MASTER

Suppression of the Primary Immune Response
in Rainbow Trout, Salmo Gairdneri,
Sublethally Exposed to Tritiated Water
during Embryogenesis

by

John Albert Strand

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of the requirements for the degree of

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University of Washington

Abstract

SUPPRESSION OF THE PRIMARY IMMUNE RESPONSE

IN RAINBOW TROUT, SALMO GAIARDNERI,

SUBLETHALLY EXPOSED TO TRITIATED WATER

DURING EMBRYOGENESIS

by John Albert Strand

Chairperson of the Supervisory Committee: Professor Allyn H. Seymour
Laboratory of Radiation Ecology

The purpose of this study was to illustrate the effects of radiation on development of the primary immune response in juvenile rainbow trout sublethally exposed to tritium during embryogenesis. Information pertaining to this subject is desired as it is highly probable that with increased application of nuclear energy, greater quantities of potentially harmful radionuclides will be released to the environment. This may be particularly true for advanced reactor designs such as CTR (Controlled Thermonuclear Reactor) where radionuclides, principally tritium, are expected to escape in both gaseous and liquid effluents in quantities significantly greater than for present PWR (Pressurized Water Reactor) and BWR (Boiling Water Reactor) designs.

Antibody synthesis in response to vaccination with a $0.1 \text{ cc } (1.8 \times 10^8 \text{ cells/cc})$ intraperitoneally injected heat-killed strain of Flexibacter columnaris was employed to investigate the effect of tritium irradiation (0, 0.04, 0.4, 4.0 and 40.0 rads total dose for 20 days during embryogenesis) on development of the primary immune response in 5-month rainbow trout, Salmo gairdneri. Rearing throughout the experimental period was conducted under essentially pathogen-free conditions. A standard tube agglutination test was applied to determine agglutinin values to F. columnaris in serum samples at 1-week prevaccination, and 3, 5, 7, 9, and 11 weeks postvaccination. Total

serum protein measurements and electrophoretic separation of blood serum proteins followed by densitometric analyses were performed to assess the potential for qualitative and quantitative changes in blood serum components which conceivably accounted for suppressed immune responsiveness in tritium-irradiated fish. Data on the biological effects of tritium on early life stages in terms of hatchability, abnormality, latent mortality, and growth were also collected.

Specific agglutinins to F. columnaris increased rapidly in both control and irradiated fish following vaccination. However, during the 9th and 11th weeks following vaccination, control fish demonstrated higher agglutinin titers, indicating suppression of the primary immune response in tritium-irradiated fish. Agglutinin levels in irradiated fish were suppressed 50% of control levels at 40.0 rads during the 9th week, and 50% of control levels at 4.0 rads during the 11th week.

While lowered total serum protein concentration was implicated as a possible cause for lowered serum agglutinins in tritium irradiated experimental fish, the decreased levels of total serum protein observed under present experimental conditions could not be correlated to increasing dose of radiation exposure.

Electrophoretic separation demonstrated four major protein fractions in the serum of both control and irradiated experimental fish. Although there occurred variability among experimental fish, fraction I usually contained four bands; fraction II contained four bands; fraction III contained five bands; and fraction IV contained two bands. Fraction II was not well-defined and stained with homogeneous heavy dye. Fraction IV was also not well-defined and stained with a light homogeneous dye. Densitometric analyses indicated that irradiated fish contained a significantly higher proportion of total available serum protein in fraction I; but differed insignificantly

from control fish in the relative proportions of total serum protein contained in fractions II, III, and IV.

Electrophoretic separation of serum proteins of both control and irradiated vaccinated fish revealed that fraction IV contained four distinct bands. Subsequently performed densitometric analyses demonstrated that fraction IV increased significantly in percent of total protein following antigenic stimulation, suggesting that fraction IV represented specific humoral antibody to the immunogen, F. columnaris. These data also revealed that percent of total protein in fractions I and II was lowest in vaccinated fish demonstrating highest agglutinin titers, suggesting that the decrease in fractions I and II occurred in compensation for the increase in fraction IV. Finally, densitometric analyses indicated that the relative percent of total protein contained in fraction IV was significantly reduced in irradiated vaccinated fish suggesting that decreased amounts of fraction IV accounted for lower antibody titers in irradiated fish demonstrated by agglutinin assay.

A review of all experiments conducted at this laboratory directed at determining the effects of early radiation exposure on the parameters of hatchability, incidence of abnormality, latent mortality and depressed growth, revealed considerable variation among similar treatments and indicated that significant effects at dose levels of 50 rads and below were not consistently demonstrated.

While present experimental results demonstrated that the primary immune response in juvenile rainbow trout was significantly suppressed following embryonic exposure to tritium at essentially the 1.0 $\mu\text{Ci/ml}$ level, and perhaps at the 0.1 $\mu\text{Ci/ml}$ level, these concentrations are no less than 5 to 6 orders of magnitude above present levels for tritium in the aquatic environment, and

at least 3 to 4 orders of magnitude above current estimates for future environmental levels.

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INTRODUCTION

It is well documented that many pollutants at less than lethal concentrations exert a "stress" on fish which serves to enhance their susceptibility to disease (Mackie and Menyies, 1938; Fish and Rucker, 1943; Bisset, 1946; Schaperclaus, 1954; Snieszko, 1957, 1958, 1962, 1964; Rucker, 1958; Kingsbury, 1961; Bullock, 1964; Haley et al., 1967; Wood, 1968; Pippy and Hare, 1969; Meyer, 1970; Collins, 1970; Shotts et al., 1972; Mawdesley - Thomas, 1972; and Mahoney et al., 1973). The subject has been recently reviewed by Wedemeyer (1970) and Snieszko (1974).

Wedemeyer (1970) discussed the increase in disease susceptibility which follows non-specific stress in terms of probable physiological and biochemical pathways involved. These included steroid hormone metabolism, inflammatory response, and antibody and interferon synthesis. Snieszko (1974) cited specific examples to show that infection occurred when susceptible fish were exposed to pathogenic microorganisms under certain conditions of environmental stress. He reviewed examples of infectious disease enhanced by stress caused by temperature, eutrophic processes, sewage, pesticides, heavy metals, other industrial pollutants, and metabolic waste products of fish. Snieszko was careful to point out, however, that most present knowledge of the subject was based on casual observation of the coincidence of stress and outbreak of infectious disease, and that few studies were systematically designed to demonstrate the interacting effects of specific pollutants and other stresses on occurrence of infectious disease in fish.

There exists, however, no comparable information base on the potentially stressing effects of radiation on susceptibility of fish to infection, either naturally acquired or experimentally induced. Information that is presently

available is limited to two recently published reports (Shechmeister et al., 1962; Strand et al., 1973). Moreover, knowledge of the effects of radiation on susceptibility to disease that does exist, stems largely from efforts undertaken with mammalian systems.

Information pertaining to this subject is desired in light of current practices and declared future intent to utilize more extensively water-borne animals and plants as food resources. With increasing application of nuclear energy, it is very likely that contamination of surface waters by radioactive materials will increase, with clear potential for harmful interaction with inherent biological systems. This may be particularly true for advanced nuclear reactor designs such as CTR (Controlled Thermonuclear Reactor) where radionuclides, principally tritium, are expected to escape in both gaseous and liquid effluents in quantities significantly greater than for present PWR (Pressurized Water Reactor) and BWR (Boiling Water Reactor) designs (Young et al., 1975).

The studies here described were directed to yield definitive information on the effects of radiation on the expression of the primary immune process in a representative teleost. The primary immune process contributes substantially to immune competence in higher animals and is, therefore, a useful indicator of the degree of susceptibility or resistance of an animal to disease-causing microorganisms. These studies were undertaken to substantiate my earlier findings suggesting that the primary immune response in rainbow trout, Salmo gairdneri, was significantly suppressed following exposure to relatively low concentrations of tritium (1.0, and 10 $\mu\text{Ci/ml}$) as tritiated water (Strand et al., 1973).

Additional studies were developed which focused attention on the nature of the stressing effect of irradiation on immune competence. These latter

studies emphasized measurement of total serum protein and electrophoretic separation of blood serum proteins to assess the potential for qualitative and quantitative changes in blood serum components which conceivably accounted for suppressed immune responsiveness in tritium-irradiated test fish.

Data on the biological effects of tritium as tritiated water on early life stages in terms of hatchability, abnormality, latent mortality, and growth were also collected.

LITERATURE REVIEW

Before undertaking a review of the literature pertinent to understanding the effects of radiation on the immune response, it should be noted that "immune competence" is a general term embracing several different forms of the immune response observed in animals inclusive of man. Any consideration of the effects of radiation must, then, be made separately for each such form of the immune response and, in some cases, for separate components of a given form of the immune response. However, this does not imply that the different forms of immunity are controlled by different mechanisms, but rather a few basic mechanisms exist within which slight variations occur as expressed in different species or under different conditions.

The two basic forms of immune processes are: 1) humoral antibody immunity, which entails the synthesis of circulating antibody molecules found in either serum or other body fluids; and 2) lymphocyte-induced cellular immunity, in which the actual site of the immune response contains both lymphoid cells and macrophages.

The morphological and haematological distinction of the immune response into humoral and cellular forms is diagrammatically illustrated in Figure 1. Differentiation of the stem cell into lymphocytic components is directed by thymic induction; whereas differentiation of plasma cells is by the bursa of Fabricius or its equivalent, although certain cells that are morphologically lymphocytes are also concerned with humoral immunity (B-lymphocytes). The complete maturation of active immunocytes of lymphoid and plasmacytic immunocompetent cells subsequently follows antigenic stimulation.

FIGURE 1. Development of the haemopoietic system in mammals from a common stem cell. The first division demonstrates a possible dichotomy involving a lymphoid stem cell. One type then differentiates into thrombocytes, erythrocytes, or granulocytes and monocytes, under the induction of platelet factors (PF), erythropoietin (EP), or colony stimulating factors (CSF). Immunocyte differentiation is from antigen (AG) induced stimulation of immunocompetent precursor cells (ICC) which have differentiated under the control of thymus or bursa equivalent (from United Nations, 1972).

My review of the subject, however, as it pertains to the present studies will necessarily concern radiation effects on humoral antibody immunity.

I. NATURE OF MAMMALIAN ANTIBODY RESPONSE

Stimulation by an antigen is generally followed by a delay of a few days in mammals before circulating antibody is detectable in serum. Several steps leading to synthesis and ultimate secretion of antibody are identified and are illustrated in Figure 2. These include: 1) transport of the antigen to immunocompetent cells, 2) proliferation of immunocompetent cells, and 3) differentiation of plasma cells which ultimately synthesize and secrete specific antibody.

Extensive studies of mammals (Good and Papermaster, 1964) have indicated that nearly all gamma globulin components serve as antibodies. Other components of mammalian serum which have properties of immunoglobulins demonstrate electrophoretic mobility of slow beta globulins.

Antibodies are subdivided into different immunoglobulin classes on the basis of salient physical, chemical, and biological properties, for example, as in Table I, for man: IgM, IgA, IgG, IgD, and IgE. Each class has in common a basic molecular structure of two light (L) and two heavy (H) polypeptide chains (Edelman and Poulik, 1961; Porter, 1959). The molecule is held together by disulfide bonds (-S-S-). In part of each chain the amino acid sequence is identical in all molecules of the same type; this is the constant region. There is also a variable region where the amino acid sequence is different from molecule to molecule. Biological properties of the molecule are controlled through sites on the constant part of the heavy chains (Fundenberg and Warner, 1970), and since the classes differ in their heavy chains, a given biological effect is usually controlled by only one or a limited

FIGURE 2. Stages of antibody formation within a mammalian lymph node. Antigen (AG) first requires processing by macrophages (M) or follicle cells (FC) (afferent stage), then is transferred in a suitable form to the thymus derived (T) and/or bursa (or bursa equivalent) derived (B) lymphocytes for induction of the immune response. This stage may require collaboration between (T) and (B) cell types. The (B) lymphocyte then becomes the progenitor of the antibody (AG) producing cell clone (efferent stage), giving rise to the immunoblasts (IB) or immature plasma cells, and then to mature plasma cells (PC) (from United Nations, 1972).

TABLE I. Human Immunoglobulin (Ig) Classes

Class	Biological Properties	No. Subclasses	Serum Conc. (Mg 1%)	S Constant	Mol. Wt.	Carbohydrate (% W/W)
IgG	Complement fixation; placental transfer	4	1200	6.6 - 7.2	150-160,000	2.9
IgA	Localized protection in external secretions	3	150	7 - 17	150-160,000 (monomer)	8
IgM	Complement fixation, early immune response	2	75	18 - 20 30 - 150	900,000 (pentamer)	12
IgD	Unknown	--	0.3 - 30	7	160-180,000	12
IgE	Reagin activity; mast cell fixation	--	0.01-0.07	8	200,000	11

number of the immunoglobulin classes. However, the constant regions do not affect the specificity of an antibody for an antigen. Instead, it is the variable region of both light and heavy chains that are responsible for recognizing and binding antigen.

More conveniently, identification is made according to biological property or the type of specific secondary reaction with which the different classes associate. An antibody is designated as a hemolysin if it lyses red blood cells in the presence of complement, a normal component of serum. It is designated a precipitin if it precipitates a soluble protein in the presence of electrolytes. It serves as an agglutinin if it elicits a clumping or suspension of bacteria, red blood cells, or other particulate materials. It may also be an anaphylactin or antitoxin. Antibodies of the same specificity may vary in physical-chemical structure, but at the same time an antibody preparation containing only one type of molecule may serve as a lysin, precipitin, or agglutinin, depending on how the antigen is manipulated and what vehicles are provided.

As it pertains to the present studies, following antigenic stimulation with certain bacterial antigens, mammals are reported to produce initially a 19S (IgM) antibody which is antigenically distinct from a 7S (IgG) antibody subsequently produced.

II. EFFECT OF RADIATION ON SUSCEPTIBILITY TO INFECTION

As early as 1909, Lwen reported that radiation injury enhanced susceptibility of rabbits to infection with Staphylococcus spp., and rats and mice to Bacillus anthracis (Taliaferro and Taliaferro, 1951). From 1909 to 1950, some sixteen researchers (Angevine and Tuggle, 1941; Burrows, et al., 1950; Clemmesen, 1939; Clemmesen, et al., 1948; Corper and Chovey, 1920; DeGara and Furth, 1945; Gear and Davis, 1942; Gowen and Zelle, 1945; Liu, et al., 1941; Morton, 1916; Murphy and Ellis, 1914; Roberts, et al., 1939; Schwienhorst, 1928; and Smith, et al., 1954) reported that irradiated animals were less capable of combating experimentally-induced infections attributed to a variety of bacteria, rickettsia, and viruses. In 1950, Miller, et al. re-emphasized the implications of infection following radiation insult. By 1954, Schechmeister, employing varying experimental designs and mammalian hosts, demonstrated that resistance to 20 different infectious agents was suppressed following exposure to ionizing radiation. At present, more than 1000 independent observations in support of these earlier findings are reported in the literature (United Nations, 1972).

III. EFFECT OF RADIATION ON MAMMALIAN ANTIBODY RESPONSE

Among the several radiosensitive immune processes of the host acting during the course of infection, the effects of radiation on antibody production are best understood.

Most early literature on the subject deals with the suppressing effect of ionizing radiation on the primary antibody response. Taliaferro and Taliaferro (1951) have conducted an exhaustive review of the literature through 1950. A more recent review conducted by Talmage and Taliaferro (1955) devoted considerable attention to radiation effects on specific secondary antibody responses. A review of the extensive efforts undertaken since 1950 is found in the recently-published report (1972) of the United Nations Scientific Committee to the General Assembly entitled "Effects of Atomic Radiation."

A. Time of Irradiation and Antigenic Stimulation

Ionizing radiation is reported to have a singular effect on formation of the antigen-antibody complex, whereas antibody synthesis is suppressed in proportion to the dose of exposure. Antibody production is suppressed in relation to total radiation dose and the time of antigenic stimulus.

Benjamin and Sluka (1908) were the first to systematically study the effect of ionizing radiation on antibody production. They studied the effects of total-body X-irradiation on precipitin formation in rabbits receiving injections of beef serum. While the precipitin reaction was inhibited when the rabbits were irradiated 3 days before injection, no effect was observed when X-irradiation was administered 3 days after injection. Also, precipitin titers in immunized rabbits were not immediately altered by X-irradiation.

In general, these earlier findings have since been substantiated by similar experiments conducted with other species. Kohn (1951) demonstrated suppression of the primary hemolysin response in rats when exposed to 300 R of X-irradiation at varying times during the week before and 5 days after injection with sheep red blood cells. X-irradiation at 175 R administered

3 days before injection elicited greater suppression of the hemolysin response than 600 R administered 2 days after injection. Kohn (1951) hypothesized several explanations to account for the observed difference in radiosensitivity: 1) development of radiation injury required time, 2) fixation of the antigen may be impaired, 3) synthesis of antibody required several steps of which the first was most radiosensitive, and 4) cells actively engaged in antibody synthesis may be less sensitive to radiation injury.

Fitch et al. (1953) studied the ability of the rat to form hemolysin when a single intravenous injection of sheep red blood cells or typhoid vaccine was administered a few days before or 1 and 6 days after receiving 500 R X-irradiation. Dixon et al. (1952) using ^{131}I labeled bovine gamma globulin, and Taliaferro et al. (1952) applying sheep red blood cells, also studied the time of antigenic stimulation in relation to X-irradiation in rabbits. All such researchers found an early radiosensitive and a later more radioresistant stage.

B. Basis of Radiation Injury

The suppressing effect of radiation on immune processes is thought not to be attributed to a direct inhibition of metabolic activities already being conducted by affected cells. Rather, the suppressing nature of radiation is more closely associated with the very general response of cells to lose their ability to reproduce or divide, as most studies concerned with the cellular basis of both the radiosensitive and radioresistant phase of antibody production suggest that proliferation is an essential feature of the early immune response. It is for this reason that the antibody forming cell is not considered relatively radiosensitive. It is a highly differentiated cell whose function is to secrete humoral antibody and it is believed not to further divide. It is indicated by a growing body of evidence (Till and

McCulloh, 1961; McCulloh and Till, 1962; Siminovitch et al., 1965; Dougherty, 1952; Caffey, 1956; Nossal, 1967; Taliaferro and Taliaferro, 1951; Claman and Chaperon, 1969; Goldie and Osoba, 1970) that the suppressing effect of radiation occurs at a time before the differentiation of the antibody cell. It is, furthermore, suggested that the sequence of differentiation is sensitive at three points of cell proliferation: 1) the haemopoietic stem cells, 2) the early differentiated cells of the thymus, and 3) the antigen stimulated immunocompetent cells.

C. Life Stage Dependence

Little information is available on the effects of irradiation on immune competence at varying life stages or age. For the most part, the review of the literature above encompasses effects on the adult animal. Information on early life stage effects are important as these stages are clearly indicated as being the most radiosensitive.

In a study of mice, rats, guinea pigs, and rabbits administered a daily exposure of 1.2 to 4.3 R for 1.5-2.0 years, the strongest suppression of natural immunity occurred in young animals, and in particular when radiation was administered during uterine development (Kiselev and Buzini, 1959). In a related study on the effects of low exposure levels 4-65 R, delivered each day for 5 days to pregnant rats with fetuses at 16 days of embryonic development, there resulted measurable inhibition of agglutinins in response to typhoid vaccine when the progeny were vaccinated at 2-5 months of age (Pelts, 1966). A reduced phagocytic activity of the blood leucocytes also occurred.

