

SERUM AND MUCUS ANTIBODIES
IN RAINBOW TROUT. (SALMO GAIIRDNERI)

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A Thesis
in
The Department
of
Biological Sciences

Presented in Partial Fulfillment of the Requirements
for the degree of Master of Science at
Concordia University
Montréal, Québec, Canada

September 1981

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ABSTRACT

SERUM AND MUCUS ANTIBODIES IN RAINBOW TROUT (SALMO GAIRDNERI)

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Rainbow trout (Salmo gairdneri) were found to be immunologically competent at both 9°C and 15°C, and produced serum and mucus antibodies in response to antigenic challenge with sheep red blood cells. Only high molecular weight antibodies, with a molecular weight of approximately 760,000 daltons, were detected. There was no evidence of production of low molecular weight antibodies for up to 133 days of weekly immunization. Two antigenically similar serum immunoglobulin populations with different electrophoretic properties were isolated. Mucus antibodies were found to be antigenically similar to serum antibodies, and immunofluorescence microscopy suggested that mucus immunoglobulin can be synthesized locally. The results are discussed with reference to their relevance to defense of rainbow trout against infection.

ACKNOWLEDGEMENTS

I thank my family and friends for their encouragement throughout this project. Sebastian's moral support was deeply appreciated. I would also like to thank my supervisor, Dr. P. Anderson for his guidance, Dr. C.K. Osterland for the use of his laboratory facilities, and Dr. N. Gilmore for his helpful suggestions. Sincere appreciations go to Yves Trudeau for his capable and patient assistance in the latter part of this project, to Dr. S. d'Apollonia for the graphs and photos; and to Claudia Povajnik and Denise Anderson for typing this thesis.

This work was supported in part by a grant from the Department of Education of Quebec (FCAC Q-10).

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INTRODUCTION

1.0 General Introduction.

The immune system is responsible for an organism's defense against infection by foreign agents, but is an often overlooked aspect when studies of various body systems of fish are carried out. There are many reasons for proposing more intensive studies of fish immunology. Many species of fish, for example, are economically important to man; some are bred in hatcheries and are released into lakes and rivers for restocking purposes. It would be of great advantage to be able to induce a long-term protective immunity against infection in these fish prior to their release from hatcheries. This is not yet feasible, and a further understanding of fish immunology is needed to determine optimal modes and conditions for conferring protective immunity.

Chemicals released by man into the aquatic environment can seriously affect reproduction, development, growth and behaviour of fish. Toxicants could also conceivably affect the immune system of fish, and some insight into the effects of these toxicants could be gained by using immunological parameters when studying populations of fish which have been exposed to pollutants and toxicants. Finally, a more thorough investigation into the immunity of fish could further our understanding of immunological developments and functions as they relate to man.

1.1 Functions of the immune response

A brief description of a well-studied, highly advanced immune response, that found in mammals, will be presented to introduce basic concepts which will be referred to in the discussion of fish immunology. The mammalian immune response is divided into humoral and cellular

components which are mediated by "B" and "T" lymphocytes respectively. The B lymphocytes are responsible for the humoral response, through the production of antibodies, and T lymphocytes mediate certain cellular responses such as rejection of grafts and immunity against viral diseases and against infection with intracellular bacteria. Both B and T lymphocyte-mediated responses are specific for a particular inducing antigen and have a memory component, such that if the same antigen is encountered after the primary response has subsided, a more potent secondary response, with a shorter latent period and a slower return to pre-exposure levels, is elicited. Specificity and memory (anamnesis) characterize the true immune response, but non-specific mechanisms, such as phagocytosis by polymorphonuclear cells and macrophages, the complement system, lymphokines, lysosomal enzymes and vasoactive amines serve to augment the immune response.

1.2 Antibody structure and classification system

Antibody molecules, or immunoglobulins, have a common structure which consists of four polypeptide chains, two identical heavy chains (H_2), and two identical light chains (L_2), which are held together by non-covalent and disulfide bonds (Figure 1). Classification of immunoglobulin depends on differences in primary structure of the heavy chains; human immunoglobulins, for example, have been assigned to five classes. The classes are IgG, IgA, IgM, IgE and IgD, their heavy chains are gamma, alpha, mu, epsilon and delta respectively. IgG, IgD and IgE exist in the serum as 7-8S monomers (H_2L_2). IgM is present as a 19S pentamer (H_2L_2)₅; a small polypeptide, the "J" or joining chain links the five subunits of IgM into a circular configuration (Figure 2).

Figure 1. Schematic representation of a monomeric 7S immunoglobulin molecule. (Adapted from Hood L.E., Weissman I.L. and W.B. Wood, in: Immunology. 1978. The Benjamin/Cummings Publishing Company, Inc., California. P.5.).

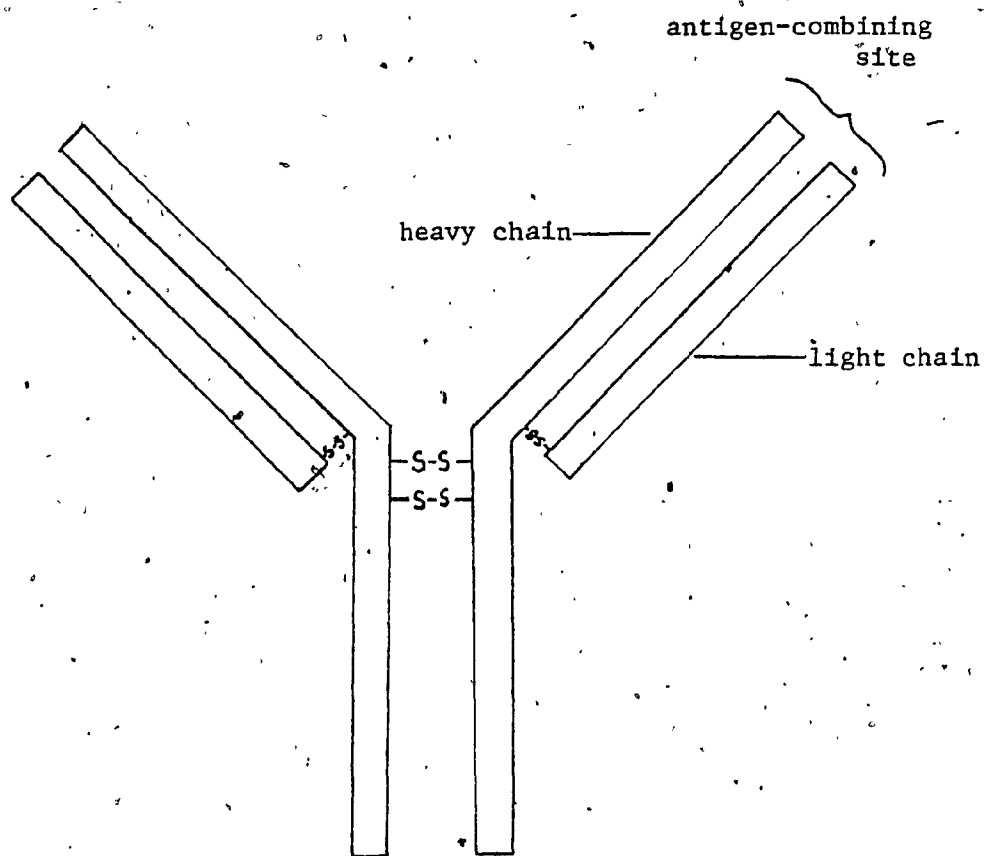
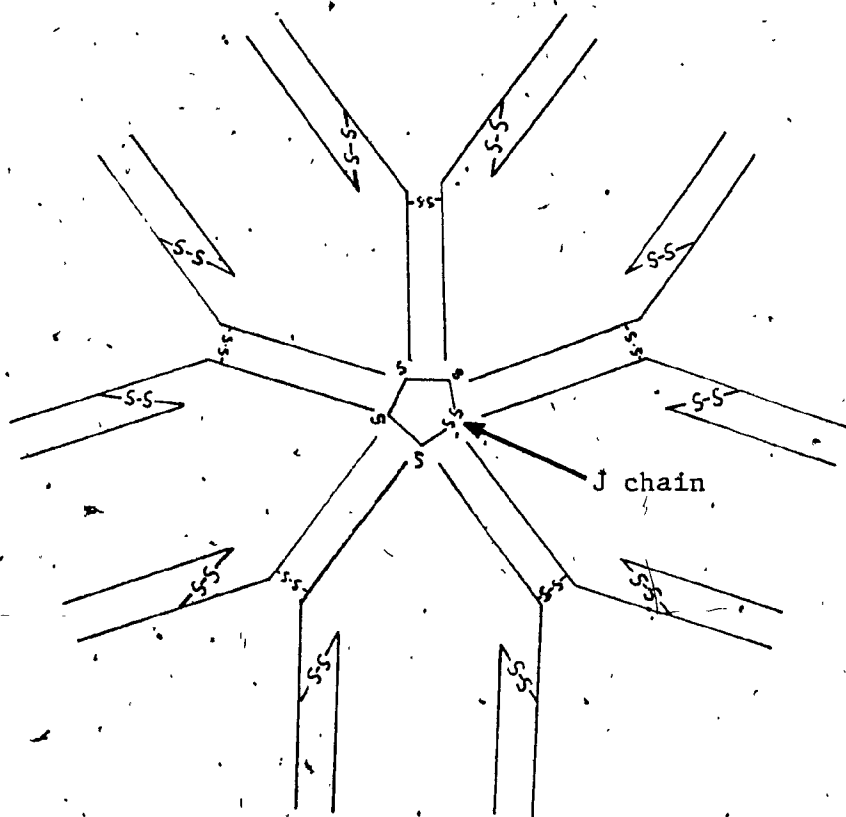


