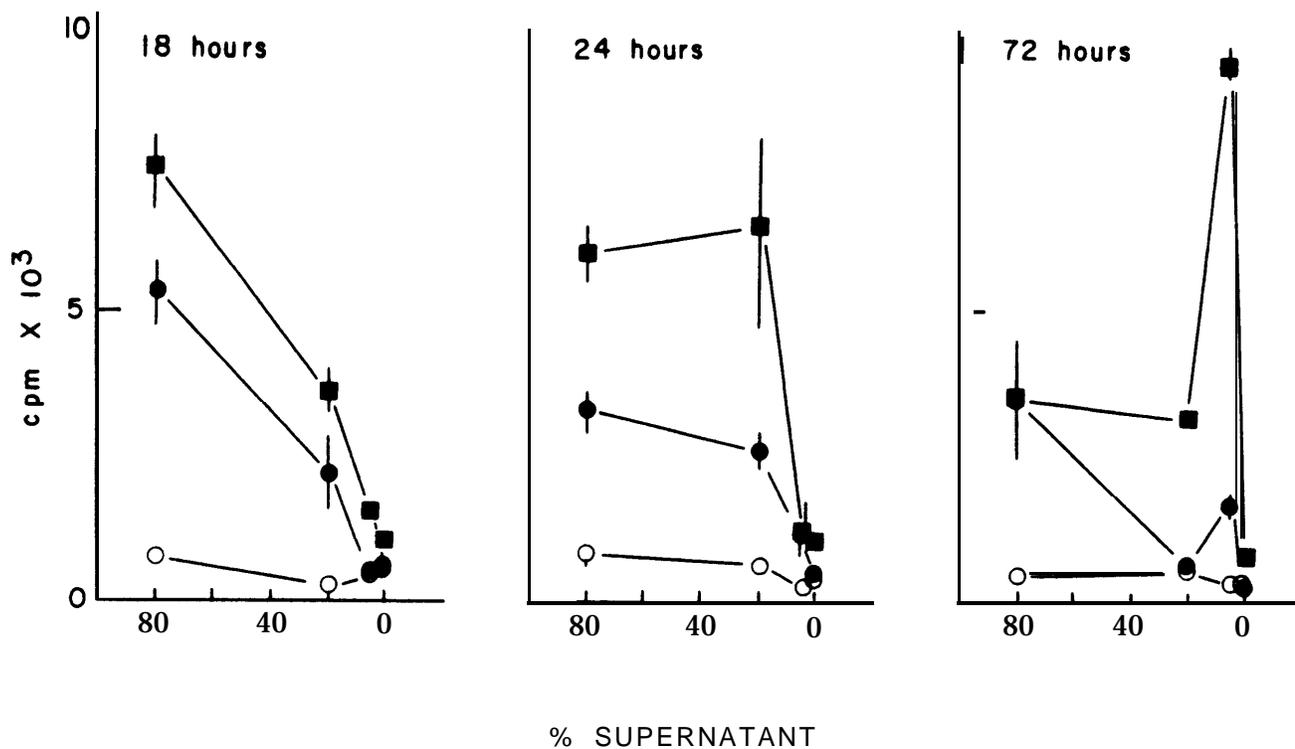


Figure C. Stimulation of spring chinook salmon (*Oncorhynchus tshawytscha*) lymphocytes by supernatants derived from lymphocyte cultures with and without phytohemagglutinin. The ability of anterior kidney lymphocytes to elaborate factors resulting in proliferation by other anterior kidney lymphocytes was examined. 18, 24, and 72 hour phytohemagglutinin induced (■), control (○), and control plus 10 ug/ml fresh phytohemagglutinin (●), supernatants were tested at 1.25, 5, 20, and 30% (v/v) in fresh RPMI 1640. Cells were harvested on day 5. Data are reported as mean cpm \pm one standard error of triplicate cultures.