There appear in the literature no accounts of radiation effects at earlier stages of development, and in particular at a time when progenitor cells

of immunocompetent cells are first differentiated. It is conceivable that very low doses could potentiate permanent injury if delivered over this stage of development.

D. Single Exposure

Studies of radiation suppression of antibody production have generally yielded D_{37} values of 50-100 rads. Thus, doses of radiation in this range, when administered to the whole animal, have usually shown some significant degree of suppression of antibody production. A 75% reduction in antibody forming plaques was found in mice given 50 rads 10 days prior to injection (Kennedy et al., 1965). Dixon et al. (1952) reported a significant reduction in antibody formation with 75 R, and 125 R gave considerable suppression. In dose-effect studies which have embraced the 50 or 100 rad range, increasing dose yielded proportionately greater suppression of antibody production.

Enhancement of the antibody production at low doses has also been observed. An elevated peak titer, reduced latent period, and increased production of antibody were all recorded in rabbits administered 25 rads 2 days to 2 hours after antigenic stimulation (Taliaferro and Taliaferro, 1954). Extended haemolysin production was also observed in rabbits given 25 rads 1 month prior to antigenic stimulation (Taliaferro and Taliaferro, 1969).

Data on the effects of single radiation doses below 50 rads are few, and in most situations no significant effects are reported as determined in the whole animal. However, a change in the morphology and motility of small lymphocytes following in vitro exposures of 2-5 R was reported by Stefani and Schrek (1964).

The problem, then, is that it is difficult to extrapolate from the effects of high doses to the effects of low doses. The immune response as a

whole is comprised of several separate components, each differing in radiosensitivity. At moderate to high doses, several or all components may be affected, but at low doses only one may be susceptible. The real question is, therefore, whether any of the components are radiosensitive below the 50 rad level.

E. Fractionated and Continuous Exposure

In considering the effects of fractionated and continuous dosage, one must first understand that haemopoietic stem cells continue to provide, throughout the life span of the animal, an input of differentiated stem cells which eventually give rise to the varying forms of immunocompetent cells. It is further understood that it is a remote possibility that the entire immune response would fail, except perhaps in the situation of extremely high dose levels.

The evidence in the literature on this particular aspect is again quite sparse, but it does provide an estimate of the exposure level that is necessary to achieve immune suppression. In rabbits administered 4-5 R per day to a cumulative exposure of 356-2039 R, no functional disturbance of antibody formation in response to injection with parathyphus vaccine was observed (Dzhikidzeh and Aksenova, 1964). Also, in monkeys, a daily exposure of 1.34 R up to a total of 675 R did not affect the production of antibody to tetanus toxin (Dzhikidzeh and Aksenova, 1964). However, in a study on pathogen-free mice exposed to 1-4 R per hour, the ability of irradiated animals to produce antibodies to some but not all antigens was inhibited at sublethal dose levels (Stoner and Hale, 1963). However, in considering the effects of fractionated doses, it is generally agreed that it is difficult to assess the actual effect of a fractionated dose that would have caused significant suppression if it had been given as a single dose.

The main issue of relevance is whether repeated small doses or low-level continuous irradiation give rise to an accumulation of injury, or whether repair following small doses is complete, and thus adaptation to repeated exposure occurs. The concept of adaptation implies that cells of the antibody forming precursor series are all equally radiosensitive. They are continuously replaced throughout the life span of the organism and there exist many more undifferentiated immunocompetent cells than are usually required to achieve a given level of immune responsiveness. Thus, if an organism normally produces only 10% of the total immunological potential, the cell population may well tolerate 90% loss from continuous exposure to radiation without concomitant loss of immune responsiveness. In studies conducted by Kennedy et al. (1965), it was suggested that the proliferative capacity of the immune system may be depleted by a thousandfold without completely losing the ability to respond to antigenic stimulation. Makinodan and Price (1973) also stress the reserve of immunocompetent cells that remain unexpressed under normal conditions of antigenic stimulation.

The only exception to this concept, however, is perhaps when irradiation is administered early in ontogeny, prior to differentiation of the haemopoietic stem cell.

F. Internal Emitters

The literature contains accounts of a great number of studies on the effects of internally deposited radionuclides on immune responsiveness. A single subcutaneous injection of 0.5 mCi per kg ^{210}Po or a single intraperitoneal injection of 0.05 mCi per kg ^{90}Sr administered to guinea pigs did not affect the primary immune response, but did lead to a marginal reduction in secondary immune responsiveness (Lvitsina, 1966). A single intraperitoneal

injection of tritium oxide at a dose of 0.3 Ci per kg (total dose 400 rads) suppressed immunologic activity significantly in dogs, and was correlated with other clinical manifestations of radiation disease (Shalmova and Zhuraveev, 1966). Howard and Jannke (1970) found that ^{90}Sr fed daily at a rate of 625 μCi per day to Pitman-Moore miniature swine over a period of 10 months markedly impaired the antibody synthesizing process in response to heat-killed Brucella abortus vaccine administered subcutaneously or intramuscularly. Dilley and McDonald (1970) reported that dogs that had inhaled aerosols of $^{238}\text{PuO}_2$ showed a significant depression in heterophile antibodies to washed sheep erythrocytes and a reduced half-life of antibodies formed.

It is generally concluded that the changes in immunogenesis attributed to internal emitters are marked by periods of suppression and periods of normalization, and occasionally stimulation. Internal irradiation is accompanied by a generally stronger suppression of secondary responsiveness. It is assumed that when animals are irradiated internally, the long periods over which dose is accumulated slows down restorative or repair processes.

IV. NATURE OF TELEOST ANTIBODY RESPONSE

The immune response in poikilothermic animals including teleost fish is well-documented (Weinreb, 1958; Hirsch, 1959; Sundberg, 1955; Engle et al., 1958; Becker et al., 1958; Pliska, 1938; Cushing, 1942; Bisset, 1948; Klontz, 1959; Post, 1963; Ridgeway et al., 1966; Hodgins, 1967). Most mechanisms of immunity found in mammalian systems can be demonstrated: agglutinating and precipitating antibody, anaphylaxis, delayed hypersensitivity, anti-viral antibody, and cellular response following antigenic challenge. Not unlike mammals, young forms have a well-developed thymus. The spleen, gut-associated peripheral lymphoid, and gonadal lymphoid tissues all demonstrate relatively advanced structure.

There appears to be a close correlation between immune response and environmental temperature. The range of temperature dependence is related to the fish's natural environment. For example, in the warm water carp, Cyprinus carpio, antibody may be induced at 20-25°C, but not at 12°C, whereas the sablefish, Anoplopoma fimbria, from colder waters in the North Pacific, produces antibody at 5-8°C. The induction phase of antibody production in fish is prolonged in relation to mammals from 18 to 30 days in cold and temperate species, although some warm water species produce detectable circulating antibody in less than 9 days. The antibody titers in fish, however, often exceed the titers obtained in mammals, and the circulating antibody can persist for much greater lengths of time, up to 1 year in some cases.

As in mammals, teleosts likely are capable of protein changes within the blood serum proteins which would account for the presence of antibodies. However, little evidence is found which provides positive identification of the type of serum protein component that contains the antibody molecule. It was believed that some teleosts did not possess circulating gamma globulin. However, the application of sensitive immunoelectrophoresis has revealed that certain species possess amounts of circulating gamma globulins or functionally analogous molecules. These species also lack large numbers of plasma cells which is usually correlated with low levels of circulating gamma globulin. The deficiency in teleosts of both plasma cells and circulating gamma globulin is thought to be due to low levels of antigenic stimulation. However, following injection with such antigens as rabbit red blood cells, freshwater teleosts produced detectable levels of immunoglobulin directed against the immunogen (Ridgeway et al., 1966). An antibody activity attributable to the 7S fraction of gamma globulin occurred in this group. Following antigenic stimulus, goldfish, Carassius auratus, produced 19S antibody, but when

water temperature was raised, a conversion to 7S antibody occurred (Abranoff et al., 1970). Rainbow trout isoimmune haemagglutinating antibodies resided in a serum fraction which contained 10.5S and 13.5S components (Hodgins et al., 1967). They migrated as B-globulins as defined by immunoelectrophoresis. However, no large to small molecule shift in production analogous to mammals and other teleost was observed with either rainbow trout isoimmune haemagglutinating or immune antibody. Several other references indicated the complete lack of a serum protein component in teleosts which has similar electrophoretic mobility to the gamma globulin component in higher mammals. The implication is also made that the lack of this serum protein fraction accounted for the moderate capacity of these fish to produce measurable antibody.

V. EFFECT OF RADIATION ON TELEOST ANTIBODY RESPONSE

In contrast to mammalian systems, the effects of radiation on disease susceptibility in teleosts are poorly understood. In recent years, only two papers embracing this subject have appeared in the literature.

The increase in susceptibility of irradiated goldfish, Carassius auratus to an experimentally induced infection, Aeromonas salmonicida, and to a naturally acquired ectoparasite trematode, Gryodactylus spp. was observed by Shechmeister et al. (1962).

In this study, it was noted that the effect of radiation on susceptibility to infection occurred as a function of radiation dose. This was indicated by the time to death of test fish injected with graded doses of A. salmonicida and exposed to 1000 and 2000 R of X-irradiation. In fish exposed to 1000 R, all mortality occurred from 4 to 20 days after injection, whereas the time of mortality after exposure to 2000 R varied from 5 to 10 days after injection. It was noted that noninjected fish irradiated at

1000 R died between 32 and 112 days; noninjected test fish exposed to 2000 R died between 10 to 59 days. No mortality was observed in noninjected-nonirradiated or injected-nonirradiated control test fish.

Exposure to radiation also affected susceptibility to the ectoparasite trematode, Gyrodactylus spp. Of nonparasitized test fish exposed to 100 R X-irradiation only 2.5% died by postirradiation day 36, whereas 78% of the parasitized fish similarly exposed died by day 36. Test fish not infected with Gyrodactylus spp. exposed to 100 R survived for 350 days. Mortality in a 1000 R nonparasitized group reached 7% in 33 days after irradiation, while 91% of parasitized group exposed to 1000 R died in 17 days. No mortality in nonirradiated-nonparasitized control fish occurred in 45 days; however, 28% of nonirradiated but parasitized control fish died in 28 days.

In more recent studies, antibody production in response to a naturally occurring fish pathogen, Flexibacter columnaris, was employed to investigate the effects of irradiation on susceptibility of disease in juvenile and adult rainbow trout. Radiation was administered over the first 20 days of embryogenesis in the form of tritiated water at 0, 1.0, and 10.0 μCi per ml, and resulted in an estimated total dose of 0, 5.8, and 58.1 rad (Strand et al., 1973).

Bimonthly agglutination assays to measure the increase in humoral antibody were initiated after juvenile test fish were 9 months of age and had received their first antigenic stimulus from naturally occurring F. columnaris pathogens in hatchery rearing waters supplied by the Columbia River. Yearling test fish, irradiated during embryogenesis the previous year, were similarly examined after receiving their second seasonal challenge to F. columnaris.

The results of agglutination assay demonstrated that the percentage of test fish with agglutinins for F. columnaris and the titers of all test groups increased over the summer-autumn test period following seasonal challenge. During the subsequent winter-spring test period, the percentages of test fish exhibiting positive titers were markedly reduced. All irradiated groups demonstrated an increase in titer over the summer-autumn test period. The control groups, however, demonstrated higher titers which suggested a suppression of immune responsiveness in irradiated test fish.

As shown in Table II, a Chi-square (χ^2) test of independence was applied to the agglutinin data to determine if irradiation of the embryos significantly lowered immune responsiveness in juvenile and yearling test fish. Analysis of the data for juvenile control and irradiated test fish revealed a significant decrease in antibody titers in the irradiated test group over the last two summer-autumn sampling periods; however, no significant difference was observed between the 1.0 and 10.0 μCi irradiated test groups. The yearling rainbow trout, irradiated at 1.0 μCi per ml as embryos, demonstrated a significant decrease in antibody titer over the last three summer-autumn sampling periods.

These data indicated a sufficient incidence of F. columnaris exposure and infection occurred during summer and autumn months to account for the high percentage of fish with agglutinins and the relatively high titers in all test groups. During winter and spring months, a low incidence of infection and exposure likely contributed to a marked decrease in the numbers of fish exhibiting positive titers. Previous studies demonstrated that antibody levels in rainbow trout were reduced significantly during winter months in response to colder Columbia River temperatures, 3.4 to 5.0°C (Fujihara and Nakatani, 1971). These results also suggested that there was a carry-over of

TABLE II. Comparison of Agglutinin Data Between Control Fish and Those Exposed to Tritium Utilizing Chi-Square Test of Independence on Titer Values Arranged in Contingency Tables (Strand et al., 1973)

Sample Date	<u>Juveniles</u>						<u>Yearlings</u>		
	<u>Cont. vs ^3H</u>			<u>1 μCi vs 10 μCi</u>			<u>Cont. vs 1 μCi</u>		
	<u>d.f.</u>	<u>χ^2</u>	<u>Level</u>	<u>d.f.</u>	<u>χ^2</u>	<u>Level</u>	<u>d.f.</u>	<u>χ^2</u>	<u>Level</u>
8-10	--	--	--	--	--	--	1	2.06	NS
8-23	3	6.46	NS	2	3.53	NS	3	6.94	NS
9-8	4	7.11	NS	2	2.22	NS	4	10.50	a
9-20	3	11.66	b	3	1.43	NS	4	13.71	b
10-7	4	33.61	b	2	0.18	NS	4	11.66	a

NS - Non-significant Chi-square at 0.05 level indicating distribution of titer values was independent of treatment.

a - Significant Chi-square at 0.05 level indicating the distribution of titer values was dependent of treatment.

b - Significant Chi-square at 0.01 level.

agglutinins produced as juveniles. The low but yet detectable titers in test fish sampled in winter months likely represented residual antibody produced in response to exposure and infection the previous summer. Finally, these results suggested that immune responsiveness was permanently altered by irradiation during embryogenesis. Both juvenile and 1 year sub-adults, after their respective first and second antigenic stimulus, demonstrated significantly lowered antibody titers than control test groups.

VI. SUMMARY

In summary of the literature, our knowledge of the effects of radiation on susceptibility to disease is based largely on studies undertaken with

mammalian systems. Among the several radiosensitive immune processes of the host acting during the course of infection, the effects of radiation on antibody production are best understood.

While radiation is reported to have singular effect on formation of the antigen-antibody complex, normal antibody production is suppressed in proportion to the dose of exposure. The degree of suppression is related to the time of antigenic stimulus, and to total radiation dose. In general, the immune response is most radiosensitive when irradiation is delivered prior to antigenic stimulation. Doses of radiation in the 5-100 rad range, if delivered to the whole animal, usually produce a significant suppression of antibody synthesis.

The suppressing nature of radiation is believed to be closely associated with the very general response of cells to lose their ability to reproduce or divide, as most studies concerned with the cellular basis of both the radiosensitive and radioresistant phase of antibody production suggest that proliferation is an essential feature of the early immune response.

The suppressing effect of radiation on antibody production occurs at a time before the differentiation of antibody-forming cells. As the haemopoietic stem provides for input of differentiated immunocompetent cells throughout the life of the animal, it is most likely that the immune response would readily adapt to low doses of irradiation. The only exception to this is whether irradiation is administered early in ontogeny. However, there appear in the literature no accounts of radiation effects over the very early stages of development, and in particular when progenitor cells of immunocompetent cells are first differentiated. It is conceivable that very low doses may result in permanent injury if delivered over this stage of development.

It is generally concluded that changes in immunogenesis attributed to internal emitters are marked by periods of suppression, normalization, and occasionally stimulation. It is assumed that when animals are irradiated internally, the long periods over which the dose is accumulated decreases restorative or repair processes.

Most immune mechanisms found in mammalian systems can be demonstrated in fish: agglutinating and precipitating antibody, anaphylaxis, delayed hypersensitivity, anti-viral antibody, and cellular response following antigenic challenge. Young forms demonstrate a well-developed thymus and other lymphoid tissues. As in mammals, fish are capable of protein changes within blood serum proteins which would account for the presence of circulating antibodies. However, positive identification of the protein component that contains the antibody molecule has not been made.

There exists no comparable information base on the effects of radiation on primary immune responsiveness in fish. It would appear that the effect of radiation on susceptibility to infection occurs as a function of radiation dose, and that enhanced susceptibility in adult forms occurs at doses as low as 100 rads. Early life stages appear to be more radiosensitive, perhaps at doses as low as 5 rads; and if irradiation occurs early in ontogeny, immune responsiveness may be permanently altered. However, few studies have been designed to systematically assess this relationship.

OBJECTIVES

A number of objectives were proposed which would yield information on the potential latent effect of radiation on primary immune responsiveness in rainbow trout attributed to early life stage exposure:

- To expose the fertilized embryos of rainbow trout to concentrations of tritium as tritiated water over a range of 0, 0.01, 0.1, 1.0, and 10.0 $\mu\text{Ci/ml}$.
- To produce immune bodies against the pathogenic microorganism, Flexibacter columnaris, in both juvenile control and irradiated rainbow trout.
- To determine the potential for suppressed levels of immune bodies in juvenile rainbow trout in relation to the dose of tritium irradiation administered during early embryogenesis.
- To establish which serum fraction contains the specific antibody and attempt to characterize the fraction into specific albumins or globulins.
- To determine the potential for qualitative and quantitative changes in blood serum components of tritium-irradiated rainbow trout which conceivably account for suppressed immune responsiveness.

These more recent studies employed an experimental approach which embraced greater control of potentially interacting environmental factors. Through the duration of these studies, the experimental organisms were reared under essentially pathogen-free conditions. Also, to stimulate primary immune responsiveness, a vaccination approach employing a heat-killed vaccine was substituted for challenge by naturally occurring pathogens in hatchery rearing waters. Moreover, the experimental design was developed to permit estimation of error attributed to pond position, and compartment position within each pond.

METHODS AND PROCEDURES

I. TEST ORGANISM

Brood stock for spawning were obtained from a Kamloop strain of rainbow trout, Salmo gairdneri, maintained at the Tacoma and McMillin hatcheries of Trout Lodge, Inc., Tacoma, Washington.

II. RADIATION EXPOSURE

Approximately 15,000 eggs were stripped from twelve 4-year female fish and fertilized immediately with sperm collected from twelve 4-year male fish. The embryos were well-mixed, divided into five equal groups, and immersed in five concentrations (0, 0.01, 0.10, 1.0, and 10.0 μCi per ml) of tritium- (biological grade) spiked spring water (pathogen free).

Rearing through 20 days of embryogenesis at $10.5 \pm 0.2^\circ\text{C}$ was facilitated within five recirculation drip incubation systems of 150 liter capacity as shown in Figure 3. Tritium-spiked rearing water was recycled at a rate of 15 liters per minute. Dual pumping capacity controlled by a hydrostatically-operated level sensing device was provided for each unit in the event of pump failure. Temperature stability to $\pm 0.2^\circ\text{C}$ was maintained by a stainless steel heat exchanger installed in the reservoir of each unit. All five units were self-contained and connected by flexible ducting to the main laboratory air exhaust system. This served to maintain negative pressure across each incubation system, thus lessening the opportunity for volatilized tritium to escape into the laboratory atmosphere.

FIGURE 3. Recirculation drip incubation system (Heath Techna - Plastics, Inc., Kent, Washington), employed in the exposure of rainbow trout embryos to tritiated water. Fresh water was pumped at a rate of 15 liters per min from reservoir (a), to level sensing tank (b), then through air equilibrator (c) into rearing trays (d), then returned to reservoir.