Figure 2. Schematic representation of circulating 19S pentameric IgM.

(Adapted from Hildemann W.H., Clark E.A. and L.L. Raison 1979. In:

Comprehensive Immunogenetics. Elsevier North Holland, Inc., New York, P.3).



Serum IgA is present in monomeric (H_2L_2), dimeric ($(H_2L_2)_2$) and trimeric ($(H_2L_2)_3$) form.

1.3 Secretory antibody

IgA is the major immunoglobulin present in external secretions of the gastrointestinal and respiratory tracts in humans. This secretory IgA is dimeric and contains a J chain similar to that found in pentameric IgM, and a protein with a molecular weight of 71,000 daltons called the secretory piece. The secretory piece probably functions to protect the secretory IgA molecules from proteolysis by the enzymes present on the mucus membranes. Secretory immunoglobulin does not originate from blood and body fluids, but is produced locally by plasma cells (mature antibody-producing B-lymphocytes) present in the lamina propria of the respiratory and gastrointestinal tracts. Secretory piece is produced by serous epithelial cells. As the secretory immunoglobulin diffuses toward the lumen, it passes between and through the epithelial cells where it becomes bound to the secretory component, and is released onto the surface of the lumen.

1.4 Historical review

1.4.1 Immunity in invertebrates

All animals are capable of defending themselves from infection in a hostile environment. The complexity and specificity of the defense mechanisms increase as animals ascend the evolutionary scale. The most primitive unicellular animals, the protozoans, phagocytose and hydrolyze foreign particles, and, while this process is a form of feeding, it

offers protection against environmental organisms as well.² Multi-cellular invertebrates show a more sophisticated defense system: cells found in hemolymph or coelomic fluids of annelids, crustaceans and insects, for example, have been shown to carry out non-specific phagocytosis of a variety of inert particles, cells and bacteria.³⁻⁸

Humoral defense mechanisms have also been reported in these invertebrates. The blood and body fluids of many different invertebrate forms bring about agglutination, precipitation and lysis of widely different antigens.^{5,6,9-12} These are probably not true B-type humoral immune responses, however, as the specificity of these responses is questionable. Molecules analogous to mammalian immunoglobulins have not been identified in any invertebrate species.^{10,12,13}

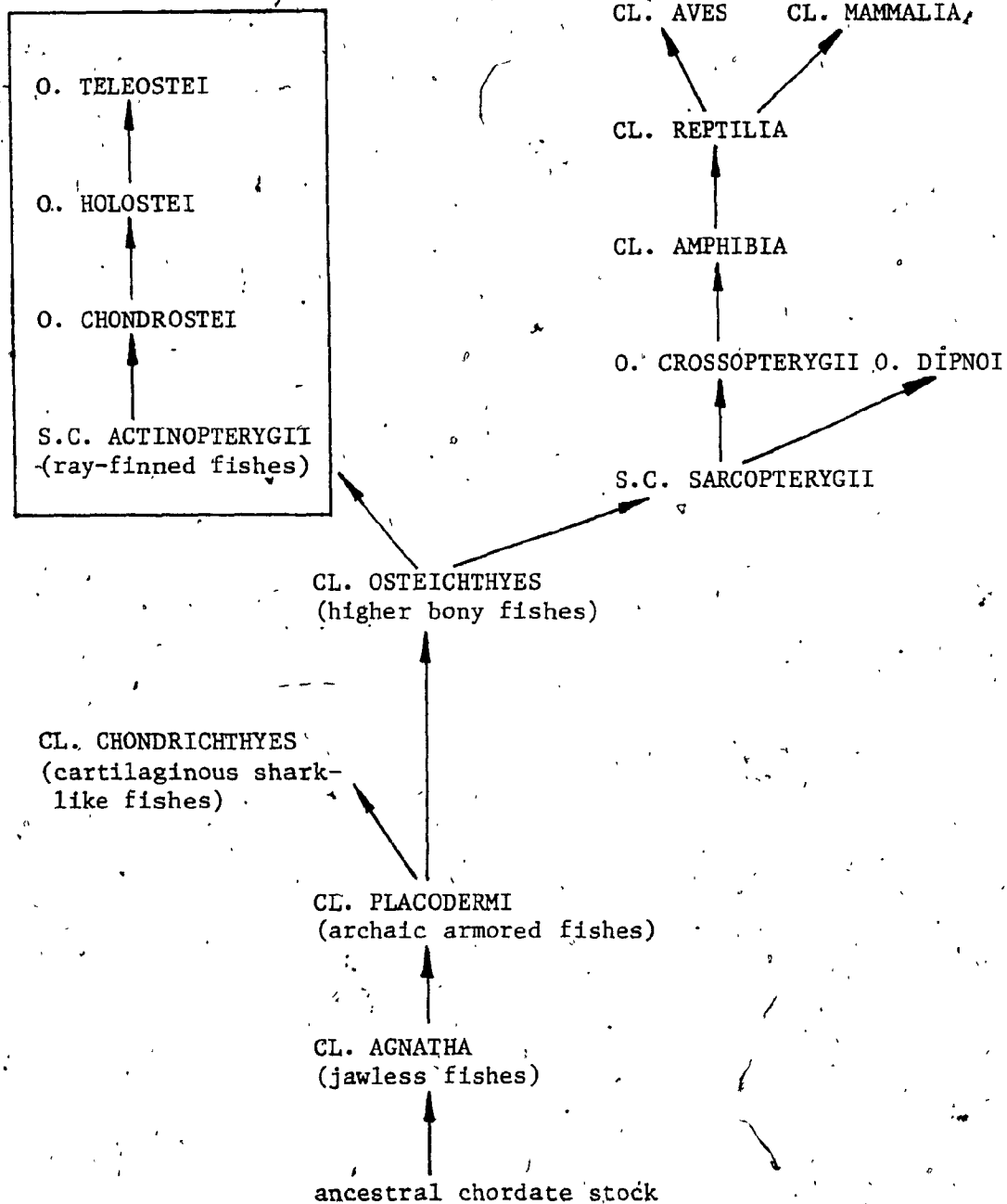
There is evidence of cellular response involving specificity and at least short-term memory in some of the advanced invertebrates. Acceptance of autografts (tissue grafted back into the original donor) and rejection of allografts (grafts between members of the same species) and xenografts (grafts between species), with accelerated rejection of second- and third-set grafts (second and third grafts from same type of donor) has been reported in annelids^{14,15} and echinoderms¹⁶. It seems, then, that specific cellular immunity appeared earlier during evolution, and preceded the capacity for humoral antibody response.^{14,15,17,18}