III. TRITIUM ACCUMULATION AND RETENTION

As the embryos developed in the five concentrations of tritiated water, five replicate embryo and water samples were collected at each of 12 sampling intervals over the 20-day exposure period to determine accumulation. Embryos were similarly sampled to determine retention after transfer to a tritium-free environment. All such embryo samples were immediately frozen and stored at -40°C until analyses.

Distinction between tritium in tissue water and in the remainder of the embryo was emphasized.

A. Incubation Water Tritium

The samples of incubation waters were analyzed for tritium by adding 0.1 ml of the aqueous sample to 19.9 ml of a liquid scintillation solution.

B. Tissue-free-Water Tritium

The tissue-free-water tritium (TFWT) was extracted in a Model 300 Tri-Carb Sample Oxidizer (Packard Instrument Corp., Inc., Downers Grove, Illinois). The membranes of each embryo were ruptured by puncture with a sharp scalpel blade. The embryo was then immediately placed within the oxidizer unit and subjected to an instrument heat of 200°C . A low flow of N_2 gas was used to sweep the evolved volatile tritium fraction into a liquid scintillation solution. This procedure was carried out for a period of 45 minutes.

C. Tissue-Bound Tritium

At the end of 45 minutes, a fresh scintillation vial was emplaced in the oxidizer unit and the residue of the embryo was combusted in an oxygen stream to obtain the tissue-bound tritium (TBT) fraction. The water of combustion was trapped directly by the liquid scintillation solution.

D. Liquid Scintillation Solution

A liquid scintillation solution prepared by dissolving 100 g of naphthalene, 5 g PPO, 0.2 g POPOP in a mixture of 720 ml of dioxane, 135 ml toluene, and 45 ml methanol was used for all analyses.

E. Liquid Scintillation Counting

Counting of samples was conducted on a Mark II liquid scintillation system (Nuclear-Chicago Corp., Des Plaines, Illinois).

IV. ESTIMATION OF DOSE

The estimated beta dose to embryos developing in tritiated water was calculated by the method described by Hine and Brownell (1956). Two basic assumptions were made: 1) tritium was uniformly distributed within the embryo, and 2) no concentration of tritium in embryos above a water equilibrium level occurred. The dose equation was as follows:

$$D_{\beta} = 2.13 \bar{\epsilon}_{\beta} C; \text{ where:}$$

$$D_{\beta} = \text{dose in rads per hour,}$$

$$\bar{\epsilon}_{\beta} = \text{average beta-ray energy in MeV (0.00569), and}$$

$$C = \text{concentration of } ^3\text{H in } \mu\text{Ci per ml.}$$

V. POSTIRRADIATION MAINTENANCE

At 20 days (eyed stage), in order to deplete tritium burdens, the embryos were transferred to a single-pass drip incubation system maintained on thermally-controlled, $10.5 \pm 0.2^{\circ}\text{C}$, dechlorinated sanitary water. Chlorine content as total residual chlorine determined by the amperometric method

(A.P.H.A., 1971) was reduced from ~300 ppb to <10 ppb by the facility described in Figure 4. Other water quality parameters inclusive of dissolved oxygen, nitrogen, and pH were monitored routinely over this period. Hatching occurred at 28 ± 1.5 days.

The effects of each of the five treatments were assessed in terms of the proportions of embryos hatching and the numbers of obviously abnormal fry produced. Anomalies scored included gross malformations of the head and body. Throughout this period the embryos were inspected each day and all dead embryos carefully removed. Infertile eggs were not removed until 20 days after fertilization or when the embryos were firmly eyed.

At 14 days after hatch, the fry were transferred to five (3 m x 0.6 m x 0.3 m) compartmented fiberglass rearing troughs also maintained on thermally-controlled dechlorinated sanitary water, Figure 5. Tritium irradiated and control treatment groups were each subdivided and arranged among the available compartments of the five trough array according to Table III. Rearing temperature was increased from $10.5 \pm 0.2^\circ\text{C}$ to $20.0 \pm 0.5^\circ\text{C}$ at a rate of $2.0 \pm 0.5^\circ\text{C}$ per week.

A final transfer of the fingerlings to five (3 m x 1.2 m x 1 m) compartmented concrete rearing ponds also maintained on thermally controlled, $20.0 \pm 0.5^\circ\text{C}$, dechlorinated sanitary water was made 3 months after hatching, Figure 6. Tritium-irradiated and control treatment groups were arranged as before. The dechlorination facility described in Figure 7 efficiently maintained total residual chlorine of incoming laboratory sanitary water at <10 ppb. Other water quality parameters inclusive of dissolved oxygen, nitrogen, and pH were monitored routinely. Bacteriological examination of rearing waters to detect the presence of Flexibacter columnaris and other potential pathogens was also conducted routinely.

FIGURE 4. Dechlorination units employed during the initial 3 months of study. This facility was fabricated onsite and consisted of two vertical cylinders (1.5 m x 0.2 m) plumbed in series. Each unit contained 0.2 m³ of activated carbon and supplied a total flow of 37.8 liters per minute to either a single pass drip incubation system or to five - 3 m x 0.6 m x 0.3 m fiberglass rearing troughs. The source of water to be dechlorinated was supplied by the conventional sanitary water treatment plant at the Hanford Atomic Energy Works.

FIGURE 5. Compartmented fiberglass rearing troughs (3 m x 0.6 m x 0.3 m) employed in the rearing of irradiated rainbow trout over the initial 3 months of study.

FIGURE 6. Compartmented rearing ponds (3 m x 1.2 m x 1 m) employed in the rearing of irradiated rainbow trout for the latter 6 months of study.

DEFIANCE BOND

TABLE III. Arrangement of Tritium-Irradiated and Control Test Groups in Five Trough or Concrete Pond Array According to Latin Square Sampling Design*

<u>Compartment</u>	<u>Trough or Concrete Pond</u>				
	<u>I</u>	<u>II</u>	<u>III</u>	<u>IV</u>	<u>V</u>
1	A	B	C	D	E
2	B	A	E	C	D
3	C	D	A	E	B
4	D	E	B	A	C
5	E	C	D	B	A

*The letters A, B, C, D, and E represent control, 0.04, 0.4, 4.0, and 40.0 rad treatment levels, respectively.

From the onset of feeding 17 days after hatching, the fish were reared on a dry pelletized diet manufactured by Moore-Clark Co., Salt Lake City, Utah. The quantity, size of pellet, and frequency of feeding followed a regime recommended by Leifritz (1959).

Rearing troughs or ponds were brush-scrubbed and flush-cleared routinely to remove excrement, unused food, and algal foul.

Mortality and the incidence of gross malformation were recorded as before. Additionally, weight and length frequencies were measured.

VI. IMMUNIZATION

A. Virulence Bioassay

Old Flexibacter columnaris cultures, isolated and lyophilized in previous years of study, were not used in current studies because of the possibility of loss of virulence characteristics during storage. New cultures

FIGURE 7. Dechlorination units employed for the latter 6 months of study. This facility was fabricated offsite by the Illinois Water Treatment Company, Rockford, Illinois, and consisted of 2 vertical cylinders containing 0.8 m^3 of activated carbon and 1.1 m^3 of crushed quartz of graded mesh size. The dechlorinators were installed to permit one unit in service with the second unit in standby. Each unit supplied a total flow of 200 liters per minute of processed water.

were necessarily isolated from heavily infected large-scale suckers, Catostomus macrocheilus, obtained from the Palouse River near Lyons Ferry, Washington, in July 1974, and tested for virulence using nonirradiated rainbow trout from current studies. It was the purpose of virulence testing to obtain the culture of F. columnaris of highest virulence to be used in preparation of the vaccine and agglutinating test antigen.

Upon isolation, 4 test cultures selected for virulence testing were grown-up with shaking at room temperature in 200 ml of tryptone (0.4%) and yeast extract (0.04%) at pH 6.8-7.0 for 18 hr. At this time the cells were in the early to mid-log phase of growth. The concentration of each culture was then adjusted by the addition of sterile distilled water to give a reading of 50.0 (O.D. = 0.1) on a Klett Colorimeter, using a green filter with a wavelength of 525 mμ. At Klett 50.0 the concentration of cells was approximately 1.8×10^8 cells per ml.

Each test culture and control were tested in duplicate using groups of 5 rainbow trout selected randomly from nonirradiated stocks used in the current studies. The test fish were on the average 9.8 g in weight and 9.4 cm in length at the time of assay. Virulence tests were conducted at $20.0 \pm 0.5^\circ\text{C}$ in thermally-controlled troughs shown in Figure 8. Placement of each test culture and control was as shown in Table IV. Exposures to the test cultures were conducted in 5 battery jars containing 1.9 liters of dechlorinated sanitary water (<10 ppb total residual chlorine). One-hundred (100) ml volumes each of the F. columnaris test cultures were introduced to the exposure jar and the fish exposed for 30 min. The addition of 100 ml culture (Klett 50.0) gave a final dilution of 1:20 and an approximate exposure concentration of 9×10^6 F. columnaris cells per ml of exposure water. The controls were

FIGURE 8. Virulence bioassay test system.

100% COTTON FIBER

TABLE IV. Arrangement of Test and Control Cultures in Thermally-Controlled Trough System Employed in Virulence Bioassay

<u>Placement of Test Culture</u>	<u>Trough</u>	
	<u>I</u>	<u>II</u>
1	Control	Strain 4
2	Strain 1	Strain 3
3	Strain 2	Strain 2
4	Strain 3	Strain 1
5	Strain 4	Control

sham-exposed with 10 ml volumes of sterile media diluted 1:30 with sterile distilled water. Stone bubblers maintained dissolved oxygen at or near saturation over the duration of exposure.

After the 30 min exposure, the fish from each jar were poured into a clean hand net, the excess exposure water removed by gently shaking the net, and the fish transferred to 10-liter holding jars. The stone bubblers used in the exposure jars were rinsed and placed in the holding jars each filled with dechlorinated sanitary water (<10 ppb total residual chlorine) thermally stabilized to $20.0 \pm 0.5^{\circ}\text{C}$. Each holding vessel was equipped with a plastic net top and sun shade.

At hourly intervals post exposure, each holding vessel was checked for mortality and maintenance of test conditions. Results are shown in Table V. On the basis of 80% mortality obtained in 24 hr and 90% mortality obtained in 48 hr, test culture or strain No. 3 was selected for use in preparation of the parenteral vaccine and agglutinating test antigen.

TABLE V. Results of Virulence Bioassay

<u>Test Culture</u>	<u>% Mortality 24 hr</u>	<u>% Mortality 48 hr</u>	<u>Pathology 48 hr</u>
Control	0	0	None
Strain 1	0	0	None
Strain 2	70	90	Gills coated with mucus; gill filaments bleached
Strain 3	80	90	Gills coated with mucus; gill filaments bleached; hemorrhage and erosion.
Strain 4	40	70	Gills coated with mucus; gill filaments bleached; hemorrhage and erosion.

B. Preparation of Vaccine

Upon completion of the virulence assay, the culture with the highest virulence was grown-up as before. The culture was then heat-killed at 65°C for 1.0 hr, cooled, and centrifuged at 7850 x g for 5 min. The media was decanted and the remaining cells washed three times with sterile 0.85% saline solution. The resulting cell suspension was adjusted to a Klett reading of 100.0 (O.D. = 0.2) at a wavelength of 525 mμ with 0.85% sterile saline. This suspension was approximately 3.6×10^8 F. columnaris cells per ml.

To 1.0 liter of heat-killed cells at Klett 100.0, 500 ml of sterile 1.7% saline solution was added, well-mixed, and 75.0 ml aliquots of the suspension measured into each of twenty 150 ml serum vials. The serum vials were capped with sterile rubber stoppers and 25.0 ml of full-strength Freund's incomplete adjuvant were introduced through the serum cap into each vial using a sterile syringe and 20 gauge needle. Each vial was then well-shaken to

thoroughly mix the adjuvant and heat-inactivated cells. The resulting preparation had a concentration of approximately 1.8×10^8 heat-inactivated cells per ml in 25% incomplete adjuvant and 0.85% saline solution.

Ten serum vials containing 100 ml each of sham vaccine were similarly prepared using 500 ml of sterile 0.85% saline, 250 ml of 1.7% saline and 250 ml of Freund's incomplete adjuvant. The sham vaccine thus contained 25% Freund's adjuvant in 0.85% saline solution.

The vaccines were kept refrigerated at 5°C until used.

C. Preparation of Agglutinating Test Antigen

The selected culture was used to grow-up 7.0 liters of F. columnaris at room temperature as before. The culture was heat-killed at 65°C for 1.0 hr, cooled, and centrifuged at $7850 \times g$ for 5 min. The media was decanted and the remaining cells washed 3 times with sterile 0.85% saline solution. The resulting cell suspension was adjusted to a Klett reading of 100.0 (O.D. = 0.2) at a wavelength of 525 m μ with 0.85% sterile saline. This suspension was approximately 3.6×10^3 F. columnaris cells per ml. The heat-inactivated antigen was put-up in 10-liter stoppered glass bottles and stored in the refrigerator at 5°C until use.

D. Vaccination of Test Fish

At 5 months after hatching, control and irradiated test fish were administered intraperitoneally 0.1 cc of the heat-inactivated antigen (1.8×10^8 cells per ml) F. columnaris in 25% Freund's incomplete adjuvant. A 0.1 cc sham vaccination containing sterile saline and 25% Freund's incomplete adjuvant was similarly administered to the second group of control fish. A third group of control fish received handling but received no injection. The

second and third test groups (vaccination controls) were maintained in separate submerged cages in each of the last two compartments of each pond. Prior to injection, test fish were sedated with tricaine methanesulfonate (Ayerst Laboratories, Inc., New York, New York), 1 part to 1000 parts water.

VII. POSTIMMUNE SAMPLING

A. Collection of Blood

Tricaine methanesulfonate (Ayerst Laboratories, Inc., New York, New York), 1 part to 1000 parts water, was administered to sedate fish prior to blood sampling.

One-half (0.5) ml was obtained by syringe from the dorsal aorta without the aid of anticoagulant, discharged into disposable plastic 0.5 ml tubes, and refrigerated for 0.5 hr to reduce serum coagulation.

B. Preparation of Serum

The chilled blood samples were centrifuged for 2 min at 20,000 rpm in the same 0.5 ml disposable plastic tubes. The serum from each blood sample was pipetted into similar disposable tubes, capped, and refrigerated or frozen at -40°C until analyses were performed. Nearly 0.25 ml serum was available from each 0.5 ml blood sample.

C. Serum Agglutination Assay

At 1 week prevaccination, at 3 weeks postvaccination, and at bimonthly intervals thereafter a standard tube agglutination test (Conn, 1957) for the specific antigen of vaccination was performed on the serum from 20 fish from each of the control and irradiated test groups, and 5 fish each from the sham-vaccinated test groups. The standard tube test was modified to use a volume of 0.45 ml physiological saline and 0.05 ml of fish sera before

dilution (Fujihara and Nakatani, 1971). Serial dilutions of 0.25 ml were made from the original saline-serum solution, and 0.25 ml of the test antigen (3.6×10^8 heat-inactivated *F. columnaris* cells per ml) was added to each of the dilution tubes to give final dilutions of 1:20 - 1:2650. The approximate concentration of heat-inactivated cells in each agglutination tube was 4.5×10^7 cells per ml. The tubes were incubated at room temperature for 1 hr, and at 5°C for 20 hr, at which time each was examined for agglutination. The highest dilution in which macroscopic agglutination was observed was recorded as the serum titer value.

D. Total Serum Protein Analysis

Total serum protein concentration was determined using a Technicon Autoanalyzer Dual-Channel Colorimeter (Technicon Instrument Corp., Terrytown, New York). Measurement of protein was by the Biuret reaction. Protein formed a colored complex with cupric ions in alkaline solution. Absorbance of the protein-Biuret complex was read against a sample blank at 550 mμ. The system was standardized using Versatol (General Diagnostics Division, Warner-Lambert Laboratories, Morris Plains, New Jersey) and SMA (Technicon Instrument Corp., Terrytown, New York) reference standards.

E. Polyacrylamide Gel Electrophoresis

The methods used for polyacrylamide gel electrophoresis are essentially as outlined in Technical Bulletin No. 128 distributed by E-C Apparatus Corporation, St. Petersburg, Florida. An E-C-490 vertical gel cell was used to separate 20 μl samples of rainbow trout serum diluted 1:3. A 5% Cyanogum-41 gel was utilized. The electrophoretic separation was performed at $18.0 \pm 0.5^\circ\text{C}$ using a tris- Na_2 -EDTA-boric acid buffer (0.18 M), pH 8.4 (Peacock et al., 1965). The buffer was discarded after each use. A drop of bromphenol blue

(0.005% W/V) solution was added to each sample as a reference point to measure relative mobility. Direct current voltage at the start of electrophoresis was 300 V. At the end of a 3 hr separation it varied between 290-310 V. The total current output at the beginning was 190 mA, and at the end it varied from 130 to 175 mA.

After removal of the gel slab from the electrophoretic cell, the dye band was cut off. The gel was then stained for 30 min with 0.25% Amido Black 10B in a solution of 5:5:1 (V|V|V) methanol, distilled water, and glacial acetic acid.

Destaining was facilitated in the E-C-489 electrophoretic destainer following methods outlined in Technical Bulletin No. 137 of the E-C Apparatus Corporation, St. Petersburg, Florida. Gels were destained for a minimum of 1 hr in the presence of the same acidified methanol solution. After destaining, the gels were cleared for 1-2 hr in a 5% aqueous glycerine solution. The gels were then examined for the presence or absence of certain serum proteins. Densitometric tracings were performed using an E-C 910 transmission densitometer, scanning between 600-625 m μ . The densitometer operated with an external integrating recorder thus facilitating simultaneous quantitation of serum protein patterns. Integrations were checked with the aid of planimetry. The gels were photographed and preserved by wrapping in Saran Wrap.

F. Agar Gel Immunodiffusion

Agar diffusion is another of many methods available for detecting the presence of antibody in serum or in other fluids. It is based on the combining of soluble antigen and antibody in a gel matrix to produce a visible precipitate. In the present studies, agar gel diffusion was employed as a first step to a subsequently planned immunoelectrophoretic approach, and

was conducted to provide information on the optimum quantities of both antigen and antibody required to produce the precipitin reaction. Through the aid of immunoelectrophoresis it was hoped that the protein or proteins acting as antibody in antisera from immunized test fish would be identified with greater assurance.

Agar diffusion was conducted on acetone, rinsed standard glass microslides (25 x 75 mm), overlaid with 3.5 ml of sterile 1% agarose made up in 0.1 M sodium barbital buffer, pH 8.2. After pouring, the slides were allowed to harden for 2 hr. A specially designed cutter was used to punch 5 small circular wells placed circumferentially and a larger circular well centrally. The small outside wells received 0.02 ml of heat-killed undiluted (3.6×10^3 cells per ml) or sonically disrupted antigen. Sonically disrupted antigen was prepared by continuous disruption of heat-killed undiluted (3.6×10^3 cells per ml) antigen in a sonifier cell disrupter (Heat Systems - Ultrasonics, Inc., Melville, New York), for 10 min at an energy input of 130 w. The larger centrally-located well received 0.1 ml of antisera diluted over a range of 1:1 to 1:5. Only antisera which had produced a strong positive reaction during agglutinin assay were used for this purpose. Each slide was then introduced to a moist chamber maintained at room temperature and observed daily for 1 week for the presence of precipitins.

G. Interfacial Ring Test

The interfacial ring test was also used to detect the presence of antibody protein in the antiserum of immunized test fish. As in the agar diffusion technique, soluble antigen and antibody combine in a liquid medium to form a visible precipitate or ring. The technique was performed in clear 6 x 50 mm glass tubing, one end sealed by heating with a micro-burner.

Antiserum, 0.1 ml, was introduced with a Pasteur pipet. An equal volume of antigen, either whole-cell or sonically-disrupted, was then carefully overlaid without mixing the two solutions. Immune sera overlaid with saline served as a control. The tubes were then incubated upright, either at room temperature, or 5°C for 24 hr, and the interface observed for development of a precipitin line or a sharp ring in the antibody layer. At the end of 24 hr the bottoms of the tubes were observed for the presence of settled antigen-antibody precipitin.

VIII. STATISTICAL ANALYSES

A. Transformation of Data

The agglutinin data was observed to be heteroscedastic; that is, there occurred some inequality of variances among the five treatment groups. For this reason, a logarithmic transformation (base_e) of the agglutinin data was performed prior to application of analysis of variance (Zar, 1974). This correction resulted in data that simultaneously demonstrated acceptable homoscedasticity and additivity characteristics.

B. Multiway Factorial Analysis of Variance

The studies outlined above were designed so that the data (agglutinin titer, total serum protein, weight, length, and latent mortality) were treated by analysis of variance. This particular design was a 5 x 5 Latin Square, Table III. Interest was focused on the main effect of irradiation level; however, two restrictions were placed on the randomization; that attributed to the trough or pond position within the sampling array, and to the compartment position within each trough or pond. This design was only possible if the number of levels of both restrictions equaled the number of

treatment levels. Not all randomization was lost in the design, as this particular Latin Square was selected from several possible Latin Squares of the required size.

The model for this design was:

$$Y_{(h)ijk} = \mu + \tau_{(h)} + \rho_i + \gamma_j + \delta_{ij} + \lambda_k + (\tau\lambda)_{(h)k} + \epsilon_{ijk}$$

where:

μ = overall mean effect in sample

$\tau_{(h)}$ = treatment effect (fixed) $h = 1, 2, \dots, 5$

ρ_i = row effect (random) $i = 1, 2, \dots, 5$

γ_j = column effect (random) $j = 1, 2, \dots, 5$

δ_{ij} = random effect for ij^{th} cell

λ_k = time effect (fixed) $k = 1, 2, \dots, K$

$(\tau\lambda)_{(h)k}$ = treatment time interaction (fixed)

ϵ_{ijk} = random effect for ij^{th} cell at time k

For a particular sampling time the model was:

$$Y_{(h)ij} = \mu + \tau_{(h)} + \rho(i) + \gamma_j + \delta_{ij}$$

C. One-Way Analysis of Variance

One-way analysis of variance was applied to demonstrate potential differences in percent of total serum protein contained in each of the four major protein fractions of rainbow trout serum in response to increasing agglutinin titer.

D. Duncan Multiple Range Test

To determine potential differences among the five treatment groups as to agglutinin titer, total serum protein, percent of total protein in each major protein fraction, weight, and length, the Duncan Multiple Range Test was used (Kramer, 1956). This test is applied a posteriori to the analysis of variance f-test to locate and indicate the extent of differences among means.

E. Chi-Square

A heterogeneity chi-square analysis of 2 x 2 contingency tables was conducted to demonstrate potential differences among the five treatment means as to hatchability, early mortality, and incidence of abnormality.

F. Significance Level

Throughout these studies, the use of the word "significant" when associated with comparisons of results implies a statistical significance at $\alpha = 0.05$ (95% level of confidence), unless otherwise stated.

RESULTS

I. TRITIUM ACCUMULATION

Tritium differences are most conveniently compared if expressed as ratios: tritium per milliliter free tissue water/tritium per milliliter environmental water, and tritium per gram wet weight of embryo/tritium per milliliter environmental water. These relationships for the 0.1 $\mu\text{Ci/ml}$ exposure level are reported in Figure 9. Each datum represents the mean for five replicate determinations.

After 1 day, tissue-free water tritium had attained approximately 90% of the tritium level in environmental waters. By 4 days, tissue-free water tritium exceeded the concentration in environmental waters by 23%; although for the remainder of the exposure period, concentration ratios remained below unity. The concentration ratio of the whole embryo was always less than the concentration ratio for tissue-free water tritium and only exceeded unity on the 4th day of exposure.

II. TRITIUM RETENTION

The retention of tritium upon transfer to inactive free-flowing water after exposure for 20 days at the 1.0 $\mu\text{Ci/ml}$ exposure level is shown in Figure 10. For both tissue-free water and tissue-bound fractions, fast and slow components of turnover were demonstrated. Based on analyses of the slopes of each curve, half-times for the tissue-free water tritium fraction were estimated to be 1 hr and 3 days. Half-times for the tissue-bound fraction were estimated at 2 hrs and 6 days. The slow component of the tissue-free water tritium fraction persisted for 17 days, although it was barely detectable at the end of the sampling period. The slow component for

FIGURE 9. Tritium concentrations in exposure water and embryos.

FIGURE 10. Retention of tritium upon transfer to inactive free-flowing water after exposure for 20 days at the 1.0 $\mu\text{Ci/ml}$ exposure level.

the tissue-bound tritium fraction, for comparison, persisted at nearly 1% of the initial TBT level at 17 days.

III. DOSE ESTIMATION

Dose estimates for each of the five concentrations of tritiated water employed in the present study are shown in Table VI. Dose estimates were calculated from mean tritium levels in each incubation system over the 20 day exposure period and not simply derived from theoretical or experimental design levels. Departure from experimental design levels ranged from 10 to 30%, undoubtedly attributable to an error in calibration or in preparation of the tritium solutions to be added to each incubation system. Sampling error and exchange of tritium with tissue-bound hydrogen in the embryos could also have contributed to lower than anticipated levels of tritium in each incubation system.

TABLE VI. Dose Estimates for Embryos of Rainbow Trout Immersed in Tritiated Water for 20 Days

<u>Mean Concentration in $\mu\text{Ci/ml}$</u>	<u>Dose (rads) Per Day</u>	<u>Accumulated Dose (rads) for 20 Days</u>
0	0	0
0.009	0.002	0.04
0.08	0.02	0.4
0.7	0.2	4.0
6.9	2.0	40.0

IV. SERUM AGGLUTINATION ASSAY

All experimental groups sampled 1 week prior to vaccination failed to demonstrate positive agglutinins to F. columnaris at the lowest test dilution.

Mean titer values (reciprocals) for specific agglutinins to F. columnaris for each experimental group in each pond over the 11-week sampling period following vaccination are graphically presented in Figures 11 through 15. Each datum represents the mean of 20 individuals. Numerical presentation of these same data is shown in Table VII. Summary statistical information is contained in Table VIII.

These data indicated that under the prescribed experimental conditions, a clearly observable and statistically significant change in mean titer values occurred during the 11-week sampling period following vaccination. Also, significant differences in mean titer values among the five treatment means were detected.

From similar analyses performed for each pond at each sampling interval, Table IX, it was found that on the 9th and 11th weeks, there occurred significant differences among the five treatment means. Subsequent statistical analyses applying the Duncan Multiple Range Test demonstrated the differences reported in Table X. During the 9th week, statistical significance was detected between the control and 0.4 rad levels and higher; between 0.04 rad and 4.0 rad levels and higher; between 0.4 rad and 40.0 rad levels; and between 4.0 rad and 40.0 rad levels. The 40.0 rad level was significantly different than all lower treatment levels. During the 11th week, statistical significance was not detected among the control, 0.04 rad, and 0.4 rad levels, or between the 4.0 rad and 40.0 rad levels; however, there occurred a significant difference between the lowest three treatment levels and the two highest treatment levels.

FIGURES 11-15. Mean titer values in Pond I-V for specific agglutinins to F. columnaris arranged according to increasing radiation dose at each sampling interval.

TABLE VII. Mean Agglutinin Titers (Reciprocals) to *F. columnaris* Arranged According to Increasing Irradiation Dose at Each Sampling Interval

POND I					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	0	0	0	0	0
3	29	29	22	19	22
5	196	117	126	132	80
7	1074	1192	720	528	508
9	1024	729	896	584	504
11	1568	1120	912	816	704

POND II					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	0	0	0	0	0
3	24	23	24	25	18
5	201	182	107	120	70
7	-	-	-	-	-
9	1072	1088	800	768	624
11	1536	1292	864	944	896

POND III					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	0	0	0	0	0
3	25	22	24	29	21
5	512	384	592	105	348
7	-	-	-	-	-
9	1312	1024	912	960	832
11	2496	2496	1888	1129	1152

POND IV					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	0	0	0	0	0
3	29	28	21	18	25
5	880	620	624	408	360
7	-	-	-	-	-
9	1213	1184	968	1152	576
11	2425	2368	2080	1312	808

POND V					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	0	0	0	0	0
3	22	31	18	22	27
5	564	577	832	504	438
7	-	-	-	-	-
9	1408	1216	1056	800	672
11	2560	2080	2368	888	1120

TABLE VIII. Summary Analysis of Variance for Agglutinin Titer ($\log_e$)

Source of Variation	Degree of Freedom	Sum of Squares	Mean Squares	F Ratio
Squares (weeks)	3	270.65	90.21	1235.86**
Ponds within weeks	16	16.56	1.03	14.18**
Compartments within weeks	16	1.65	0.10	1.41 ^{N.S.}
Treatment within weeks	16	5.77		
<hr/>				
Treatment	4	4.40	1.10	15.10**
Treatment week	12	1.36	0.11	1.56 ^{N.S.}
<hr/>				
Error within weeks	48	3.48	0.07	
Total	99	298.12		

* - Significant F Ratio value at the 0.05 level.

** - Significant F Ratio value at the 0.01 level.

N.S. - Nonsignificant F Ratio value at the 0.05 level.

TABLE IX. Analysis of Variance for Agglutinin Titer ($\log_e$)
at Each Sampling Interval

3 WEEKS

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	0.35	0.08	1.23 N.S.
PONDS	4	0.06	0.01	0.23 N.S.
COMPARTMENTS WITHIN PONDS	4	0.39	0.09	1.35 N.S.
RESIDUAL	12	0.86	0.07	
TOTAL	24			

5 WEEKS

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	1.70	0.42	2.94 N.S.
PONDS	4	14.49	3.62	25.07 **
COMPARTMENTS WITHIN PONDS	4	0.99	0.24	1.72 N.S.
RESIDUAL	12	1.73	0.14	
TOTAL	24			

9 WEEKS

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	1.13	0.28	12.83 **
PONDS	4	0.30	0.07	3.47 *
COMPARTMENTS WITHIN PONDS	4	0.03	0.01	0.39 N.S.
RESIDUAL	12	0.26		
TOTAL	24			

11 WEEKS

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	2.57	0.64	12.46 **
PONDS	4	1.68	0.42	8.14 *
COMPARTMENT WITHIN PONDS	4	0.23	0.05	1.11 N.S.
RESIDUAL	12	0.62	0.05	
TOTAL	24			

* SIGNIFICANT F VALUE AT 0.05 LEVEL.

** SIGNIFICANT F VALUE AT 0.01 LEVEL.

N.S. NONSIGNIFICANT F VALUE AT 0.05 LEVEL.

TABLE X. Duncan Multiple Range Test Comparisons of Mean Titer ($\log_e$) Values, 9th and 11th Weeks, Arranged According to Increasing Irradiation Dose¹

	0 rad	0.04 rad	0.4 rad	4.0 rad	40.0 rad
9th Week	7.00	6.84	6.73	6.61	6.37
11th Week	7.75	7.41	7.23	6.82	6.73

¹Differences are significant at 0.05 level.

TABLE XI. Comparison of Mean Values for Agglutinin Titer ($\log_e$) for All Ponds, 5th, 9th, 11th Weeks, by Duncan Multiple Range Test¹

	<u>Pond I</u>	<u>Pond II</u>	<u>Pond III</u>	<u>Pond IV</u>	<u>Pond V</u>
5th Week	4.46	4.48	5.53	6.07	6.24
9th Week	6.50	6.67	6.78	6.78	6.79
11th Week	6.78	6.88	7.31	7.34	7.41

¹Differences are significant at 0.05 level.

The analyses shown in Table VIII and IX also disclosed a significant pond effect. Subsequent statistical analyses applying the Duncan Multiple Range Test demonstrated the differences reported in Table XI. The fish sampled in Ponds I and II demonstrated significantly lower agglutinin titers than fish sampled in Ponds III, IV, and V. The failure to detect a significant effect on agglutinin titer attributed to position by compartment in each pond was also noted.

Serum agglutinins to F. columnaris in saline-adjuvant injected and non-injected control fish as determined 1 week prior to vaccination, and 3 and 5 weeks following vaccination are reported in Tables XII and XIII. Although a small percentage of the fish in these experimental groups demonstrated a positive titer, mean values for all positive titers were significantly lower than mean values of all positive titers in the antigen-adjuvant injected control groups.

Sampling during the 7th week was not conducted in Ponds II through V due to a malfunction of the dechlorination system. Total residual chlorine elevated to ~70 ppb in makeup water entering the pond array on October 25, 1974, producing observable stress in all experimental groups. Although total residual chlorine as determined in each individual pond did not exceed 35 ppb, feeding was diminished, and a low mortality persisted over the next 2 weeks. The malfunction in the dechlorination system was corrected immediately, and all ponds were flush-cleared.

V. TOTAL SERUM PROTEIN ANALYSIS

Mean total serum protein (g%) concentrations as determined in each pond over the 12-week sampling period and presented according to increasing irradiation dose are reported in Table XIV. Summary statistical information is contained in Table XV.

TABLE XII. Serum Agglutinins to F. columnaris in Caged Saline-Adjuvant Injected Control Fish

TIME IN WEEKS	POND I		POND II		POND III		POND IV		POND V	
	NO. POSITIVE TITER	MEAN POSITIVE TITER	NO. POSITIVE TITER	MEAN POSITIVE TITER	NO. POSITIVE TITER	MEAN POSITIVE TITER	NO. POSITIVE TITER	MEAN POSITIVE TITER	NO. POSITIVE TITER	MEAN POSITIVE TITER
0	0/6	1:0	0/6	1:0	0/7	1:0	0/7	1:0	0/6	1:0
3	0/5	1:0	0/5	1:0	0/5	1:0	1/5	1:20	1/5	1:20
5	2/5	1:20	2/5	1:20	1/5	1:20	3/5	1:25	2/5	1:30

TABLE XIII. Serum Agglutinins to F. columnaris in Caged Noninjected Control Fish

TIME IN WEEKS	POND I		POND II		POND III		POND IV		POND V	
	NO. POSITIVE TITER	MEAN POSITIVE TITER	NO. POSITIVE TITER	MEAN POSITIVE TITER	NO. POSITIVE TITER	MEAN POSITIVE TITER	NO. POSITIVE TITER	MEAN POSITIVE TITER	NO. POSITIVE TITER	MEAN POSITIVE TITER
0	0/6	1:0	0/6	1:0	0/7	1:0	0/7	1:0	0/6	1:0
3	1/5	1:20	0/5	1:0	0/5	1:0	2/5	1:20	2/5	1:20
5	1/5	1:20	0/5	1:0	1/5	1:0	2/5	1:50	0/1	1:0

TABLE XIV. Mean Total Serum Protein (g%)
At Each Sampling Interval

POND I					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	3.78	3.71	3.35	3.28	3.22
3	3.65	3.54	3.15	3.24	3.52
5	2.90	2.99	3.31	3.13	3.96
7	3.81	3.89	3.75	3.89	3.89
9	3.07	3.08	3.20	3.41	3.45
11	3.33	2.86	3.38	3.32	2.97

POND II					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	3.65	3.59	3.39	3.63	3.65
3	3.67	3.22	3.09	3.36	3.52
5	3.63	3.16	3.59	3.44	3.52
7	-	-	-	-	-
9	3.22	3.07	3.31	3.24	3.32
11	3.23	3.17	3.43	3.10	3.26

POND III					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	3.61	3.24	3.68	3.64	3.49
3	2.72	2.95	2.83	3.62	2.99
5	3.58	3.52	3.38	3.57	3.16
7	-	-	-	-	-
9	3.49	3.56	3.50	3.66	3.48
11	3.01	2.87	2.82	3.41	2.78

POND IV					
TIME IN WEEKS	0 RAD	0.1 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	3.59	3.61	3.63	3.41	3.33
3	2.89	3.05	3.14	3.10	2.94
5	3.82	3.45	3.55	3.78	3.46
7	-	-	-	-	-
9	3.70	3.65	3.74	3.77	3.29
11	2.93	3.01	3.20	3.35	2.77

POND V					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	4.10	3.75	3.85	3.74	3.58
3	3.28	2.90	3.27	2.74	3.00
5	3.53	3.43	3.62	3.35	3.42
7	-	-	-	-	-
9	3.70	3.58	3.88	3.54	3.73
11	3.17	2.94	2.89	3.08	3.10

TABLE XV. Summary Analysis of Variance for Total Serum Protein (g%)

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F Ratio
Squares (weeks)	4	4.27	1.06	25.59**
Ponds within weeks	20	2.63	0.13	3.15*
Compartments within weeks	20	0.89	0.04	1.06 ^{N.S.}
Treatments within weeks	20	0.77		
<hr/>				
Treatment	4	0.30	0.77	1.85 ^{N.S.}
Treatment x weeks	16	0.47	0.02	0.70
<hr/>				
Error within weeks	60	2.50		
Total	124	11.08		

* - Significant F value at the 0.05 level
 ** - Significant F value at the 0.01 level
 N.S. - Nonsignificant F value at the 0.05 level

These data indicated that under the prescribed experimental conditions, a significant difference in mean total serum protein concentrations among the five treatment levels was not detected. However, a clearly observable and statistically significant effect over time was measured. In general, total serum protein concentrations decreased by approximately 12% in all experimental groups over the 12-week sampling period.

The analyses in Table XV also disclosed a significant pond effect. From a similar analyses performed for each pond at each sampling interval, Table XVI, it was found that on the 9th week there occurred significant differences among the pond means. Subsequent statistical analyses applying the Duncan Multiple Range Test demonstrated the differences reported in Table XVII. Total serum protein concentrations in fish from Ponds I and II were significantly lower than in fish from Ponds III, IV, and V. However, analyses as shown in Table XVI failed to detect this relationship either before or after the 9th week. Also these data failed to detect a significant effect on total serum protein concentration attributed to position by compartment in each pond.

VI. POLYACRYLAMIDE GEL ELECTROPHORESIS

Serum protein patterns for 5-month nonvaccinated control and irradiated experimental fish are shown in Figure 16. The origin is on the right; the most rapidly migrating bands on the left. All bands were generally well-defined and mobilities did not change significantly from pattern to pattern. Inconstant bands were encountered in certain patterns and dissimilarities in dye intensity were often observed.