1.4.2 Immunity in fishes (see Figure 3 for phylogeny)

1.4.2.1 Agnatha

Specific immunological responsiveness has been definitely demonstrated in the hagfish, a representative of one of the most primitive

Figure 3. Diagrammatical tree of the phylogeny of the vertebrate classes. The subclass Actinopterygii has been expanded and blocked off for emphasis. (Adapted from Acton R.T. et al 1971⁴⁵ and from Romer A.S. 1962. Who's who among the vertebrates, Ch. 3, in The Vertebrate Body, Shorter Version. W.B. Saunders Co., Philadelphia).



vertebrates. Hagfish have been shown to be capable of recognizing and rejecting skin allografts; the rejection process is specific and shows immunologic memory.^{19,20} Hagfish produce specific serum antibody in

response to injections of a number of cellular and soluble antigens.^{19,21-23}

The antibody, a macromolecule, was first described as being similar to the IgM of higher vertebrates.^{19,22} Later,

DeLoannes and Hildemann (1975)²⁴ were unable to find any evidence of a mu-like heavy chain component in hagfish immunoglobulin, and proposed that the immunoglobulin is a multimer of light chain components alone.

Raison et al (1978)²³ reported that hagfish have both high molecular weight and low molecular weight antibody, and that the high molecular weight molecules dissociate into the low molecular weight forms. They found that the heavy chains of hagfish antibody were similar to the mu-chains of mammalian IgM in molecular weight, but that the light chains were significantly heavier than those of their mammalian counterparts.

Conflicting results have also been reported in studies of the structure of lamprey immunoglobulin. Marchalonis and Edelman (1968)²⁵ reported that two antigenically similar forms of immunoglobulin, a 7S and a 14S molecule exist in the lamprey. The 7S protein could be broken down into heavy and light chain components, and the heavy chain was found to be similar to the IgM heavy chain of higher vertebrates in molecular weight, multichain structure and diversity. They found the immunoglobulin molecule to be extremely labile and postulated that lamprey immunoglobulin consists of two light and two mu-like heavy chains which are lacking interchain disulfide bonds. The 14S molecule was thought to be an aggregate of 7S molecules. Although

Litman et al. (1970)²⁶ and Pollara et al (1970)²⁷ agreed that lamprey immunoglobulin lacks interchain disulfide linkages between its major subunits, they found the antibody to be confined to a 9S fraction, with no evidence of counterparts to either heavy or light chains of higher vertebrates.

1.4.2.2 Chondrichthyes

The immunoglobulins of the more highly evolved cartilaginous fishes have been somewhat better characterized. Sharks have both high molecular weight (19S) and low molecular weight (7S) forms of antibodies.²⁸⁻³⁵ Both forms of antibody are very similar to mammalian immunoglobulins: they are composed of equimolar amounts of light and heavy chain, the light chains are similar to those found in immunoglobulins of higher vertebrates, and the heavy chains of both resemble those of mammalian IgM. The 19S and 7S antibodies belong to the same IgM-like class; the 7S form is a monomer (H_2L_2) and the 19S form is a pentamer (H_2L_2)₅ of 7S subunits.^{28,29,30-35} McCumber and Clem (1976)³⁶ were able to isolate and characterize what appeared to be the J chain from the high molecular weight immunoglobulin of the nurse shark.

It has been postulated that, although both monomeric and pentameric antibodies belong to the same immunoglobulin class, they function and are distributed throughout the body as if they were of different classes.³²

There appears to be no extracellular interconversion of 19S and 7S antibody, either in vivo or in vitro.³² It has been reported that sequential antibody production occurs in sharks, that the first antibodies produced in response to antigenic stimulation are of the high molecular weight type, and later, after months of immunization, of the

7S type. ^{28,34,37} Sigel et al (1972) ³⁸ found that, after prolonged immunization, there was a significant increase in the binding affinities of nurse shark 7S IgM, but not of the 19S IgM.

It has also been recorded that some sharks produce only 19S IgM antibody in response to immunization, while other sharks, sometimes from the same group of fish, produce both 19S and 7S IgM. ^{39,40} In the sharks producing only 19S IgM, 7S antibodies were not detected even after prolonged immunization, while in sharks producing both 19S and 7S IgM there was no increase in affinity of either form of immunoglobulin over a period of many months after immunization.

There have been reports of the occurrence of shark immunoglobulins which differ from the typical IgM-like antibodies. Gitlin et al (1973) ⁴¹ reported that sharks have at least two, and as many as four, distinct classes of immunoglobulins, some of which are present as both high molecular weight and low molecular weight components. They did not, however, isolate and characterize the individual immunoglobulin classes to determine whether or not any resembled those of higher vertebrates. Fuller et al, (1978) ⁴² found that in immunized nurse sharks the heavy chains of the majority of the 7S antibodies were not similar to those of mammalian IgM, but seemed to be more closely related to the gamma chain of mammalian IgG; 7S immunoglobulin with a mu-like heavy chain seemed to constitute a minority. These findings have yet to be substantiated by other authors.

Johnston et al (1971), ⁴³ working with another chondrichthyan, the stingray, found that antibody activity was confined to high molecular weight material only. They speculated that low molecular weight immunoglobulin probably does exist in the stingray, but was not

detected by their methods. The stingray macroglobulin was found to be functionally, physicochemically and ultrastructurally very similar to the IgM macroglobulin found in mammals, and electron microscopy confirmed the pentameric structure of the 19S protein. Marchalonis and Schonfeld (1970)⁴⁴ reported that although high molecular weight antibody is present in the serum of stingrays, the major immunoglobulin is a dimer $(H_2L_2)_2$ with a sedimentation coefficient of 11S. The heavy chains of both forms of immunoglobulin were found to be similar to the mu-chains of human IgM in molecular weight and electrophoretic mobility.

1.4.2.3 Chondrostei

The paddlefish, Polyodon spathula, a representative of the primitive ray-finned chondrosteans, has one form of antibody. This 14S molecule can be broken down into light and heavy chains, the amino acid sequences of which are similar to those of mammalian IgM. Electron microscopy shows that the 14S molecule is a tetrameric $(H_2L_2)_4$, rather than a pentameric, form of IgM.⁴⁵

1.4.2.4 Holostei

Similar results have been reported in holostean fish; the gar, Lepisosteus, produces only high molecular weight type of antibody to a variety of antigens.⁴⁵⁻⁴⁸ The isolated 14S immunoglobulin molecule could be broken down into eight heavy and eight light chain components, and the molecular weight, amino acid sequences and proportion of heavy and light chains were similar to those of mammalian IgM.⁴⁶ The tetrameric structure of this 14S molecule has been confirmed by electron microscopy.^{45,46}

1.4.2.5 Teleostei

It appears that some teleosts have only tetrameric IgM-like immunoglobulin while others have both tetrameric and monomeric immunoglobulin. A single high molecular weight antibody has been described in catfish⁴⁵ and the gray snapper.⁴⁹ Mestecky et al (1975)⁵⁰ were able to identify and isolate catfish J chain, which has a molecular weight of approximately 15,000 and appears to be covalently attached to the tetramer by disulfide bonds. Goldfish have been reported to have two populations of high molecular weight 14S antibodies which are distinguishable from each other by net charge only;^{51,52} there was apparently no size difference between these two forms of immunoglobulin, and they were antigenically similar. Uhr et al (1962)⁵³ reported both high molecular weight and low molecular weight antibody in goldfish, with a shift from 19S to 7S with time. Marchalonis (1971),⁵⁴ however, found evidence that the low molecular weight immunoglobulin of cyprinoid carp and goldfish is an in vitro degradation product of the 19S immunoglobulin, and is brought about by storage at 4°C. Both high molecular weight (16S) and low molecular weight (7S) IgM-like immunoglobulin have been described in groupers, margates and bluegill sunfish.^{37,55-58}

1.4.2.5.1 Salmonidae

Salmonids have generally been described as having only high molecular weight immunoglobulin, a 16-18S tetramer of IgM-like 7S monomers. Cisar and Fryer (1974)⁵⁹ have reported that there are actually two populations of this tetrameric antibody, that they are antigenically related, but are distinguishable from each other by different electrophoretic mobilities on agar gel electrophoresis. Others have reported

immunoglobulin of only one electrophoretic mobility.⁶⁰⁻⁶⁴ There appears to be no shift from high molecular weight to low molecular weight antibody in brown trout; antibody remains in the high molecular weight fraction in both primary and secondary responses.⁶⁵ Alexander et al (1970)⁶⁶ have reported the presence of both high molecular weight 19S and low molecular weight 7S immunoglobulin in the serum of healthy and diseased salmon. They stated that they were able to identify 7S antibody in the serum of all of these fish, but that 19S antibody was lacking in some.

1.4.2.6 Dipnoi

Clear evidence of the emergence of a second class of immunoglobulin distinct from IgM-like molecules is first seen in dipnoid lungfish.⁶⁷ High molecular weight (19S) IgM-like immunoglobulin is maintained and an additional smaller 5.9S antibody molecule is found. The heavy chains of this new immunoglobulin do not resemble those from any of the major immunoglobulin classes of vertebrates.

1.5 Effect of temperature on antibody production

Ambient temperature has been found to have a profound effect on the immune response in poikilothermic vertebrates. Studies carried out in the wild have shown that incidence of disease in fish varies with seasonal changes in water temperature.^{68,69} Controlled studies carried out under laboratory conditions have shown that poikilotherms are more resistant to initial infection, and if already infected, are better able to rid themselves of disease at higher temperatures.⁷⁰

Poikilotherms which have been infected with live or killed bacteria

seek out a higher than normal temperature when possible.⁷¹ Early experiments, in which poikilotherms were injected with antigens at various temperatures, showed substantial antibody production at high temperatures and little or none at low temperatures.⁷²⁻⁷⁵ Others have shown that antibody production often does occur at low temperatures,⁷⁶ but that the rate of production occurs more slowly,^{69,77,78} such that many weeks or months may elapse before detectable titers are demonstrable.⁷⁹

1.6 Mucus: its functions and involvement in the immune response

The outer surface of fish is covered by a layer of slimy mucus, to which a number of functions have been ascribed. Rosen and Cornford (1971)⁸⁰ postulated that fish mucus assists in locomotion by reducing surface friction of the fish as it swims. The continuous replacement of mucus probably also protects the fish against colonization by parasites, fungi, and bacteria: it has been noted that fins of trout and char have significantly fewer mucus-producing cells than the rest of the body, and that in an outbreak of Saprolegnia, infected fish developed fungal patches on the fins before the rest of the body was affected.⁸¹ It has been suggested that mucus may play a part in maintaining osmoregulation in fish.⁸² The reported coagulation of suspended mud particles by fish mucus could conceivably be of survival value, especially at the level of the gills, where such precipitation would better allow fish in turbid water to obtain a continuous supply of oxygen.

That this skin mucus may provide more than a passive barrier from infection is seen by studies carried out on the Amazonian discus fish.⁷⁹ Newly-hatched fry feed on a mucus secretion derived from parental

epidermis; the fry die in the absence of the parents, but survive if they are reared in water to which broad-spectrum antibiotics have been added.⁷⁹ It seems, then, that the parental mucus is able to confer a protective immunity upon the fry. Lysozymes and lymphocytes have been described in catfish mucus⁸³ and specific antibodies in response to antigenic stimulation have been induced in the holostean gar⁸⁴ and in the teleostean plaice.⁸⁵ These antibodies are of high molecular weight and appear to be related to serum antibodies; it has been postulated that a secretory system similar to that found in mammals is involved in the synthesis of these mucus antibodies.^{84,85}

1.7 Purpose of this study

The present study was undertaken to study some of the humoral aspects of the immune system in a primitive cold water teleost, the rainbow trout (Salmo gairdneri). The effect of temperature on the production of serum antibodies was investigated. Attempts were made to identify high molecular weight and low molecular weight antibody in rainbow trout. Serum and mucus antibodies were identified and their relationship to each other was investigated. An attempt was made to determine whether or not mucus antibodies are produced locally: immunofluorescence procedures were employed for identification of antibody-secreting cells at the level of the skin in rainbow trout.

MATERIALS AND METHODS

2.0 Materials and methods

2.1 Experimental fish

Rainbow trout (Salmo gairdneri) were purchased from the Lac à l'Eau Claire hatchery in St-Alexis-des-Monts, Quebec. Only those trout weighing more than 500 grams were used in these studies, as small rainbow trout have been reported to respond poorly to antigenic stimulation.⁸⁶ Experimental trout were maintained in tanks with a continuous flow of dechlorinated water, and were fed daily with a commercial pelleted dry food.

2.2 Temperature studies

Groups of seven fish were kept at either $9^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ or at $15^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. Care was taken so that temperature was the only variable: both groups of fish received the same amount and type of light, water flow was the same in both tanks, and both groups of fish were fed daily at the same time.

2.3 Collection of samples from trout

2.3.1 Collection of serum

Trout were anaesthetized with tricaine methane sulfonate, (Sandoz or Sigma), and bled from the dorsal aorta with a 23-gauge needle. The blood was allowed to clot for one to two hours at room temperature, and overnight at 4°C . Serum was separated from the clot by centrifugation and, if not used immediately, stored at -20°C .

2.3.2 Collection of mucus

The body surface of anesthetized trout was rinsed with normal saline or water. Mucus was gently scraped from the surface of the body;

care was taken not to include blood or other body fluids in the sample. Sterile saline was stirred into the viscous mucus samples, the mixtures were centrifuged, and the supernatants were stored at -20°C until used.