TABLE XVI. Analysis of Variance for Total Serum Protein (g%) at Each Sampling Interval

PRE VACCINATION				
SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	0.21	0.05	1.72 ^{N. S.}
PONDS	4	0.34	0.08	2.71 ^{N. S.}
COMPARTMENTS WITHIN PONDS	4	0.05	0.01	0.40 ^{N. S.}
RESIDUAL	12	0.37	0.03	
TOTAL	24	0.99		
3 WEEKS				
SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	0.07	0.01	0.25 ^{N. S.}
PONDS	4	0.81	0.20	2.90 ^{N. S.}
COMPARTMENTS WITHIN PONDS	4	0.23	0.05	0.84 ^{N. S.}
RESIDUAL	12	0.84	0.07	
TOTAL	24	1.96		
5 WEEKS				
SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	0.12	0.03	0.54 ^{N. S.}
PONDS	4	0.32	0.08	1.36 ^{N. S.}
COMPARTMENTS WITHIN PONDS	4	0.30	0.07	1.30 ^{N. S.}
RESIDUAL	12	0.70	0.05	
TOTAL	24	1.46		
9 WEEKS				
SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	0.07	0.01	1.21 ^{N. S.}
PONDS	4	0.92	0.23	16.12 ^{**}
COMPARTMENTS WITHIN PONDS	4	0.17	0.04	3.07 ^{N. S.}
RESIDUAL	12	0.17	0.01	
TOTAL	24	1.34		
11 WEEKS				
SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	0.28	0.07	2.15 ^{N. S.}
PONDS	4	0.22	0.05	1.64 ^{N. S.}
COMPARTMENTS WITHIN PONDS	4	0.11	0.02	0.87 ^{N. S.}
RESIDUAL	12	0.40	0.03	
TOTAL	24	1.03		

* SIGNIFICANT F VALUE AT 0.05 LEVEL

** SIGNIFICANT F VALUE AT 0.01 LEVEL

N. S. NONSIGNIFICANT F VALUE AT THE 0.05 LEVEL

FIGURE 16. Serum electrophoretic patterns of 5-month nonvaccinated control rainbow trout (a), (b), and (c), as compared to serum patterns of 5-month nonvaccinated irradiated (40.0 rad) rainbow trout (d), (e), and (f), and human (g), and (h). Separations were conducted simultaneously in 5% polyacrylamide gel.

TABLE XVII. Comparison of Mean Values for Total Serum Protein (g%) for All Ponds, 9th Week, by Duncan Multiple Range Test¹

<u>Pond I</u>	<u>Pond II</u>	<u>Pond III</u>	<u>Pond IV</u>	<u>Pond V</u>
3.23	3.24	3.54	3.63	3.68

¹Differences are significant at 0.05 level.

As shown in Figure 16, slab electrophoresis with 5% polyacrylamide separation gel shows the presence of 4 major fractions. Although there occurred variability between animals, fraction I usually contained four bands; fraction II contained four bands; fraction III contained five bands; and, fraction IV contained two bands. Fraction II was not well-defined and stained with a homogeneous heavy dye. Fraction IV also was not well-defined and stained with a homogeneous light dye. However, no significant qualitative differences between control and irradiated test groups were observed. A comparison of these results with those of the human conducted under the same conditions is also reported in Figure 16. The most rapidly migrating bands in the rainbow trout, on the left, were found to be as mobile as human serum albumin.

Electrophoretic patterns for representative control and irradiated non-vaccinated experimental fish were also subjected to densitometric analyses. Percent of total serum protein contained in each of the four major fractions was calculated by planimetry and is presented in Table XVIII. A one-way analysis of variance was applied to these data to detect potential differences attributed to radiation history. Results are reported in Table XIX and indicated that the two experimental groups differed only in the percent of total protein contained in fraction I.

TABLE XVIII. Mean Percent of Total Serum Protein in Fractions I, II, III, and IV in Nonvaccinated Control and Irradiated (40.0 rads) Experimental Fish

	<u>I</u>	<u>II</u>	<u>III</u>	<u>IV</u>
<u>Control</u>				
n	10	10	10	10
Mean	25.32	47.04	25.32	2.24
Standard Deviation	4.76	3.67	4.86	1.08
Standard Error	1.50	1.16	1.53	0.34
95% C.I.				
Upper	28.67	49.62	28.75	3.00
Lower	21.96	44.45	21.88	1.47
<u>Irradiated</u>				
n	10	10	10	10
Mean	31.31	45.09	22.01	1.59
Standard Deviation	4.59	5.48	7.13	0.99
Standard Error	1.45	1.73	2.25	0.31
95% C.I.				
Upper	34.54	48.95	27.03	2.28
Lower	28.07	41.22	16.98	0.89

TABLE XIX. One-Way Analysis of Variance Comparison of Percent of Total Serum Protein in Fractions I, II, III, and IV in Nonvaccinated Control and Irradiated (40.0 rad) Experimental Fish

<u>Fraction</u>	<u>Source Variation</u>	<u>Degrees of Freedom</u>	<u>Sum of Squares</u>	<u>Mean Squares</u>	<u>F Ratio</u>
I	Among Treatments	1	179.40	179.40	8.19*
	Within Treatments	18	394.26	21.90	
	Total	19	573.66		
II	Among Treatments	1	19.01	19.01	0.87 ^{N.S.}
	Within Treatments	18	391.93	21.77	
	Total	19	410.94		
III	Among Treatments	1	54.78	54.78	1.46 ^{N.S.}
	Within Treatments	18	671.44	37.30	
	Total	19	726.22		
IV	Among Treatments	1	2.11	2.41	1.96 ^{N.S.}
	Within Treatments	18	19.39	1.07	
	Total	19	21.50		

* - Significant f Ratio value at the 0.05 level.

** - Significant f Ratio value at the 0.01 level.

N.S. - Nonsignificant f Ratio value at the 0.05 level.

Serum protein patterns (fraction III, IV) for 6 1/2-month control and irradiated vaccinated experimental fish are shown in Figure 17. As before, no qualitative differences between control and irradiated experimental groups were observed. However, fraction IV in the vaccinated experimental fish, either control or irradiated, demonstrated four distinct components. Also, there appeared to be a clearly observable quantitative difference in the expression of fraction IV between control and irradiated experimental fish.

To test this relationship the percent of total serum protein contained in each fraction was calculated as before. Selected results are reported in Table XX. A one-way analysis of variance was applied to these data and indicated as shown in Table XXI that significant differences between the two experimental groups were encountered in the expression of fractions I and IV. In the control group, fraction I contained a lower percent of the total serum protein; while fraction IV contained a higher percent of the total.

It was also observed that fraction IV was more pronounced in nearly all vaccinated experimental fish, either control or irradiated, which demonstrated agglutinin titers of at least 1:40; and, was most pronounced in vaccinated experimental fish demonstrating the highest agglutinin titers measured, 1:1280 to 1:2560.

To test this relationship, the percent of total serum protein contained in each major protein fraction in relation to increasing agglutination titer, Table XXII, was subjected to one-way analysis of variance, and subsequently the Duncan Multiple Range Test. Results shown in Table XXIII and XXIV indicated that fraction IV accounted for an increasing percentage of the total available serum protein as agglutinin titers increased. Concomitantly, fractions I and II were observed to contain lower percentages of the total

TABLE XX. Mean Percent of Total Serum Protein in Fractions I, II, III, and IV in Vaccinated Control and Irradiated (40.0 rad) Experimental Fish

	<u>I</u>	<u>II</u>	<u>III</u>	<u>IV</u>
<u>Control</u>				
n	12	12	12	12
Mean	27.27	43.10	23.94	5.60
Standard Deviation	4.90	4.44	4.00	3.54
Standard Error	1.41	1.28	1.15	1.02
95% C.I.				
Upper	30.35	45.89	26.46	7.83
Lower	24.19	40.30	21.42	3.37
<u>Irradiated</u>				
n	12	12	12	12
Mean	31.79	42.00	23.47	2.66
Standard Deviation	3.79	4.54	5.92	1.30
Standard Error	1.09	1.31	1.70	3.77
95% C.I.				
Upper	34.17	44.86	27.19	3.48
Lower	29.40	39.15	19.75	1.84

TABLE XXI. One-Way Analysis of Variance Comparison of Percent of Total Serum Protein in Fractions I, II, III, and IV in Vaccinated Control and Irradiated (40.0 rad) Experimental Fish

<u>Fraction</u>	<u>Source Variation</u>	<u>Degrees of Freedom</u>	<u>Sum of Squares</u>	<u>Mean Squares</u>	<u>F Ratio</u>
I	Among Treatments	1	122.40	122.40	6.37*
	Within Treatments	22	422.73	19.21	
	Total	23	545.13		
II	Among Treatments	1	7.15	7.15	0.35 ^{N.S.}
	Within Treatments	22	443.80	20.17	
	Total	23	450.95		
III	Among Treatments	1	1.30	1.30	0.51 ^{N.S.}
	Within Treatments	22	562.45	25.56	
	Total	23	563.75		
IV	Among Treatments	1	51.92	51.92	7.26*
	Within Treatments	22	157.19	7.14	
	Total	23	209.11		

* - Significant F value at 0.05 level

** - Significant F value at 0.01 level

N.S. - Nonsignificant F value at 0.05 level

TABLE XXII. Mean Values for Percent of Total Serum Protein in Fractions I, II, III, and IV Arranged According to Increasing Agglutinin Titer

FRACTION I									
	1:0	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
MEAN	28.91	31.90	28.98	28.40	30.70	25.76	25.17	23.51	26.62
n	22	13	7	7	8	8	9	6	5
STANDARD DEVIATION	5.61	3.24	3.62	3.96	7.15	3.97	5.61	3.82	5.43
STANDARD ERROR	1.19	0.91	1.37	1.50	2.53	1.40	1.87	1.55	2.43
95% C. I.									
LOWER	26.43	29.96	25.74	24.85	24.61	22.52	20.94	19.70	20.37
UPPER	31.40	33.85	32.22	31.94	34.28	29.00	29.41	27.33	32.86

FRACTION II									
	1:0	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
MEAN	46.10	42.73	44.35	42.58	39.61	43.46	45.73	39.98	40.86
n	22	13	7	7	8	8	9	6	5
STANDARD DEVIATION	4.44	3.28	2.44	4.88	3.55	2.78	6.04	4.42	3.49
STANDARD ERROR	0.94	0.91	0.92	1.84	1.25	0.98	2.01	1.80	1.56
95% C. I.									
LOWER	44.13	40.77	42.17	38.22	36.71	41.19	41.17	35.55	36.84
UPPER	48.06	44.70	46.54	46.94	42.50	45.83	50.29	44.40	44.87

FRACTION III									
	1:0	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
MEAN	23.17	22.84	23.45	24.78	24.13	25.61	23.34	25.25	21.14
n	22	13	7	7	8	8	9	6	5
STANDARD DEVIATION	6.09	3.86	4.14	7.80	2.39	3.37	2.95	5.49	3.62
STANDARD ERROR	1.29	1.07	1.56	2.95	0.84	1.19	0.98	2.24	1.62
95% C. I.									
LOWER	20.48	20.53	19.75	17.80	22.18	22.85	21.11	19.75	16.97
UPPER	25.87	25.15	27.15	31.76	26.08	28.36	25.57	30.74	25.30

FRACTION IV									
	1:0	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
MEAN	1.76	2.46	3.17	4.12	5.50	5.15	5.67	11.26	11.60
n	22	13	7	7	8	8	9	6	5
STANDARD DEVIATION	0.23	0.38	0.78	1.29	0.99	0.73	0.60	1.85	3.78
STANDARD ERROR	0.23	0.38	0.78	1.29	0.99	0.73	0.60	1.85	3.78
95% C. I.									
LOWER	1.26	1.64	1.31	1.07	3.86	3.45	4.30	6.71	1.87
UPPER	2.26	3.29	5.03	7.18	9.63	6.84	7.05	15.81	21.32

FIGURE 17. Serum electrophoretic patterns (fraction III, IV) of 6-1/2 month vaccinated control rainbow trout (a), (b), (c), (d) and (e), as compared with serum patterns of 6-1/2 month vaccinated irradiated (40.0 rad) rainbow trout (g), (h), (i), (j), (k), and (l). Separations were conducted simultaneously in 5% polyacrylamide gel.

TABLE XXIII. One-Way Analysis of Variance Comparison of Percent of Total Serum Protein in Fractions I, II, III, and IV, Arranged According to Increasing Agglutinin Titer

<u>Fraction</u>	<u>Source Variation</u>	<u>Degrees of Freedom</u>	<u>Sum of Squares</u>	<u>Mean Squares</u>	<u>F Ratio</u>
I	Among Treatments	8	480.94	60.11	2.43*
	Within Treatments	76	1876.28	24.68	
	Total	84	2357.22		
II	Among Treatments	8	442.08	55.26	3.21**
	Within Treatments	76	1305.68	17.18	
	Total	84	1747.76		
III	Among Treatments	8	103.01	12.87	0.53 ^{N.S.}
	Within Treatments	76	1821.61	23.96	
	Total	84	1924.62		
IV	Among Treatments	8	813.27	101.65	11.35**
	Within Treatments	76	680.15	8.94	
	Total	84	1493.42		

* - Significant F value at 0.05 level

** - Significant F value at 0.01 level

N.S. - Nonsignificant F value at 0.05 level

TABLE XXIV. Duncan Multiple Range Test Comparison of Mean Values for Percent of Total Serum Protein in Fractions I, II, III, and IV Arranged According to Increasing Agglutinin Titer

FRACTION I**ORDERED
MEANS**

31.90 30.70 28.98 28.91 28.40 26.62 25.76 25.17 23.51

**ORDERED
TITER**

1:20 1:160 1:40 1:0 1:80 1:2560 1:320 1:640 1:1280

FRACTION II**ORDERED
MEANS**

46.10 45.73 44.35 43.46 42.73 42.58 40.86 29.98 39.61

**ORDERED
TITER**

1:0 1:640 1:40 1:320 1:20 1:80 1:2560 1:1280 1:160

FRACTION III**ORDERED
MEANS**

25.61 25.25 24.78 24.13 23.45 23.34 23.17 28.84 21.14

**ORDERED
TITER**

1:320 1:1280 1:80 1:160 1:40 1:640 1:0 1:20 1:2560

FRACTION IV**ORDERED
MEANS**

1.76 2.46 3.17 4.12 5.15 5.50 5.67 11.26 11.60

**ORDERED
TITER**

1:0 1:20 1:40 1:80 1:320 1:160 1:640 1:1280 1:2560

serum protein as agglutinin titers increased. Fraction III remained nearly constant. This relationship is further illustrated by arranging densitometric tracings of serum protein patterns by increasing agglutinin titer as shown in Figures 18, 19, and 20.

This same relationship is clearly demonstrated if the agglutinin titers for the fish analyzed in Table XX are revealed. While the mean agglutinin titer for the control group was 1:395, the mean titer of the irradiated group was only 1:70.

Densitometric analyses also confirmed that fraction IV in vaccinated experimental fish was comprised of four distinct components, yielding a total of 17 components for the entire serum pattern, Figure 21. However, at the highest agglutinin titers measured, 1:1280 to 1:2560, only two of the four components were consistently observed.

VII. AGAR GEL IMMUNODIFFUSION

Immunodiffusion as conducted in 1% agarose gel in the presence of 0.1 M sodium barbital buffer failed to demonstrate a zone of precipitation, when either whole-cell or a sonicated antigen preparation was used. Tests employing varying dilutions of both antigen and antisera also produced negative results.

VIII. INTERFACIAL RING TEST

The basic approach was repeated applying the interfacial ring test which serves as one of the most sensitive methods available to detect small quantities of antibody protein. Results are shown in Table XXV.

In no trial was there demonstrated a visible ring of precipitin formed at the liquid-liquid interface of the principal reactants. However, in all trials in Series 1 and 2, a white precipitate formed at the bottom of each

FIGURES 18, 19, 20. Densitometric profiles of vaccinated rainbow trout demonstrating the increase in serum protein fraction IV in response to increasing agglutinin titer, and the concomitant decrease in fractions I and II.

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FIGURE 21. Schematic of electrophoretic pattern for immature vaccinated rainbow trout serum proteins applying 5% polyacrylamide separation gel. Bands are numbered following increasing mobility. Inconstant bands detected included numbers 2, 9, 11, and 14.

(-)

(+)

TABLE XXV. Results of Interfacial Ring Tests

TRIAL	FISH NO.	SOURCE ¹	AGGLUTININ TITER	ANTIGEN	PRECIPITIN INTERFACE	PRECIPITIN TUBE BOTTOM
SERIES 1						
1	19	IV-A	1:640	SONICATED	NEGATIVE	POSITIVE
2	20	IV-A	1:1280	SONICATED	NEGATIVE	POSITIVE
3	19	III-E	1:640	SONICATED	NEGATIVE	POSITIVE
4	20	III-E	1:1280	SONICATED	NEGATIVE	POSITIVE
5	19	IV-A	1:640	WHOLE-CELL	NEGATIVE	POSITIVE
6	20	IV-A	1:1280	WHOLE-CELL	NEGATIVE	POSITIVE
7	19	III-E	1:640	WHOLE-CELL	NEGATIVE	POSITIVE
8	20	III-E	1:1280	WHOLE-CELL	NEGATIVE	POSITIVE
SERIES 2						
1	7	IV-A	1:1280	SONICATED	NEGATIVE	POSITIVE
2	18	IV-A	1:640	SONICATED	NEGATIVE	POSITIVE
3	3	III-E	1:640	SONICATED	NEGATIVE	POSITIVE
4	13	III-E	1:1280	SONICATED	NEGATIVE	POSITIVE
5	7	IV-A	1:1280	WHOLE-CELL	NEGATIVE	POSITIVE
6	18	IV-A	1:640	WHOLE-CELL	NEGATIVE	POSITIVE
7	3	III-E	1:640	WHOLE-CELL	2	2
8	13	III-E	1:1280	WHOLE-CELL	NEGATIVE	POSITIVE
SERIES 3						
1	1	IV-A	1:640	SONICATE-SUPERNATANT	NEGATIVE	NEGATIVE
2	4	IV-A	1:1280	SONICATE-SUPERNATANT	NEGATIVE	NEGATIVE
3	1	III-E	1:640	SONICATE-SUPERNATANT	3	3
4	14	III-E	1:1280	SONICATE-SUPERNATANT	NEGATIVE	NEGATIVE
5	4	IV-A	1:640	SONICATE-SUPERNATANT	NEGATIVE	NEGATIVE
6	8	III-A	1:1280	SONICATE-SUPERNATANT	NEGATIVE	NEGATIVE
7	9	III-A	1:640	SONICATE-SUPERNATANT	NEGATIVE	NEGATIVE
8	9	III-A	1:640	SONICATE-SUPERNATANT	NEGATIVE	NEGATIVE
9	11	III-A	1:1280	SONICATE-SUPERNATANT	NEGATIVE	NEGATIVE
10	11	III-A	1:1280	SONICATE-SUPERNATANT	NEGATIVE	NEGATIVE
11	11	IV-A	1:1280	SONICATE-SUPERNATANT	NEGATIVE	NEGATIVE

¹ SERUM COLLECTED ON 11/12/74 - 11/15/74² NO ANTI SERA ADDED³ BUBBLE IN TUBE

tube, usually within 1 hour after introducing the reactants. This was found to occur with both whole-cell and sonicated antigen preparations, although the reaction occurred earlier and was more complete in the case of sonicated antigen. Dark field microscopic examination under low power revealed that the precipitate formed was not unlike the previously described agglutination reaction.

Additional interfacial ring tests were performed with the supernatant from sonicated antigen centrifuged at 7850 x g for 5 min, but produced negligible precipitation. Such results suggested that antigenicity was retained in the cellular debris of the centrifugate.

IX. OTHER BIOLOGICAL EFFECTS OF TRITIUM IRRADIATION

A. Hatchability

The results of exposure to the five treatment levels as determined throughout embryogenesis (30 days) are shown in Table XXVI. Chi-square (χ^2) values for each treatment compared with the control indicated that under the described experimental conditions, a statistically significant reduction in hatching percentage occurred in the 0.4, 4.0, and 40.0 rad treatment levels. A slight enhancement in hatching efficiency was observed in the 0.04 rad treatment group. However, no demonstrable delay in hatching for any of the four radiation levels as compared to the control was detected.

B. Early Mortality

Mortality as recorded for the immediate period following hatching (60 days) is reported in Table XXVII. Chi-square (χ^2) values for each treatment compared with the control indicated that a significant increase in the incidence of mortality occurred in the 0.04, 4.0, and 40.0 rad treatment

TABLE XXVI. Effect of ^3H on Hatching Percentage of Rainbow Trout Embryos

TREATMENT LEVEL (rads)	NO. EGGS	% HATCH	DEGREES FREEDOM	χ^2	LEVEL
CONTROL	2885	95.84	-	-	-
0.04	3102	96.82	1	3.91	*
0.4	2915	94.53	1	4.96	*
4.0	2641	92.74	1	23.88	**
40.0	2791	90.98	1	49.19	**

TABLE XXVII. Effect of Embryonic Exposure to ^3H on Expression of Posthatch Mortality, Day 30 - 90

TREATMENT LEVEL (rads)	FRY	MORTALITY	DEGREES FREEDOM	χ^2	LEVEL
CONTROL	2537	18.13	-	-	-
0.04	2796	22.60	1	10.80	**
0.4	2547	17.90	1	0.31	N. S.
4.0	2237	23.69	1	14.68	**
40.0	2328	23.71	1	15.04	**

* SIGNIFICANT χ^2 AT 0.05 LEVEL** SIGNIFICANT χ^2 AT 0.01 LEVELN. S. NONSIGNIFICANT χ^2 AT 0.05 LEVEL

levels. However, a significant increase in post-hatch mortality was not encountered in the 0.4 rad treatment group.

C. Abnormality

The numbers of larvae hatching abnormally and the numbers of abnormally formed larvae are shown in Table XXVIII. As before, statistical comparisons were made on the basis of a Chi-square test of independence performed for each treatment group and the control. Over the 30-day period of embryogenesis, while a significant increase in early embryos and those that hatched head-first was observed in the 0.4, 4.0, and 40.0 rad treatment levels, no significant difference in the incidence of abnormally-formed larvae in any of the irradiated groups as compared to the control was detectable.

D. Late Mortality

Mortalities as encountered in each treatment group over the latter 6 months of the study are recorded in Table XXIX. For convenience, these data are reported for two continuous 90-day time periods; day 90-180, and day 181-270. The data are presented in terms of percent mortality of the total available number of fish as determined at the beginning of each period of observation. Summary statistical information is contained in Table XXX.

These data indicated that increasing irradiation dose as administered over 20 days of embryogenesis did not significantly affect expression of late mortality. As well, these data demonstrated no interacting effect on late mortality caused by position of the test groups in the sampling array, either by pond or by compartment within each pond.

TABLE XXVIII. Effect of ^3H on Incidence of Abnormal Hatching of Rainbow Trout Embryos and Abnormal Larvae of Rainbow Trout

EFFECT OF ^3H ON INCIDENCE OF ABNORMAL HATCHING OF RAINBOW TROUT EMBRYOS.

TREATMENT LEVEL (rads)	NO. EMBRYOS	EARLY EMBRYOS	DEGREES FREEDOM	χ^2	LEVEL	HATCH HEAD-FIRST	DEGREES FREEDOM	χ^2	LEVEL
CONTROL	2885	10	-	-	-	0	-	-	-
0.04	3102	19	1	2.17	N. S.	0	1	0.00	N. S.
0.4	2915	35	1	13.52	**	7	1	6.92	**
4.0	2641	24	1	77.03	**	25	1	27.17	**
40.0	2791	40	1	18.84	**	18	1	18.54	**

EFFECT OF ^3H ON INCIDENCE OF ABNORMAL LARVAE OF RAINBOW TROUT

TREATMENT LEVEL (rads)	NO. EMBRYOS	HEAD MALFORMED	DEGREES FREEDOM	χ^2	LEVEL	BODY MALFORMED	DEGREES FREEDOM	χ^2	LEVEL
CONTROL	2885	3	-	-	-	7	-	-	-
0.04	3102	0	1	3.22	N. S.	9	1	0.12	N. S.
0.4	2915	0	1	3.02	N. S.	10	1	0.49	N. S.
4.0	2641	4	1	0.24	N. S.	3	1	1.26	N. S.
40.0	2791	6	1	1.10	N. S.	9	1	0.32	N. S.

*SIGNIFICANT χ^2 VALUE AT 0.05 LEVEL

**SIGNIFICANT χ^2 VALUE AT 0.01 LEVEL

N. S. NONSIGNIFICANT χ^2 VALUE AT 0.05 LEVEL

TABLE XXIX. Mortality, Day 90 - 180
and Day 180 - 270

DAY 90 - 180

COMP.	TREATMENT (RADS)	NO. JUVENILES	NO. MORTALITY	% MORTALITY	NO. SAMPLED	NO. REMAIN
POND I						
A	CONTROL	347	0	0.0	30	317
B	0.04	350	1	0.6	21	328
C	0.4	340	8	2.3	20	312
D	4.0	331	6	1.8	20	305
E	40.0	372	9	2.4	20	343
POND II						
B	0.04	380	6	1.5	20	354
A	CONTROL	345	1	0.2	31	313
D	4.0	342	9	2.6	20	313
E	40.0	342	8	2.3	20	314
C	0.4	308	12	3.8	20	276
POND III						
C	0.4	365	13	3.5	20	332
E	40.0	312	16	5.1	20	276
A	CONTROL	338	2	0.5	31	305
B	0.04	350	6	1.7	20	324
D	4.0	260	9	3.4	20	231
POND IV						
D	4.0	300	5	1.6	20	275
C	0.4	347	9	2.5	20	318
E	40.0	339	13	3.8	20	306
A	CONTROL	325	11	3.3	14	304
B	0.04	344	12	3.4	20	312
POND V						
E	40.0	349	5	1.4	20	324
D	4.0	343	5	1.4	20	318
B	0.04	327	6	1.8	20	301
C	0.4	348	2	0.5	20	326
A	CONTROL	342	18	5.2	30	294

DAY 180 - 270

COMP.	TREATMENT	NO. JUVE- NILES	NO. VACCI- NATED	NO. VACCINATION MORTALITY	% VACCI- NATION MORTALITY	NO. SAMPLED	NO. OTHER MORTALITY	% OTHER MORTALITY	NO. REMAIN	% MORTALITY DAY 90-270
POND I										
A	CONTROL	317	277	36	12.9	215	32	11.5	34	19.5
B	0.04	328	328	7	2.1	110	22	6.8	189	8.5
C	0.4	312	312	12	3.8	120	21	7.0	159	12.0
D	4.0	305	305	6	1.9	119	16	5.3	164	8.4
E	40.0	343	343	40	1.1	155	7	2.3	141	15.0
POND II										
B	0.04	354	354	1	0.2	91	51	14.4	205	15.2
A	CONTROL	313	273	7	2.5	150	42	13.4	114	14.4
D	0.4	313	313	2	0.6	89	27	8.6	195	11.1
E	40.0	314	314	1	0.3	94	13	4.1	206	6.4
C	4.0	276	276	2	0.7	101	8	2.9	165	7.1
POND III										
C	0.4	232	232	11	3.3	92	17	5.2	212	11.2
E	40.0	276	276	1	0.3	91	27	9.8	157	15.0
A	CONTROL	305	265	11	4.1	140	29	11.4	125	12.4
B	0.04	324	324	5	1.5	95	35	10.9	189	13.1
D	4.0	231	231	1	0.4	99	15	6.5	116	9.6
POND IV										
D	4.0	275	275	5	1.8	93	15	5.5	162	8.9
C	0.4	318	318	4	1.2	97	46	14.6	171	17.0
E	40.0	306	306	4	1.3	103	28	9.2	171	13.2
A	CONTROL	304	264	8	3.0	134	28	10.9	134	14.4
B	0.04	312	312	11	3.5	97	22	7.3	182	13.0
POND V										
E	40.0	324	324	14	4.3	90	30	9.6	190	14.0
D	4.0	318	318	5	1.5	88	17	5.4	208	7.8
B	0.04	301	301	2	0.6	91	22	7.3	186	9.1
C	0.4	326	326	1	0.3	98	88	11.4	189	11.7
A	CONTROL	294	251	0	0.0	86	179*	71.3*	29	57.6*

*MORTALITY DUE TO DISSOLVED OXYGEN DEPLETION - MALFUNCTION OF AERATOR SYSTEM.

TABLE XXX. Summary Analysis of Variance for Mortality, Day 90 - 180
and Day 180 - 270 DAY 90 - 180

SOURCE OF VARIATION	DEGREE OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENT	4	5.06	1.26	0.66 ^{N. S.}
PONDS	4	7.74	1.93	1.01 ^{N. S.}
COMPARTMENTS	4	12.74	3.18	1.67 ^{N. S.}
RESIDUAL		22.83	1.90	
TOTAL	24	48.39		

DAY 180 - 270

SOURCE OF VARIATION	FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	94.57	23.64	2.22 ^{N. S.}
PONDS	4	25.95	6.48	0.60 ^{N. S.}
COMPARTMENTS	4	41.55	10.38	0.97 ^{N. S.}
RESIDUAL	12	127.66	10.63	
TOTAL	24	289.75		

* SIGNIFICANT F VALUE AT 0.05 LEVEL

** SIGNIFICANT F VALUE AT 0.01 LEVEL

N. S. NONSIGNIFICANT F VALUE AT 0.05 LEVEL

E. Growth

Mean weights (g) for each treatment group as recorded 1 week prior to vaccination and at four intervals following vaccination are reported in Table XXXI. Mean lengths (cm) are reported in Table XXXII. Summary statistical information for each parameter are contained in Table XXXIII.

These data clearly demonstrated that considerable growth occurred among all treatment groups over the 12-week experimental period. However, these analyses failed to detect a significant difference among means attributable to increasing radiation dose.

These data also indicated a significant pond effect. From similar statistic analyses performed for each sampling interval, Table XXXIV, it was found that a significant pond effect was detected at the time of pre-vaccination sampling, and again 3 weeks following vaccination. Subsequent statistical analyses applying the Duncan Multiple Range Test demonstrated the differences reported in Tables XXXV and XXXVI. At the time of pre-vaccination sampling, the fish maintained in Ponds II, III, and V weighed less and were smaller in length than the fish in Ponds I and IV. Three weeks following vaccination, both weights and lengths of fish in Ponds I and II were significantly lower than fish in Ponds III, II, and V. However, a significant pond effect on weight or length was not detected in subsequent samplings. Also, these data demonstrated the lack of an effect attributed to position by compartment in the pond array.

TABLE XXXI. Mean Weights (g) at Each Sampling Interval

POND I					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	21.97	26.66	22.87	22.75	22.35
3	24.62	23.35	19.62	18.02	21.92
5	41.65	34.52	26.87	31.00	33.82
7	40.40	35.29	33.97	37.52	34.68
9	34.55	38.00	38.00	37.70	37.52
11	49.80	39.52	42.07	42.55	43.37

POND II					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	23.75	21.37	23.05	21.08	22.90
3	22.12	19.27	24.22	24.42	20.85
5	22.50	22.42	29.97	33.57	29.72
7	-	-	-	-	-
9	37.50	33.82	36.87	36.50	34.35
11	47.47	36.22	40.05	44.15	38.82

POND III					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	19.42	20.62	22.57	23.20	22.55
3	29.20	25.82	23.45	25.02	29.83
5	35.77	31.92	31.62	38.30	35.32
7	-	-	-	-	-
9	42.65	31.75	37.75	45.25	32.25
11	39.40	36.65	36.72	55.05	41.60

POND IV					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	25.37	24.54	27.10	27.47	22.45
3	32.67	29.10	33.27	30.00	29.90
5	37.20	29.15	36.82	36.92	34.60
7	-	-	-	-	-
9	53.26	33.85	42.25	41.92	33.80
11	57.84	39.47	49.60	43.00	44.92

POND V					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	21.10	24.32	22.45	23.20	23.35
3	30.42	30.60	24.65	25.57	30.22
5	29.36	31.95	32.97	29.47	36.30
7	-	-	-	-	-
9	48.68	33.80	33.02	33.15	34.07
11	40.64	48.55	46.20	38.65	49.90

TABLE XXXII. Mean Lengths (cm) at Each Sampling Interval

POND I					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	12.53	13.45	12.86	13.00	12.79
3	12.27	12.38	13.24	11.82	12.27
5	14.53	14.34	12.54	13.09	13.87
7	14.49	14.19	14.21	14.73	14.11
9	14.38	14.96	14.93	14.93	15.14
11	15.76	15.16	15.54	15.53	15.35

POND II					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	12.85	12.43	12.89	12.66	12.87
3	12.04	11.58	12.81	12.88	12.35
5	11.97	12.26	13.60	14.41	13.74
7	-	-	-	-	-
9	14.54	14.18	14.43	14.81	14.53
11	15.84	14.57	14.98	16.02	15.20

POND III					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	12.31	12.40	12.55	12.72	12.62
3	13.30	12.77	12.78	12.80	13.66
5	14.49	13.94	13.93	14.84	14.51
7	-	-	-	-	-
9	15.58	14.18	15.01	15.66	14.30
11	15.20	15.14	14.75	17.14	15.74

POND IV					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	13.14	13.13	13.41	13.21	12.77
3	13.72	13.55	14.20	13.82	13.54
5	14.35	13.54	14.64	14.41	14.49
7	-	-	-	-	-
9	16.68	14.50	15.53	15.60	14.63
11	17.41	15.44	16.17	15.85	15.91

POND V					
TIME IN WEEKS	0 RAD	0.04 RAD	0.4 RAD	4.0 RAD	40.0 RAD
0	11.84	12.78	12.64	12.53	12.55
3	13.28	13.55	12.91	12.88	13.55
5	13.10	13.94	14.12	13.84	15.05
7	-	-	-	-	-
9	14.99	14.64	14.26	14.62	14.62
11	14.96	16.59	15.75	15.47	16.57

TABLE XXXIII. Summary Analysis of Variance for Weight (g) and Length (cm)

WEIGHT (g)				
SOURCE OF VARIATION	DEGREE OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
SQUARES (WEEKS)	4	7093.76	1773.44	89.97**
PONDS WITHIN WEEKS	20	721.97	36.09	1.83*
COMPARTMENTS WITHIN WEEKS	20	113.28	5.66	0.29 ^{N. S.}
TREATMENT WITHIN WEEKS	20	498.54		
TREATMENTS	4	214.07	17.78	2.71 ^{N. S.}
TREATMENTS x WEEKS	16	284.47	17.78	0.90 ^{N. S.}
ERROR WITHIN WEEKS	60	1182.74		
TOTAL	124	9610.32		

LENGTH (cm)				
SOURCE OF VARIATION	DEGREE OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
SQUARES (WEEKS)	4	155.47	38.86	104.77**
PONDS WITHIN WEEKS	20	17.98	0.89	2.42*
COMPARTMENTS WITHIN WEEKS	20	2.19	0.11	0.30 ^{N. S.}
TREATMENT WITHIN WEEKS	20	6.32		
TREATMENTS	4	1.91	0.47	1.29 ^{N. S.}
TREATMENTS x WEEKS	16	4.40	0.27	0.74 ^{N. S.}
ERROR WITHIN WEEKS	60	22.31	0.37	
TOTAL	124	204.28		

* SIGNIFICANT F VALUE AT THE 0.05 LEVEL

** SIGNIFICANT F AT THE 0.01 LEVEL

^{N. S.} NONSIGNIFICANT F VALUE AT THE 0.05 LEVEL

TABLE XXXIV. Analysis of Variance for Weight (g) at Each Sampling Interval

PRE VACCINATION				
SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	6.78	1.69	0.76 ^{N. S.}
PONDS	4	39.01	9.75	4.39*
COMPARTMENTS WITHIN PONDS	4	18.74	4.68	2.11 ^{N. S.}
RESIDUAL	12	26.64	2.22	
TOTAL	24	91.18		
3 WEEKS				
SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	32.65	8.16	1.32 ^{N. S.}
PONDS	4	326.77	81.69	13.27**
COMPARTMENTS WITHIN PONDS	4	20.12	5.03	0.81 ^{N. S.}
RESIDUAL	12	73.87	6.15	
TOTAL	24	453.41		
5 WEEKS				
SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	64.06	16.01	0.72 ^{N. S.}
PONDS	4	175.07	43.76	1.97 ^{N. S.}
COMPARTMENTS WITHIN PONDS	4	17.48	4.37	0.19 ^{N. S.}
RESIDUAL	12	265.72	22.14	
TOTAL	24	522.34		
9 WEEKS				
SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	266.43	66.60	2.85 ^{N. S.}
PONDS	4	77.30	19.32	0.82 ^{N. S.}
COMPARTMENTS WITHIN PONDS	4	52.15	13.03	0.55 ^{N. S.}
RESIDUAL	12	279.86	23.32	
TOTAL	24	675.76		
11 WEEKS				
SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	128.62	32.15	0.71 ^{N. S.}
PONDS	4	108.80	27.20	0.58 ^{N. S.}
COMPARTMENTS WITHIN PONDS	4	4.78	1.19	0.02 ^{N. S.}
RESIDUAL	12	536.63		
TOTAL	24	773.83		

* SIGNIFICANT F VALUE AT 0.05 LEVEL.

** SIGNIFICANT F VALUE AT 0.01 LEVEL.

N. S. NONSIGNIFICANT F VALUE AT THE 0.05 LEVEL.

TABLE XXXV. Summary Analysis of Variance for Length (cm)

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	0.38	0.09	1.57 ^{N. S.}
PONDS	4	1.54	0.38	6.38 ^{**}
COMPARTMENTS WITHIN PONDS	4	0.36	0.09	1.51 ^{N. S.}
RESIDUAL	12	0.72	0.06	
TOTAL	24	3.02		

3 WEEKS

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	0.62	0.15	0.94 ^{N. S.}
PONDS	4	7.35	1.83	11.14 ^{**}
COMPARTMENTS WITHIN PONDS	4	1.08	0.25	1.56 ^{N. S.}
RESIDUAL	12	1.97	0.16	
TOTAL	24	10.98		

5 WEEKS

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	1.92	0.48	0.68 ^{N. S.}
PONDS	4	4.47	1.11	1.60 ^{N. S.}
COMPARTMENTS WITHIN PONDS	4	0.19	0.04	0.06 ^{N. S.}
RESIDUAL	12	8.38	0.69	
TOTAL	24	14.98		

9 WEEKS

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENTS	4	1.96	0.49	1.58 ^{N. S.}
PONDS	4	2.35	0.58	1.90 ^{N. S.}
COMPARTMENTS WITHIN PONDS	4	0.14	0.03	0.11 ^{N. S.}
RESIDUAL	12	3.71	0.30	
TOTAL	24	8.18		

11 WEEKS

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARES	F RATIO
TREATMENT	4	1.41	0.35	0.56 ^{N. S.}
PONDS	4	2.25	0.56	0.90 ^{N. S.}
COMPARTMENTS WITHIN PONDS	4	0.45	0.11	0.18 ^{N. S.}
RESIDUAL	12	7.50	0.62	
TOTAL	24	11.63		

* SIGNIFICANT F VALUE AT 0.05 LEVEL

** SIGNIFICANT F VALUE AT 0.01 LEVEL

N. S. NONSIGNIFICANT F VALUE AT THE 0.05 LEVEL

TABLE XXXVI. Comparison of Mean Weights (g) for All Ponds, Prevaccination and 3rd Week, by Duncan Multiple Range Test¹

	<u>Pond I</u>	<u>Pond II</u>	<u>Pond III</u>	<u>Pond IV</u>	<u>Pond V</u>
Prevaccination	21.68	22.43	22.89	23.32	25.39
3rd Week	21.51	22.18	26.67	28.30	30.99

¹Differences are significant at 0.05 level.