2.3.3 Collection of mucus cells and preparation of smears

Smears of trout mucus were made on glass microscope slides with a cotton-tipped applicator stick and allowed to air-dry. A reference slide was stained with buffered differential Wright's stain (CAMCO Quikstain, Scientific Products). Slides to be used for immunofluorescence studies were fixed for 15 minutes in 5% (vol/vol) glacial acetic acid in absolute ethanol at -12°C , a procedure which has been used successfully for fixation of cytoplasmic immunoglobulin.⁸⁷ The slides were washed in phosphate-buffered saline pH 7.4 and processed for immunofluorescence as described in the section on indirect immunofluorescence (Section 2.11).

2.3.4 Preparation of trout skin for immunofluorescent studies

Specimens of skin were removed from rainbow trout, quick-frozen in isopentane on dry ice, and stored at -70°C until used. Tissue blocks were cut into 4-micron-thick sections on a microtome-cryostat (IEC model CTF) and transferred onto glass microscope slides. For orientation purposes, several sections from each block were stained with a hematoxylin-and-eosin stain for frozen sections.⁸⁸ Slides to be used for immunofluorescence were allowed to air-dry for one hour and were either used the same day or stored at -70°C until use. Slides were processed for immunofluorescence as described in Section 2.11.

2.4 Immunization of trout

2.4.1 Human gamma globulin

A six percent solution of human gamma globulin was obtained from the Swiss Red Cross (3000 Berne 22, Wankdorf Strasse 10, Switzerland). The solution was diluted to 20 mg per ml and mixed with an equal amount of complete Freund's adjuvant (Calbiochem). Initially, a volume of 0.1 ml, containing 1 mg of protein, was injected intramuscularly into each fish. Subsequently, the trout were boosted with 1 mg of the gamma globulin solution in saline at weekly intervals for ten weeks, then at two or three week intervals thereafter. Fish were bled (Section 2.3.1) and tested for serum antibodies (Section 2.5.1) starting the second week after the first injection.

2.4.2 Sheep red blood cells

Intraperitoneal injections of sheep red blood cells have been reported to induce production of specific anti-sheep red blood cell antibodies in rainbow trout.^{86, 89} Immunization with sheep red blood cells was carried out as follows. Sheep red blood cells in Alsever's solution (Institut Armand-Frappier) were washed three times with phosphate-buffered saline pH 7.2. The washed packed cells were made up to a 20 per cent solution in phosphate-buffered saline, and 0.5 ml of this was injected intraperitoneally into trout at weekly intervals.

2.5 Testing for induced antibody in immunized trout

2.5.1 Antibody to human gamma globulin

In the passive hemagglutination technique, red blood cells are coated with a protein antigen and then reacted with dilutions of test

serum. If the test serum contains antibodies directed against the antigen which coats the red blood cells, the antibody will react with its antigen, bringing about visible clumping of the cells.

Formalinized, tanned human group O red blood cells were coated with human gamma globulin, Cohn II⁹⁰ fraction (Pentex) and used in passive hemagglutination tests for determination of anti-human gamma globulin activity in serum samples from fish which had been immunized with human gamma globulin. The procedure was as follows: freshly-drawn heparinized human group O red blood cells were washed five times with physiological saline and packed after the final wash. A volume of 50 ml of formalin pH 6.0 was introduced into a dialysis sac, submerged into 200 ml of a 12.5 per cent suspension of washed, packed cells in phosphate-buffered saline, and gently agitated for approximately three hours at room temperature. The dialysis sac was then punctured to allow the formalin to escape and mixing was continued overnight. The suspension of formalinized red cells was filtered through muslin and washed five times with physiological saline. A volume of 0.6 ml of packed formalinized cells was suspended in 10 ml of phosphate-buffered saline; 10 ml of tannic acid solution (0.1 mg per ml) were added to the contents, mixed well, and incubated fifteen minutes at 37°C. The preparation was washed with phosphate-buffered saline and resuspended to 10 ml with phosphate-buffered saline. Ten milliliters of human gamma globulin (2 mg per ml) in phosphate-buffered saline were added to the formalinized tanned cells, and the suspension was mixed and incubated thirty minutes at 37°C. The cells were washed three times with stabilized phosphate-buffered saline (containing one per cent heat-inactivated and test-red-blood-cell-absorbed normal

rabbit serum) and resuspended to 50 ml with stabilized phosphate-buffered saline to give a one per cent suspension.

Testing for antibody was done in Takatsy microtiter plates with a V-shaped bottom (Cooke Laboratory Products). Sera to be tested were first heat-inactivated and absorbed with the test cells. One drop of a one per cent suspension of formalinized, tanned, and coated cells was added to one drop of a series of dilutions of test sera. The plates were incubated at 4°C overnight and checked for agglutination the following day. Controls consisted of the following: formalinized tanned uncoated cells (negative), saline (negative), and commercially-obtained anti-human IgG (Hyland) antiserum (positive).

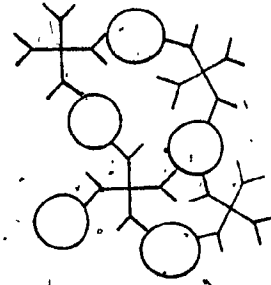
2.5.2. Antibody to sheep red blood cells

2.5.2.1 Direct hemagglutination

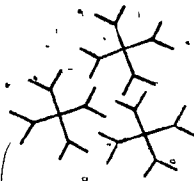
A direct hemagglutination assay was used for detection of high molecular weight antibody to sheep red blood cells. When sheep red blood cells are reacted with a sample which contains a multimeric antibody directed against sheep red blood cells, antigen-antibody interaction will be indicated by a visible clumping of the red blood cells (Figure 4).

The procedure was carried out as follows. Sheep red blood cells in Alsever's solution (Institut Armand-Frappier) were washed at least three times with physiological saline, until the supernatant became clear. The test was carried out in 10 x 75 mm test tubes as follows: two drops of a two per cent solution of sheep red blood cells were added to two drops of serially diluted test samples. After approximately 15 minutes at room temperature, and again after overnight

Figure 4. Diagrammatic representation of the direct antiglobulin test for the detection of "complete" multimeric antibody.

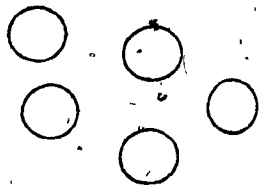


agglutination



tetrameric trout anti-
sheep-red-blood-cells

+



sheep red
blood cells

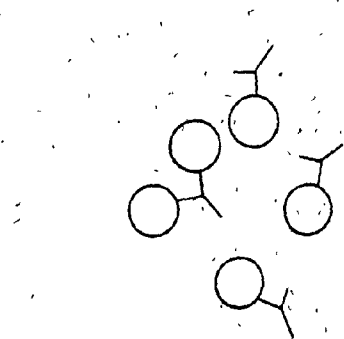
(14-18 hours) incubation at 4°C, the tubes were centrifuged for 15-30 seconds in a Sero-Fuge (Clay Adams) centrifuge and the sedimented red cells were checked for agglutination. Saline was used as a negative control and a commercially-obtained rabbit anti-sheep hemolysin (Difco) was used as a positive control. The titers were generally one dilution higher after overnight incubation at 4°C, and these are the ones reported in this study.