TABLE XXXVII. Comparison of Mean Lengths (cm) for All Ponds, Prevaccination and 3rd Week, by Duncan Multiple Range Test¹

	<u>Pond I</u>	<u>Pond II</u>	<u>Pond III</u>	<u>Pond IV</u>	<u>Pond V</u>
Prevaccination	12.47	12.52	12.74	12.93	13.14
3rd Week	12.33	12.38	13.06	13.24	13.77

¹Differences are significant at 0.05 level.

DISCUSSION AND CONCLUSIONSI. TRITIUM ACCUMULATION AND RETENTION

Concentration ratios for tissue-free water of less than unity as encountered from day 12 to day 20 are perhaps due to counting error. It was found that a significant loss of tritium activity could occur if the embryos were stored for more than two weeks in the frozen state prior to analyses. Presumably, this loss in tritium activity was due to dehydration of the embryo by sublimation.

Since tissue-free water tritium was in equilibrium with the exposure medium for part of this period, the concentration ratios of less than 1 for the whole embryo were due to a lower concentration of tritium in the tissue-bound fraction as compared with free-tissue water. Also, because there is undoubtedly less hydrogen per unit weight of embryo than there is in a comparable weight of water, there are less sites available in the tissue-bound fraction for tritium exchange. Additionally, exchange rates of tissue-bound tritium are known to be significantly slower than for tissue-free water hydrogen.

Accordingly, it may be concluded that accumulation of tritium was rapid in both tissue-free water and tissue-bound fractions of the trout embryo. While tritium in tissue-free water reached equilibrium with tritium in the exposure medium, discrimination against incorporation of tritium in the tissue-bound fraction was demonstrated. Elimination of tritium from both tissue-free water and tissue-bound fractions was also rapid. Half-times for both fast and slow components of the tissue-free water fraction were estimated to be 1 hr and 3 days; half-times for both fast and slow components of the tissue-bound fraction being 2 hrs and 6 days. The finding of no concentration of

tritium in the embryo above the water equilibrium level, in part, justified application of the Hine and Brownell (1956) method of dose calculation.

II. THE EFFECT OF TRITIUM IRRADIATION ON PRIMARY IMMUNE RESPONSIVENESS

The findings demonstrating that rainbow trout were capable of producing detectable levels of agglutinins (titers $\geq 1:20$) in 3 weeks, with peak agglutinin levels (titers $\geq 1:2560$) in 11 weeks following a single injection of a heat-killed bacterin preparation, confirmed by belief that vaccination was a suitable substitute for natural challenge in studying the effect of radiation stress on primary immune responsiveness.

For example, in the previously reviewed studies of Strand, et al. (1973) employing a natural challenge approach, at 5 weeks following first signs of infection, the range of positive titers of all surviving fish was only 1:20 to 1:160. Mortality occurred in excess of 50%. In the present study embracing the vaccination approach, at 5 weeks the range of positive titers was 1:640 to 1:2560; and mortality was negligible.

In essence, the vaccination approach was found to offer several advantages over the previously studied natural challenge approach; in that, agglutinins were produced in all fish at a more uniform and, in general, faster rate manifested by a shorter induction period and higher peak titer production. Most important, the experimental fish did not succumb to disease.

It should also be noted that for most effective utilization, the vaccination technique should be used in conjunction with a stable temperature regime and essentially disease-free conditions.

The selection of 20°C as a test temperature was based on previous studies conducted by Fujihara and Nakatani (1971) and Strand, et al. (1973) which

indicated that specific agglutinins to F. columnaris were readily induced over a temperature range of 15°-20°C. Below 15°C, agglutinin synthesis was significantly depressed.

That no disease was encountered during the duration of present studies can only be attributed to employment of the dechlorinated sanitary water source previously described. In fact, as evidenced by routine bacteriological analyses, no potential pathogen was ever isolated from trough or pond rearing waters. Use of only young-of-the-year experimental fish was also designed to preclude confounding problems of disease interaction, as it had been earlier reported by Fujihara and Nakatani (1971), that adult fish surviving initial challenge by F. columnaris became immune carriers of the disease and were fully capable of infecting other fish.

Although under routine operating conditions, TRC levels in rearing waters were efficiently reduced from ~300 ppb to <10 ppb, essentially a "safe" level for rainbow trout (Michigan Dept. of Natural Resources, 1971), the dechlorination facility shown in Figure 7 malfunctioned during the 7th week of sampling resulting in exposure of all experimental fish to TRC levels of at least 35 ppb. The malfunction was believed caused by channeling of the activated carbon matrix due to operating at flow rates near or in excess of design specifications. Channeling likely reduced both the required contact time and the required surface area to efficiently remove TRC to less than the 10 ppb "safe" level. Although the malfunction was easily corrected by back-flushing, exposure to higher than normal levels of TRC during the 7th week likely accounted for the lower than anticipated agglutinins values demonstrated by all experimental fish during the 9th week of sampling. Although readily observed in Pond I, Figure 11, this relationship is not so easily detected in

Ponds II through V, Figures 12-15, because of the lack of sampling in Ponds II through V during the 7th week.

With respect to the relatively small number of caged saline-adjuvant injected and noninjected control fish demonstrating positive titers for E. columnaris during the 3rd and 5th weeks sampling, there was evidence to suggest that some interchange between caged control fish and pond fish occurred in Pond IV and Pond V; although this explanation does not account for caged fish in Ponds I, II, and III demonstrating positive titers against the specific antigen of vaccination. However, it is entirely possible that excess vaccine leaked from the site of injection when antigen injected fish were returned to the ponds following vaccination and was sufficient to stimulate antibody synthesis in the caged fish, but was insufficient in concentration over time to produce a sustained response resulting in higher titers. This latter explanation assumes that vaccine in rearing pond waters was orally ingested or otherwise absorbed by the caged saline-adjuvant and noninjected control fish. Nevertheless, it is clearly seen that agglutinin development in either the saline-adjuvant or the noninjected control group did not follow the kinetics demonstrated in antigen-adjuvant injected control groups. The agglutinin response observed in each respective group, then, must be considered independent. It may also be concluded that adjuvant contained in the vaccine received by antigen-adjuvant injected fish had little influence on the agglutinin response observed in these fish. As well, the lack of a pronounced agglutinin response in noninjected control fish indicated that experimental fish were maintained under essentially disease-free conditions.

The finding of significantly lower agglutinin values in fish from Ponds I and II over the last three sampling periods is more difficult to explain, although chlorine toxicity is again implicated.

It may be argued that because Ponds I and II are closer on the supply header to the source of dechlorinated sanitary water than Ponds III, IV, and V, there was opportunity for higher levels of chlorine to enter Ponds I and II. However, use of a pressurized supply system as employed in the present study, and knowledge that flow rates to all ponds were methodically checked on a minimum daily basis, precludes this explanation from consideration. Furthermore, total residual chlorine values in Ponds I and II were never found to differ significantly from Ponds III, IV, and V.

A more likely explanation requires knowledge of the location of the experimental ponds with respect to a well-traveled footpath through the Hanford hatchery facility. All too often, hatchery staff members and visitors on their route from the main laboratory facility to the laboratory annex used this footpath that passes between Ponds I and II. It is entirely possible, then, that this nearly constant use of the footpath elicited a higher level of metabolic activity in fish maintained in Ponds I and II, through either a "startle" response or feeding response, that ultimately resulted in a higher catabolic than anabolic rate of metabolism. In the absence of more energy for protein synthesis from increased food intake, a higher catabolic rate could result in a lowered rate of protein synthesis, hence lower levels of antibody proteins.

Nevertheless, statistical analyses as presented in Table X confirmed that specific agglutinins to F. columnaris increased rapidly in both control and tritium-irradiated experimental fish following vaccination. As well, during the 9th and 11th weeks, control fish demonstrated significantly higher agglutinin levels, which may be interpreted as suppression of agglutinin synthesis in tritium-irradiated fish.

Statistical analyses as presented in Table X, furthermore, indicated that during the 9th week, fish irradiated at 40.0 rads demonstrated significantly

lower levels of specific agglutinins than all other irradiated and control fish; whereas, during the 11th week, fish irradiated at both 4.0 and 40.0 rads demonstrated significantly lower levels of specific agglutinins than other irradiated and control fish.

A similar relationship is observed if a comparison is made of the percent suppression encountered at each irradiation level with that of control agglutinin levels in each pond over each sampling interval. During the 9th week, agglutinin levels in irradiation test fish were suppressed 50% of control levels at 40.0 rads; while during the 11th week, agglutination levels were suppressed 50% of control levels at 4.0 rads.

These results are likely best interpreted to mean that immune suppression, as here measured, was dependent upon both the level of agglutinins and time; and that opportunity for development of higher agglutinin levels beyond 11 weeks, if provided in the experimental design, may have resulted in demonstrating significant immune suppression at the next lowest dose level. That is, differences among treatment means would likely have become more pronounced as agglutinin levels in treatment groups continued to increase beyond 11 weeks. It is also likely for this reason that attempts to model these data for predictive purposes applying both linear and curvilinear schemes, yielded less than satisfactory results.

However, it may be concluded that under the described experimental conditions, the primary immune response in juvenile rainbow trout was significantly suppressed following irradiation of the embryonic life stage at dose levels as low as 4.0 rads; and as such, provided conclusive evidence to support earlier findings suggesting sensitivity of the primary immune response at essentially the 5 rad dose level (Strand, et al., 1971).

III. NATURE OF TRITIUM IRRADIATION INDUCED SUPPRESSION OF PRIMARY IMMUNE RESPONSIVENESS

A. Effect on Total Serum Protein

Total serum protein as determined over 12 weeks of sampling averaged 3.35 g% with a range of 2.27 to 4.10 g%, which is in good agreement with previous values recorded for this strain of rainbow trout (Strand and Fujihara, 1974). Wedemeyer and Chatterdon (1970) found 2 to 6 g% to be the "normal" range for yearling New Castle rainbow trout. While perhaps these more recent values are on the low side of what may be considered the "normal" range, it is generally assumed that the young of a species demonstrates a lower total serum protein level than the adult.

The only factor found to significantly alter accuracy of the measurement was the presence of hemolysis. This problem, however, tended to be negligible if care was exercised to draw blood slowly into the syringe and to discharge the blood slowly from the syringe to the collecting vial. Drawing of air and blood together also increased opportunity for hemolysis. An error of nearly 2.00 g% was associated with highly hemolyzed samples. It is known that the color produced in the Biuret reaction by 1 mg hemoglobin is equivalent to 1.9 mg serum protein (Henry, 1967).

In the absence of an effect on total serum protein attributed to increasing radiation dose, it is difficult to explain the general trend of decreasing concentrations of total serum protein demonstrated by all experimental groups over the 12-week sampling period, although again, chlorine toxicity as encountered during the 7th week of sampling is suspect. However, a decrease in total serum protein immediately following exposure to elevated chlorine, as disclosed by the 9th week's sample, would have been expected. Instead, the data shown in Table XIV indicate that total serum protein values for all groups were significantly higher during the 9th week than the previous 5th week sampling; and that

a significant decrease did not occur until the 11th week's sample. It still may be the case that the effect of chlorine on total serum protein was delayed until the 11th week, and that the increase in total serum protein encountered during the 9th week reflected an initial over-compensation for sublethal injury. But then, a concomitant decrease in agglutinin levels during the 9th and 11th weeks would also have been anticipated. While there was indication in Pond I that the rate of increase in agglutinins decreased during the 9th week, agglutinin levels demonstrated a marked increase at the 11th week's sampling. Although we might expect agglutinins to increase with a concomitant decrease in total serum protein under certain disease conditions, no disease was encountered in experimental fish during the study period; and in fact, no potential pathogen was ever isolated from the dechlorinated sanitary water source here employed.

The finding of significantly lower total serum protein in fish from Pond I and II during the 9th week, although possibly due to chlorine toxicity, is more likely attributable to increased physical activity of the fish maintained in Ponds I and II in response to nearly constant use of the footpath by hatchery personnel and visitors.

In conclusion, while lowered total serum protein concentration was implicated as a possible cause for lowered agglutinin titers in tritium irradiated experimental fish, the decreased levels of total serum protein as here measured could not be correlated to increasing radiation dose. However, it still may be the case that the Biuret technique here employed was not sufficiently sensitive to detect changes in concentration of specific immune proteins, which are known to constitute only a small fraction of the total available serum protein.

B. Effect on Specific Immune Proteins

At the beginning of the present studies, both cellulose acetate strip and polyacrylamide gel slab electrophoretic approaches were evaluated. Initially,

for any one sample, the cellulose acetate technique provided for better replication. However, the polyacrylamide gel technique provided for separation of a greater number of protein components, and for this reason, was selected as the desired approach for future studies. Approximately 6 months were then spent in evaluating different buffers, gel concentrations, migration times, stains, staining times, and destaining times before attaining the degree of resolution and replication demonstrated in Figure 16.

In general the results shown in Figures 16 and 17 are in good agreement with those obtained by Perrier, et al. (1973) and Reichenback-Klinke (1973) who employed polyacrylamide disc electrophoresis to classify the plasma proteins of rainbow trout. It is interesting to note that Perrier, et al. (1973) observed a band (No. 13) which showed no mobility in 9% polyacrylamide gel, but was subsequently divided into three components migrating close to the origin using 5.5% separation gel. It is, then, highly likely that bands 15, 16, and 17 of fraction IV found during the present study are the same as those detected by Perrier, et al. (1973) employing the disc approach. It is, therefore, not unreasonable to assume that the patterns shown in Figures 18, 19, and 20 may be considered, at least qualitatively to represent the normal polyacrylamide slab gel electrophoresis pattern for immature rainbow trout proteins. However, these results may not represent rigorously quantitative estimates of the relative concentrations of major protein groups, as densitometric scanning always imparts some degree of error due to the inability to establish an accurate baseline.

In the present study, the main variations between experimental animals in any treatment group occurred in fractions I and III. Such differences, however, did not correlate with radiation history, and then must represent polymorphisms inherent to the strain of experimental fish here used.

Other factors such as diet, method of capture for sampling, and in general all other experimental conditions were rigorously controlled; that is, all treatment groups in the sampling array were subjected to near identical conditions over the experimental period. It is unlikely, then, that any such altered experimental condition occurred in one or more but not all treatment groups to account for observed differences in serum protein patterns between nonvaccinated and vaccinated control and irradiated experimental fish. It is equally unlikely that sex, gonadal development, body weight, or length accounted for the observed differences, as all fish used in the present study were immature and of the same weight, length, and age at each interval of sampling. It must, therefore, be concluded that the shifts in percent of total protein observed in vaccinated experimental fish, both control and irradiated, occurred as a result of vaccination; and the differences observed between control and irradiated vaccinated experimental groups were, in fact, attributable to radiation history.

Statistical analyses as shown in Table XXIII and XXIV verified that fraction IV increased as a function of agglutination titer following antigenic stimulation; and as such, suggested that fraction IV represented specific humoral antibody to the immunogen, Flexibacter columnaris. These results are not unlike those described by Summerfelt (1965) who found that the amount of serum protein in nonvaccinated golden shiners, Notemigonus crysoleucas, having an electrophoretic mobility of human gamma globulin was relatively small (2.7 to 5.0%), but increased markedly (6.6 to 8.8%) when the fish were vaccinated with heat-killed suspensions of the bacterial pathogen, Aeromonas liquefaciens.

Statistical analyses as shown in Table XXIII and XXIV also verified that the relative amounts of fast migrating protein fractions, I and II, were lowest in vaccinated fish demonstrating higher agglutinin titers. Summerfelt (1965) also observed a decrease in the relative amounts of rapidly migrating proteins

following vaccination. This response likely occurred as a compensation for synthesis of fraction IV and the requirement to maintain a constant osmoregulatory environment. This response may also be related to recorded decreases in serum albumins associated with many infectious diseases (Flemming, 1958; Wuhrmann and Wunderly, 1960; Dunn and Pearce, 1961).

Finally, statistical analyses as shown in Table XXI indicated that the relative percent of total serum protein contained in fraction IV was significantly reduced in irradiated vaccinated experimental fish, suggesting that decreased amounts of fraction IV in irradiated vaccinated experimental fish accounted for the lowered antibody titers in these fish, demonstrated by agglutinin assay.

It was also interesting to note the finding of a significantly higher percent of total serum protein contained in fraction I of nonvaccinated irradiated experimental fish, as compared with nonvaccinated control experimental fish, shown in Table XVIII. Although a significantly lower percent of total serum protein contained in fraction IV in the irradiated experimental fish was not demonstrated, the 30% lower value recorded for fraction I suggested the clear potential for radiation induced suppression of naturally occurring antibodies, which are also likely contained in fraction IV.

However, positive identification of the protein or proteins serving as antibody in the rainbow trout may not be realized from the above results; that is, by reference to analogies of electrophoretic mobility. This uncertainty is illustrated by Figure 16, in that few if any of the separated serum proteins of rainbow trout may be directly correlated with those of the human by comparisons of their respective mobilities. Perhaps the only exception to this rule is the serum albumins contained in fraction I. Klontz, et al., (1965), Mulcahy (1970), Perrier, et al., (1973), and Hattingh (1974) have all previously

stressed the inapplicability of human nomenclature to identify fish serum or plasma proteins. Obviously, proper classification may not be made without additional information on immunological crossreactivity, molecular weight, and carbohydrate content.

The attempts to conduct immunodiffusion and subsequently immunoelectrophoresis, although for the most part inconclusive, were directed to meet this need. However, in review of these results it was suggested that the antigen or antigens of F. columnaris are highly insoluble, thus accounting for their failure to produce precipitins in either gel or liquid mediums. Moreover, they are likely associated with the cell wall or cell membrane structures of the bacterial cell. If the antigen or antigens were associated with bacterial cell contents, i.e., lipopolysaccharides, enzymes, other proteins, or cellular organelles, retention of some measure of antigenicity in the supernatant would have been expected.

Ordal (1975) also is of the opinion that the antigen or antigen of F. columnaris are insoluble and may be localized in cell wall structure, although to his knowledge no other researcher has attempted to characterize the antigen or antigens of this fish pathogen.