2.5.2.2 Indirect hemagglutination.

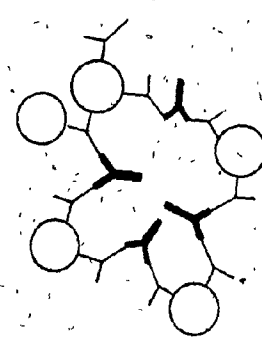
Monomeric antibodies are far less efficient agglutinators than multimeric antibodies, and give weak or negative results when used in test systems which depend on agglutination.^{91, 92} The indirect anti-globulin test was developed in 1945 by Coombs et al⁹³ for detection of these so-called "incomplete" antibodies, and a modification of this technique was used to test for the presence of trout 7S antibody. If sheep red blood cells are reacted with a trout serum containing antibodies against the sheep red blood cells, "complete" or multimeric antibodies will cause clumping of the cells after this first step (Figure 4), while "incomplete" monomeric antibody, if present, will recognize and coat the cells but will not be able to bring about agglutination of the red cells (Figure 5). If a rabbit anti-trout immunoglobulin is added to cells which are coated with trout 7S immunoglobulin, intercellular bridges will be formed by the anti-immunoglobulin, linking the red cells together to bring about visible agglutination (Figure 5).

The following modification of the Coombs indirect antiglobulin test was used for detection of trout 7S antibody to sheep red blood

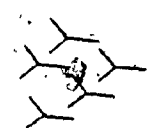
Figure 5. Diagrammatic representation of the indirect antiglobulin test for the detection of "incomplete" monomeric antibody.



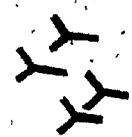
cells are coated with antibody, but give weak or negative agglutination



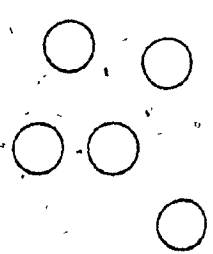
agglutination



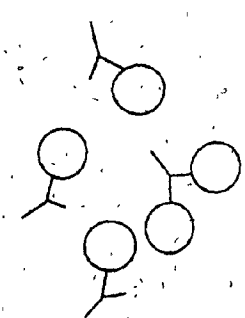
monomeric trout anti-sheep-red-blood-cells



rabbit anti-trout-immunoglobulin



STEP 1 sheep red blood cells



STEP 11 sheep red blood cells coated with monomeric antibody

coated with monomeric antibody

cells.⁹³ Serum samples were taken from trout which had been immunized with sheep red blood cells and separated on Sephacryl S-300 (Section 2.6.3). Fractions from each peak were reacted with a 2 per cent suspension of sheep red blood cells as for the direct hemagglutination technique (Section 2.5.2.1). After a one-hour incubation at room temperature, the tubes were checked for antibody activity, then washed three times with normal saline. Two drops of heat-inactivated rabbit anti-whole-trout-serum or rabbit anti-trout-immunoglobulin were added to the washed cells in each tube. After 30 minutes the tubes were centrifuged and checked for agglutination.

2.6 Purification of trout immunoglobulin

There was not enough antibody-containing trout serum available to carry out immunoglobulin purification procedures with this serum alone, and mixtures of immune trout serum and normal trout serum were used for antibody isolation. At all times, enough antibody-containing serum was present to allow identification of antibody-containing fractions following each procedure. Rainbow trout serum was first precipitated in 40 per cent ammonium sulfate (Section 2.6.1). The redissolved and dialyzed precipitate was then subjected to recycling G-200 (earlier experiments, Section 2.6.2) or Sephacryl S-300 (later experiments, Section 2.6.3) chromatography. Antibody-containing fractions from these molecular sieve procedures were pooled, concentrated, dialyzed and separated according to charge, either by DEAE anion-exchange chromatography (Section 2.6.4) or preparative zone electrophoresis in agar gel (Section 2.6.5). The final antibody-containing fractions were concentrated (Section 2.13) and checked

for purity by immunoelectrophoresis (Section 2.8.2) against rabbit anti-whole-trout-serum.

2.6.1 Ammonium sulfate precipitation

Precipitation of proteins in ammonium sulfate is a commonly-used initial step in the purification of proteins.⁹⁴ To determine a suitable ammonium sulfate concentration for precipitation of trout immunoglobulin, aliquots of pooled normal trout serum were precipitated in varying concentrations of ammonium sulfate, ranging from 25 to 55 per cent. The effectiveness of each concentration in isolating the excluded peak from G-200, which was previously shown to contain antibody, was determined by subjecting each of the redissolved precipitates to G-200 column chromatography (Section 2.6.2). The optimum ammonium sulfate saturation for precipitation of trout immunoglobulin was determined to be 40 to 45 per cent. Subsequent ammonium sulfate precipitation was done as follows. Serum was precipitated in 40 per cent ammonium sulfate at pH 7.0, allowing the resulting turbid solution to settle overnight at room temperature, then centrifuging fifteen minutes at 12,500 g to pack the precipitate. The supernatant was discarded, and the precipitate was washed twice with 40 per cent ammonium sulfate solution at pH 7.0. The washed precipitate was dissolved in distilled water and dialyzed against phosphate-buffered saline pH 7.4, with several changes of the dialyzing solution.

2.6.2 Sephadex G-200 gel filtration

Sephadex G-200 is a molecular sieve gel, and separates molecules according to size, such that the largest molecules are eluted first, and the smallest last. Sephadex G-200 fractionates proteins over a

range of 5,000 to 600,000 molecular weight.⁹⁵ Proteins larger than 600,000 daltons elute in the void or excluded volume, which is determined by eluting a solution of blue dextran (average molecular weight 2×10^6 daltons) through the column and measuring the volume of elution of the blue solution.

The columns were prepared as follows: 2.5 x 100 cm columns (Pharmacia) were packed with Sephadex G-200 (Pharmacia) in phosphate-buffered saline pH 7.4, and void volumes were determined with blue dextran 2000 (Pharmacia). Samples to be chromatographed were dialyzed against phosphate-buffered saline at 4°C and applied to the column. Originally, columns were kept at 4°C, but when it was found that chromatography at room temperature did not alter antibody activity, additional columns were set up and used at room temperature. Dialyzed samples were applied to the columns, which were equipped with flow adaptors, and eluted using upward flow. Flow rates were 11-15 ml per hour. Fractions of 3-4 ml were collected in a Brinkmann Linear II fraction collector; fractions were tested for antibody activity (Section 2.5.2.1) and the positive tubes were pooled and concentrated for further work.

2.6.3 Sephacryl S-300 gel filtration

Sephacryl S-300 (Pharmacia) is a relatively new separation gel, and has several advantages over G-200.⁹⁵ It fractionates proteins over a range of 1×10^4 to 1.5×10^6 daltons, and can be run at a faster flow rate than G-200. It was found that S-300 does not pack further after initial packing, so that the gel bed volume remains

constant. The surface of the gel is not as easily disturbed as that from G-200, allowing more even sample application. S-300 was used instead of G-200 in later experiments.

A 2.5 x 100 cm column (Pharmacia) was packed with Sephacryl S-300 (Pharmacia) which had previously been equilibrated with .02M phosphate-buffered saline pH 7.4. Samples were eluted with downward flow, at 20-30 ml per hour. Fractions were collected, analyzed and stored as for G-200 gel fractionation (Section 2.6.2).

2.6.4 DEAE anion exchange chromatography

Ion exchange chromatography is used to separate molecules according to charge⁹⁶. DE-52 cellulose (Whatman) has positively-charged groups bound to the matrix and exchanges negatively-charged ions (anions). After a sample has been allowed to adsorb onto the matrix, and unadsorbed molecules are washed out, an elution buffer of gradually increasing ionic strength is passed through the column, causing competition between buffer and sample ions for binding sites. There is a gradual decrease in the strength of binding of each ion, such that the least negatively-charged ions elute first.