IV. OTHER BIOLOGICAL EFFECTS OF TRITIUM IRRADIATION

A. Early Effects on Hatchability, Early Survival, and Abnormality

The results that demonstrated reduced hatchability in embryos and decreased survival in fry under present experimental conditions are difficult to explain, as they are in clear conflict with previous experiments, (Strand, et al., 1971) employing near identical experimental designs. Ichikawa and Suyama (1974) also reported the lack of significant reduction in hatchability of embryos of puffer, Fugu niphobles, or flounder, Paralichthys olivaceus, at concentrations of tritium up to 10^{-2} Ci/l (10-20 rads). Walden (1971)

found no significant effect on mortality of stickleback embryos, Gasterosteus aculeatus at concentrations of tritium of 0.5, 1.0, and 2.0 mCi/ml (total dose 980 to 3920 rads). Erickson (1971) also failed to detect a significant effect on mortality of guppy, Poecilia reticulata, irradiated during gestation for a period of 17 days at concentrations of tritium of 0.025 and 0.50 mCi/ml (total dose 90 to 2500 rads). Blaylock, et al., (1971) found no significant difference in hatching percentage of carp, Cyprinus carpio, embryos exposed to tritium at levels of 0.52 mCi/ml during a 72-hour period (total dose of 636 rads). Also, Brown and Templeton (1966) did not observe a consistent indication of reduced hatchability when embryos of brown trout Salmo trutta, were reared until hatching in water with ^{90}Sr over a concentration of 10^{-4} to 10^{-2} $\mu\text{Ci/ml}$ (total dose 4.0 to 181.0 rads), or when embryos of plaice, Pleuronectes platessa, were similarly exposed to concentrations of ^{90}Sr from 10^{-7} to 10^{-1} $\mu\text{Ci/ml}$ (total dose 0.004 to 230 rads). As well, Brown and Templeton (1966) failed to detect a significant reduction in hatchability when these same species were chronically irradiated with a ^{137}Cs source over a range of 0.6 to 500 rads.

Also, present results on the incidence of abnormality are not in agreement with my 1971 studies. While an increased incidence of abnormality was encountered at all tritium levels (0.05 to 58.1 rads) in this earlier study, no significant difference in the occurrence of abnormal larvae in any of the irradiated groups as compared to the control was detected under present experimental conditions.

Ichickawa and Suyama (1974) also found that a significant reduction in eye diameter and deformities of the body occurred only at high concentrations of tritium, 1 and 10 Ci/l (total dose 2160 and 15,600 rads). A reduction in eye diameter was reported by Walden (1971) working with stickleback embryos

exposed to tritium, but in each case the abnormality was observed at high concentrations of tritium, 1 and 2 Ci/l (total dose 1960 and 3920 rads). Erickson (1971) reported that development of male characters were generally depressed in guppy, but only at 0.5 and 1.0 Ci/l of tritium (total dose 3400 and 4700 rads). Also, Brown and Templeton (1966) did not report finding a significant increase in the production of abnormal larvae of brown trout and plaice exposed to ^{90}Sr over a concentration range of 10^{-4} to 10^{-10} Ci/l (0.004 to 230 rads), or exposed to gamma radiation from a ^{137}Cs source at 500 rads. McGregor and Newcomb (1968) found a significant increase in the incidence of abnormal larvae of rainbow trout exposed to x-irradiation, but at only relatively high doses of 1000 rads.

In conclusion, a review of both the most recent experiments and past experiments conducted in this laboratory reveals considerable variation among similar treatments, and indicates that significant adverse effects from early life stage exposures to tritium at dose levels of 50 rads and below cannot be consistently demonstrated. Nevertheless, there is a distinct possibility of significant radiation damage occurring at dose levels of less than 50 rads, but this can only be detected and assessed by parameters more sensitive than mortality and abnormality.

B. Late Effects on Survival and Growth

The finding of significantly lower weights and lengths in fish maintained in Ponds I and II during the early sampling intervals is again likely due to increased physical activity in relation to use of the footpath that passes between the two ponds.

However, late effects in terms of decreased survival or depressed growth as expressed in juvenile fish in response to early radiation exposure to

tritium were not observed under present experimental conditions, nor were they anticipated. In all earlier attempts to detect latent effects in juvenile and adult fish as a result of exposure to tritium over embryogenesis (total dose 0.05 to 58.1 rads), and inclusive of determinations of growth rate, relative performance to predation, and time to equilibrium loss and death, no consistent indication of impairment was demonstrated (Strand, et al., 1971).

V. SIGNIFICANCE OF EXPERIMENTAL RESULTS

Proper perspective of these findings may only be gained from a comparison with the concentrations of tritium that now or likely will occur in the environment as a result of operational or accidental releases from nuclear power plants or other nuclear facilities.

Siting on lakes and impoundments which demonstrate slow flushing times and allow for potential buildup of tritium likely represent "worst case" situations. Concern for buildup of tritium is relatively minor in rivers and streams which demonstrate fast flushing times and sufficient dilution capacities.

Peterson, et al., (1969) provided a hypothetical example of siting a 700 MW(e) PWR (pressurized water reactor) on a small lake impounding a natural creek, which they considered reasonably typical for the United States. The volume of this lake was $1.34 \times 10^9 \text{ ft}^3$, with an average outfall of $169 \text{ ft}^3/\text{sec}$. Assuming an annual rate of discharge for tritium of 3820 Ci/yr, the annual average concentration at equilibrium was estimated to be $1.7 \times 10^{-5} \text{ } \mu\text{Ci/ml}$. With no outflow, the estimated annual average concentration was increased to $1.8 \times 10^{-3} \text{ } \mu\text{Ci/ml}$.

More recently, Pagett, et al., (1975) determined that the concentration of tritium in the vicinity of the 700 MW(e) Robinson Plant (Carolina Power and Light Co.) was on the order of $4.5 \times 10^{-6} \text{ } \mu\text{Ci/ml}$. The Robinson Plant is located

on a small reservoir which provides opportunity for recirculation of dilution waters and the potential for buildup of radioactive wastes.

Larger lakes and impoundments have considerably greater dilution capacity; however, this capacity may not be sufficient if several nuclear power plants are installed at one location. For such a larger body of water, Peterson, et al., (1969) selected the hypothetical situation of siting ten 700 MW(e) PWRs on Lake Michigan. Assuming an annual discharge rate for tritium of 5 Ci/yr MW(e), they estimated that by the year 2000, the tritium concentration in lake waters would approach 6×10^{-6} $\mu\text{Ci/ml}$.

In the past, the Public Health Service maintained a nationwide tritium surveillance network. The results reported by this network were published periodically (Chesnutt, et al., 1966; Moghissi and Porter, 1966; US-PHS, 1968) and indicated that for the years 1964-1968, tritium concentrations downstream from major nuclear installations did not exceed a range of 2×10^{-7} to 1×10^{-5} $\mu\text{Ci/ml}$. More recent data provided by the Environmental Protection Agency, who now maintain the surveillance network, indicated that present levels of tritium in surface waters are generally less than 1×10^{-5} $\mu\text{Ci/ml}$ (USEPA, 1974).

It is obvious, then, while present experimental results indicate significant sensitivity of the primary immune response of rainbow trout at essentially the 1.0 $\mu\text{Ci/ml}$ level, and perhaps at the 0.1 $\mu\text{Ci/ml}$ level, these concentrations are no less than 5 to 6 orders of magnitude above present levels for tritium in the aquatic environment, and at least 3 to 4 orders of magnitude above current estimates of future environmental levels.

SUMMARY OF RESULTS

1. The purpose of this study was to illustrate the effects of irradiation on development of the primary immune response in juvenile rainbow trout sublethally exposed to tritium during embryogenesis.
2. Embryos of rainbow trout, Salmo gairdneri were exposed to tritium as tritiated water at concentrations of 0, 0.01, 0.1, 1.0, and 10.0 $\mu\text{Ci/ml}$ for 20 days during embryogenesis. Embryo and water samples were collected routinely to determine concentrations of tritium pursuant to dose calculation.
3. Rearing throughout the experimental period was conducted under essentially pathogen-free conditions.
4. At 5 months after hatching, control and irradiated experimental fish were administered intraperitoneally a 0.1 ml injection of a heat-killed strain of Flexibacter columnaris (1.8×10^8 cells/ml) in 25% Freund's incomplete adjuvant.
5. A standard tube agglutination test was applied to determine agglutinin values to F. columnaris in serum samples at 1 week prevaccination, and 3, 5, 7, 9, and 11 weeks postvaccination.
6. Total serum protein measurements and electrophoretic separation of blood serum proteins followed by densitometric analyses were performed to assess the potential for qualitative and quantitative changes in blood serum components which conceivably accounted for suppressed primary immune responsiveness in tritium-irradiated fish.
7. Data on the effects of embryonic exposure to tritium on hatchability, early survival, abnormality, late mortality, and growth, were also collected.

8. The results were as follows:

- a) Accumulation of tritium was rapid in both tissue-free water and tissue-bound fractions of the trout embryo. While tritium in tissue-free water reached equilibrium with tritium in the exposure medium, discrimination against incorporation of tritium in the tissue-bound fraction was demonstrated. Elimination from both tissue-free water and tissue-bound fractions was also rapid. Half-times for both fast and slow components of the tissue-free water fraction were estimated to be 1 hr and 3 days; half-times for both fast and slow components of the tissue-bound fraction being 2 hrs and 6 days.
- b) Dose estimates for the five concentrations of tritium were calculated to be 0, 0.04, 0.4, 4.0, and 40.0 rads, respectively.
- c) Specific agglutinins to E. columnaris increased rapidly in both control and irradiated fish following vaccination. However, during the 9th and 11th weeks following vaccination, control fish demonstrated higher agglutinin titers, indicating suppression of the primary immune response in tritium-irradiated fish. Agglutinin levels in irradiated fish were suppressed 50% of control levels at 40.0 rads during the 9th week, and 50% of control levels at 4.0 rads during the 11th week.
- d) While lowered total serum protein concentration was implicated as a possible cause for lowered agglutinin titers in tritium-irradiated experimental fish, the decreased levels of total serum protein as here measured could not be correlated to increasing dose of radiation exposure. However, it still may be the case that the Biuret technique here employed was not sufficiently sensitive to detect changes in

specific immune proteins which are known to constitute only a small fraction of the total available serum protein.

- e) Electrophoretic separation demonstrated four major protein fractions in the serum of both control and irradiated nonvaccinated experimental fish. Although there occurred variability among experimental fish, fraction I usually contained four bands; fraction II contained four bands; fraction III contained five bands; and fraction IV contained two bands. Fraction II was not well delimited and stained with a heavy homogeneous dye. Fraction IV was also not well delimited and stained with a light homogeneous dye. Densitometric analyses indicated that irradiated fish contained a significantly higher proportion of total available serum protein in fraction I, but differed insignificantly from control fish in the relative proportion of total serum protein contained in fractions II, III, and IV.
- f) Electrophoretic separation of serum proteins of both control and irradiated vaccinated experimental fish revealed that fraction IV contained four distinct bands. Subsequently performed densitometric analyses demonstrated that fraction IV increased significantly in percent of total protein following vaccination, suggesting that fraction IV represented specific humoral antibody to the immunogen F. columnaris. These data also revealed that percent of total protein in fractions I and II were lowest in vaccinated experimental fish demonstrating highest agglutinin titers, suggesting that the decrease in fractions I and II occurred in compensation for the increase in fraction IV. Finally, densitometric analyses indicated that the relative percent of total protein contained in fraction IV was significantly reduced in irradiated vaccinated experimental fish suggesting that decreased

amounts of fraction IV accounted for the lower antibody titers in irradiated fish demonstrated by agglutinin assay.

- g) A review of all experiments conducted at this laboratory directed at determining the effects of tritium irradiation on the parameters of hatchability, early survival, abnormality, latent mortality, and growth, revealed considerable variation among similar treatments, and indicated that significant effects at dose levels of 50 rads and below could not be consistently demonstrated.
- h) While present experimental results indicated that the primary immune response in juvenile rainbow trout was significantly suppressed following embryonic exposure to tritium at essentially the 1.0 $\mu\text{Ci/ml}$ level, and perhaps at the 0.1 $\mu\text{Ci/ml}$ level, these concentrations are no less than 5 to 6 orders of magnitude above present levels for tritium in the aquatic environment, and at least 3 to 4 orders of magnitude above current estimates for future environmental levels.

FUTURE RESEARCH NEEDS

Of obvious immediate concern, consideration should be given to establishing a more accurate dose-response relationship by following development of the immune response for a longer period of time after irradiation and subsequent antigenic stimulation. The experimental design should also provide for the assessment of potential effects of irradiation of lymphocyte-induced cellular immunity. Comparisons of the effects on both lymphocyte-induced cellular and humoral antibody immunity in response to other radiation sources should also be conducted to determine the relative biological effectiveness of tritium irradiation. Finally, present experimental design needs to provide for a secondary and perhaps tertiary antigenic stimulation in order to assess the potential for permanent damage to the immune processes.

Of longer-term interest, studies need to be conducted to determine at what particular stage or stages of ontogenetic development radiosensitivity of the immune processes are manifest. Also, studies conducted under less artificial conditions aimed at determining the ecological consequences of radiation-induced immune suppression need to be undertaken. As well, serious consideration should be given to study of the potential transmission of suppressed immune competence to offspring of parental stocks irradiated over early life stages.

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APPENDIX A - TERMINOLOGY

Acquired immunity - Antibody dependent immunity specifically acquired as the result of the introduction into the body of foreign substances.

Adjuvant - An agent which when injected with an antigen causes the immunized animal to produce above-normal amounts of antibody to the antigen.

Agglutinin - A sensitizing antibody which causes agglutination or clumping of bacteria or other cells in a suspension.

Albumin - A protein found in nearly every animal and in many vegetable tissues, and characterized by being soluble in water and coagulable by heat.

Anamnestic response - A recall response. It designates an antibody response to any reinjection of an antigen administered some time after the primary response at a time when any previous response has passed its peak and has dropped to a low grade. It is usually marked by a shorter latent period, a faster response, and, with many antigens, a higher peak titer than in the previous response.

Anaphylactin - An antibody that reacts with its antigen in vivo to produce anaphylaxis (contraction of smooth muscle resulting in sneezing, difficulty in breathing, incontinence, and death).

Antibody - A specific substance which is produced in an animal in response to the presence of an antigen, and which reacts with the antigen in some observable way.

Antigen - Almost any material foreign to the body, although chiefly proteins, which when injected into the animal results in the production of antibodies.

Antitoxin - An antibody which is specifically antagonistic (neutralizing) to some particular toxin.

Bursa of Fabricius - A lymphoid organ attached to the intestine near the cloaca, found only in birds.

Complement - Five protein fractions in serum which are needed to combine with certain antigen-antibody complexes, such as hemolysin-sensitized red cells, to cause lysis of the cells. It occurs in normal serums and is usually obtained from guinea pigs. It is heat-labile.

D₃₇ - Terminology used to express the sensitivity of an organism to radiation. It is the dose at which 37% of the population survives.

Densitometry - For the registration of site, shape, and intensity of precipitation bands or lines in agar plates or tubes. It may be used with other mediums as well.

Destainer - An apparatus to remove excess stain not bound to protein in the agar or polyacrylamide gel medium used in electrophoresis.

Electrophoresis - The movement of particles, including molecules in an electric field. The rate of migration is related to the total electrical charge of the molecule at the pH in question. Under standard conditions, each molecule has a characteristic rate of movement. In serum, the rate of migration of albumin is fastest and gamma-globulin is slowest.

Erythrocyte - A red blood cell.

50% endpoint - Within a controlled standardized carefully performed titration, this point gives the least statistical and technical error.

Freund's adjuvant - A commonly used adjuvant composed of a lanolin-like substance emulsified with a suspension of tubercle bacilli (complete) or without a suspension of tubercle bacilli (incomplete). It is used mixed or emulsified with the antigen.

Globulin - A class of proteins characterized by being insoluble in water, but soluble in saline solutions.

Granulocyte - Any cell containing granules, especially a leukocyte containing neutrophil, basophil or eosinophil granules in its cytoplasm.

Hemagglutinin - An antibody that causes agglutination of red blood cells.

Hemolysin - An antibody that in the presence of complement lyses red blood cells.

Humoral - Pertaining to any fluid or semifluid of the body.

Immunodiffusion - A technique to detect the presence of antibody in serum. It is based on the combining of soluble antigen and antibody in a gel matrix to produce a visible precipitate.

Induction period - The interval between the injection of antigen and the first detection of newly synthesized antibody. This period has also been termed the latent period.

Interfacial ring test - A technique to detect the presence of antibody in serum. It is based on the combining of soluble antigen and antibody in a liquid medium to form a visible precipitate.

Leukocyte - Any colorless ameboid cell mass such as a white blood cell, lymphocyte, or wandering connective tissue cell.

Lymphocyte - A variety of white blood cells which arise in the reticular tissues of lymph glands or lymph nodes.

Macrophage - A large mononuclear wandering phagocytic cell.

Monocyte - A large leukocyte, 9 to 12 μ in diameter with an oval or indented nucleus and having more protoplasm than a lymphocyte.

Parenteral - Not intestinal, by some way other than the digestive tract, i.e., intravenous, intramuscular.

Plasma cell - A specialized lymphoid cell that synthesizes and secretes specific antibody.

Platelet - One of the small colorless corpuscles in the blood of all mammals in the form of an oval or round biconcave disk $3\ \mu$ in diameter. The platelet is concerned in coagulation of blood.

Poikilothermic - Pertaining to animals whose body temperatures correspond to the environment (cold-blooded).

Precipitin - An antibody which acts on its antigen to produce a precipitate.

Primary response - The antibody response to the first injection or series of closely placed injections of an antigen.

Rad - A unit of absorbed dose from radiation exposure equal to 100 ergs per gram, or 0.01 J/kg.

Roentgen - A unit of radiation exposure from x or γ -emissions, where 1 roentgen (R) equals 2.58×10^{-4} coulomb per kilogram of air.

S - Svedberg or sedimentation constant that denotes the relative sedimentation rate of molecules under high speed centrifugation. Thus, 7S molecule is of lower molecular weight than an 18S molecule.

Serum - The fluid portion of the blood separated from its more solid elements, especially the clear liquid separated in the clotting of blood. Serum does not contain fibrinogen.

Sonicator - An apparatus that will disrupt or lyse cells by sonic vibration.

Thrombocyte - A blood platelet.

Thymus - A ductless gland located in the chest just below the sternum in mammals. It may also be found in other vertebrates. It usually reaches maximum development during the juvenile life-stage.

Tissue-bound tritium - Tritium remaining in a biological tissue after removal of the tissue-free-water tritium.

Tissue-free-water tritium - Tritium present in the water of a biological tissue removed by lyophilization, vacuum distillation, or by a similar technique.

Titer - The amount of antibody per unit of serum, usually based on the highest dilution which gives the endpoint, i.e., agglutination, precipitation.

Total residual chlorine - Inclusive of free chlorine residuals (chlorine, hypochlorous acid, hypochlorite ion) and combined chlorine residuals (chloramines).

Total serum protein - Essentially the albumin and globulin components of blood serum, where grams of protein per hundred milliliters (g%) is the unit of measurement.

Virulence - The relative infectiousness of a microorganism.

VITA

John Albert Strand, III, was born on [REDACTED] in Red Bank, New Jersey. He lived in Red Bank for 20 years with his parents, Theodora and John Strand, Jr. John received a B.A. in Biology from Lafayette College, Easton, Pennsylvania, in 1960. He received his M.S. in Biology in 1962 from Lehigh University. Mr. Strand served in the Navy from 1963 to 1967.

From 1964 to 1969, while a scientific investigator with the Biological and Medical Sciences Division at the Naval Radiological Defense Laboratory, San Francisco, his research covered numerous marine biological studies. His interests in radioecology, marine physiology and fisheries biology were further developed at Battelle-Northwest.

Mr. Strand joined Battelle-Northwest, Richland, Washington, in 1969. He has conducted research dealing with determining the biological uptake and incorporation of tritium by embryonic life stages of the rainbow trout, the irradiation dose imparted by both adsorbed and absorbed tritium, and the radiosensitivity of embryonic, fry, and juvenile life stages to tritium. Additionally, Mr. Strand assisted in the development of a marine research program to be conducted at the Battelle-Northwest Marine Research Laboratory at Sequim, Washington.