A 0.9 x 30 cm column (Pharmacia) was packed with DE-52 cellulose (Whatman) which had previously been equilibrated with 0.015M Tris-HCl pH 8.0. Samples to be chromatographed were dialyzed against starting buffer (0.015M Tris-HCl pH 8.0). DEAE-cellulose chromatography was performed at room temperature, and elution was accomplished with a linear NaCl gradient prepared in a Pharmacia Gradient Mixer, Model GM, from 0.015M Tris-HCl pH 8.0 to 0.015M Tris-HCl 0.4M NaCl pH 8.0, at a flow rate of 20-25 ml per hour. Conductivity of column eluants was

measured in a conductivity bridge (Yellow Springs Instrument Co. Model 31), and optical densities were measured at 280 nm in a spectrophotometer (Bausch & Lomb Spectronic 70).

2.6.5 Preparative zone electrophoresis on agar gel

One hundred and twenty milliliters of one per cent Ionagar (Colab Laboratories) solution in barbital buffer pH 8.6 (ionic strength .075) was poured onto a glass tray (11 x 24.5 x 5 cm), and allowed to harden. A trough was made by cutting out a vertical 1 cm-wide strip from the short axis of the gel, about 9 cm from the cathode end of the plate. Samples to be electrophoresed were dialyzed against barbital buffer overnight at 4°C. Approximately 2 ml of dialyzed sample was mixed with an equal volume of melted 2 per cent gel in barbital buffer and applied to the trough. The electrophoretic run was carried out in a water-cooled Bromma Multiphor electrophoresis chamber (LKB), using barbital buffer pH 8.6 (ionic strength .075). The plate was run at 90-100 V, 25-35 mA, applied through Exaphor (Orion Diagnostics) electrophoretic wicks, until migration, as seen by the distance traveled by a marker dye (bromophenol blue) was judged to be sufficient (18-20 hours). Following electrophoresis, the gel was divided into strips of approximately 7 mm width which were cut out perpendicular to the direction of the run, and placed into individual tubes. Elution of the strips was accomplished by freezing and thawing twice, which caused most of the liquid to be released from the gel. The supernatants from antibody-containing fractions were pooled and concentrated (Section 2.13) for further work.

2.7 Estimation of the molecular weight of trout immunoglobulin

The molecular weight of trout immunoglobulin was estimated by S_{200} column chromatography. Elution conditions were as described in Section 2.6.3. Standard proteins used in calibrating the column were thyroglobulin (molecular weight 630,000), bovine serum albumin (molecular weight 66,000), ribonuclease A (molecular weight 13,700), human IgM (molecular weight 950,000), and human IgG (molecular weight 150,000). A calibration curve was constructed from the distribution coefficient (K_{av}) values and the log-distributed molecular weights of each of these proteins. The K_{av} values were determined from the formula:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \dots\dots(1)$$

where V_o is the void volume, V_e is the elution volume of the protein, and V_t is the total volume of the gel bed. A straight line was fitted to the data by the method of least squares. Several samples of immune trout serum were run through the column, the average K_{av} value of the peaks containing antibody was determined, and the molecular weight of trout immunoglobulin was determined from the calibration curve.

2.8 Analysis of protein fractions

2.8.1 Ouchterlony double diffusion

Diffusion in gel is a technique used for analysis of immuno-precipitates. In Ouchterlony double diffusion, antibody and antigen

are placed in opposing wells and are allowed to diffuse toward and react with each other in an agar or agarose gel medium. The complexes resulting from each antigen-antibody interaction precipitate and become immobilized in the gel matrix, forming individual sharp bands of visible precipitate. One band is formed for each reaction of antibody with its specific antigen.

Ouchterlony double diffusion plates were set up as follows. Three milliliters of melted 0.9 per cent agarose (Miles Laboratories) in 0.01M phosphate-buffered saline pH 7.4 were poured onto Hyland immunodiffusion plates (approximately 73 x 23 x 2 mm) and allowed to cool. A pattern of six wells 3 mm in diameter and equidistant (5 mm) from a central 3-mm well was punched out of the gel. Wells were filled with antigen and appropriate antiserum, and precipitin lines were allowed to develop for 18-24 hours in a humidity chamber at room temperature. The gels were washed 10 hours with two changes of 2 per cent NaCl and 1 hour in distilled water. They were then transferred to Gelbond film (Marine Colloids), and allowed to dry. The dried slides were stained with 0.5 per cent amido black in water:methanol:glacial acetic acid (9 parts:9 parts:2 parts), destained with a water:methanol:acetic acid solution, and dried.

2.8.2 Immuno-electrophoresis

Immuno-electrophoresis combines the technique of gel electrophoresis (section 2.6.5) with that of double diffusion (section 2.8.1), and allows identification of antigenic constituents by their electrophoretic mobility. The constituents of a heterogeneous protein sample are first separated according to charge and size by unidirectional

electrophoresis in an agar or agarose gel medium. Following electrophoresis, longitudinal troughs are cut into the gel and filled with a corresponding antiserum, which diffuses into the medium at right angles to the direction of electrophoresis. The reaction of the antiserum with one or more of the separated proteins results in formation of visible arcs of precipitated antigen-antibody complexes. The number of precipitation arcs indicates the number of immuno-reactive constituents in the test serum.

Immunoelectrophoresis was done according to the micro-method developed by Scheidegger,⁹⁹ using a Gelman Micro-Immunoelectrophoresis system. Molten 1.25 per cent Difco Certified agar gel (ionic strength 0.025) in pH 8.6 barbital buffer was layered onto 75 x 25 mm glass microscope slides, and allowed to solidify. Sample wells were punched into the gel, and sample was applied and electrophoresed for 2 hours in cold barbital buffer pH 8.6 (ionic strength 0.05). A current of 5 mA per frame of six slides was applied through filter paper strips. Following the electrophoretic run, horizontal troughs were cut from the gel parallel to the direction of the electrophoretic field and filled with the appropriate antiserum. Slides were incubated for 16 to 20 hours in a humid chamber at room temperature to allow development of lines of precipitation. The slides were washed for at least 10 hours in two changes of 2 per cent NaCl and 1 hour in distilled water, and dried. The dried slides were stained as described in Section 2.8.1 for Ouchterlony double diffusion gels.

2.9 Immunization of rabbits

2.9.1 Immunization with whole trout serum

Rabbit antiserum against whole trout serum was prepared as follows: a pool of normal trout serum was filter-sterilized and diluted with sterile normal saline to give a protein concentration of 2 mg per ml. Equal volumes of diluted normal trout serum and complete Freund's adjuvant (Calbiochem) were emulsified in a Sorvall Omni-Mixer. A volume of this emulsion containing 1 mg of protein was injected subcutaneously into each of three rabbits. Booster injections of 1 mg protein in saline were given subcutaneously over a period of one year. Rabbits were bled (Section 2.10) 7 days after each boost, and the serum was tested for reactivity against whole trout serum by immunoelectrophoresis (Section 2.8.2).

2.9.2 Immunization with purified trout immunoglobulin

Rabbit antiserum against purified trout immunoglobulin was prepared as follows. An emulsion of equal volumes of purified protein and complete Freund's adjuvant (Calbiochem) was prepared. A volume of emulsion containing 0.1 mg of protein was injected into several sites by the intradermal route into each of three rabbits. Where sufficient antigen remained, rabbits were boosted 4 weeks following initial immunization as follows. A volume of emulsion of equal amounts of complete Freund's adjuvant and antigen containing 0.1 mg of protein was injected subcutaneously into each rabbit. Rabbits were bled (Section 2.10) weekly following immunization, and sera were tested for reactivity against whole trout serum by Ouchterlony double diffusion (Section 2.8.1)

2.10 Collection of serum from immunized rabbits

A butterfly infusion set equipped with a 19-gauge needle (Abbott Laboratories) was used to draw blood from the central ear artery of each rabbit. Blood was allowed to clot for two hours at 37°C and overnight at 4°C. Serum was separated from the clot by centrifugation and, if not used immediately, stored at -20°C.

2.11 Immunofluorescence microscopy of trout mucus and skin

The technique of using fluorescein-labeled antibodies in fluorescence microscopy was developed for the purpose of localizing specific antigen in situ.^{100,101} When fluorescein-tagged antiserum is reacted with its antigen in tissue, the subsequently-formed antigen-antibody complexes are seen as bright green fluorescence under a microscope which is equipped with an ultraviolet light source.

In the present study, an indirect technique was used; trout tissue was reacted with an unlabeled rabbit antiserum against trout immunoglobulin. Since the rabbit antibodies were not labeled, antigen-antibody complexes could not be detected by fluorescence microscopy. A fluorescein-tagged goat antiserum against the rabbit antibodies was reacted with the complexes; these labeled antibodies bound to the rabbit antibodies which were already bound to the trout immunoglobulin, allowing localization of trout immunoglobulin in the tissues.

Preliminary tests were carried out in which normal rabbit serum was substituted for rabbit anti-trout-immunoglobulin serum. It was found that normal rabbit serum cross-reacted with trout skin components, causing a fair amount of non-specific fluorescence. The non-specific

fluorescence was reduced by a 10-minute pre-treatment of the skin sections in 0.1M glycine-HCl pH 2.3, and by absorption of rabbit serum and antiserum with pieces of fresh frog skin. The absorptions were done as follows: frog skin was cut into small pieces and mixed into the rabbit sera in a 1 to 1 ratio (vol:vol). After a one-hour incubation at room temperature, the skin sections were removed from the sera by centrifugation.

Slides to be used in immunofluorescence studies were processed as follows. Slides were immersed in phosphate-buffered saline pH 7.4, and care was taken to prevent the tissue from drying throughout the following steps. One drop of absorbed rabbit antiserum to trout immunoglobulin was placed on each tissue section or smear and allowed to react for 30 minutes in a humid chamber. At the end of the incubation period, slides were washed for 30 minutes in three changes of phosphate-buffered saline. One drop of a 1 in 5 dilution of a commercial fluorescein isothiocyanate-tagged goat antiserum to rabbit immunoglobulin (Cappel Laboratories) was applied to each sample and incubated in a moist chamber for 30 minutes, following which the slides were again washed in three changes of phosphate-buffered saline. Slides were mounted in a glycerol:phosphate-buffered saline medium (9 parts glycerol to 1 part phosphate-buffered saline), coverslipped, and examined for fluorescence in a Zeiss fluorescent microscope, equipped with a BG 12 exciter, FT 510 beam splitter, barrier 50, and BG red-attenuation filters. The light source was a mercury vapour lamp (HBO-50W, Osram, Germany). Photomicrographs were taken with a Zeiss Photomicroscope III camera which was equipped with an automatic exposure timing device. Kodak Tri-X pan film (ASA 400) was used.

2.12 Protein measurements

Quantitative protein measurements were done according to the method of Lowry et al.¹⁰² Commercial bovine gamma globulin (Protein Standard I, Bio-Rad Laboratories) was used as a standard.

2.13 Concentration of samples

* Samples were concentrated by positive pressure in an Amicon concentrator, using Diaflo XM-50 or XM-100 ultrafiltration membranes (Amicon).

RESULTS

3. Results

3.1 Antibody production in rainbow trout

3.1.1 Antibody response following immunization with human gamma globulin

Rainbow trout kept at 9°C failed to produce detectable serum antibodies to intramuscularly-injected human gamma globulin for up to thirty-one weeks after the first injection. The passive hemagglutination test (Section 2.4.1) used for detection of these antibodies was judged to be adequate, as controls of saline and uncoated cells were negative, while commercial anti-human gamma globulin consistently gave positive results at a 1/512 dilution. Ouchterlony double diffusion assays (Section 2.8.1) were set up to test for precipitating antibodies to human gamma globulin, but these tests were also negative. The ambient water temperature was increased from 9°C to 14°C, but the trout remained unresponsive. The failure to produce antibodies to human gamma globulin was not due to immune incompetence in these fish, as they were able to produce specific hemagglutinating antibodies in response to intraperitoneal immunization with sheep red blood cells. These results suggested that, under the conditions described in Section 2.4.1, injections of human gamma globulin induced a state of tolerance or specific immune unresponsiveness in these trout.

3.1.2 "Natural antibodies" in rainbow trout

3.1.2.1 Natural hemagglutinins

Naturally-occurring agglutinating antibodies against a variety of vertebrate red blood cells and bacteria have been described in representatives from all classes of fish. 22, 23, 25, 29, 34, 38, 42, 60, 69, 108-111

Natural hemagglutinating antibodies against sheep red blood cells were not detected in non-immunized rainbow trout in the present study.

3.1.2.2 Natural hemolysins

Low titers (up to 8) of natural hemolysin for sheep red blood cells were found in both immunized and non-immunized groups of trout. Natural hemolytic activity was destroyed by heat-inactivation for 30 minutes at 56°C, and did not increase appreciably following immunization of the trout with sheep red blood cells.

3.1.3 Induced antibodies to sheep red blood cells

The majority of rainbow trout given weekly intraperitoneal injections of sheep red blood cells produced detectable agglutinating antibodies against sheep red blood cells within two months after the first injection. There was considerable individual variation among trout which responded to antigenic stimulation (Table 1). Production of antibodies to sheep red blood cells was transient in some fish and long-lasting in others. Some fish responded with production of high titers (as high as 4096) of antibody, others produced antibody in low amounts.

3.2 Effect of temperature on antibody production

Rainbow trout produced serum antibodies against sheep red blood cells at both 9°C and 15°C, but the latent period prior to the appearance of detectable antibody was significantly shorter (Student's t-test, $P < 0.02$) at 15°C than at 9°C. Antibodies were detected as early as the fourth week in serum samples taken from the 15°C group

Table 1. Long-term studies on serum antibody production in a representative group of 6 trout, kept at 10-15°C and given weekly 0.5 ml intraperitoneal injections of 20% sheep red blood cells for 31 weeks, then monthly for 2 months. Fractions represent the highest serum dilution which agglutinates sheep red blood cells. The presence of natural hemolysins interfered with interpretation of hemagglutination at low dilutions, and sera which showed no hemagglutination at a 1/8 dilution were considered to be negative.

Weeks	4	8	18	24*	30	51
Fish #1	neg	1/32	1/16	ND	1/32	neg
#2	neg	1/64	neg	1/16	*	
#3	neg	1/128	1/128	ND	1/64	neg
#4	neg	1/128	1/128	*		
#5	neg	1/256	1/8	ND	neg	neg
#6	neg	1/512	1/512	ND	1/4096	1/512

* dead

ND test not done

of fish; no serum antibodies were found before the seventh week in fish kept at 9°C (Tables 2,3). Tremendous individual variation in response was again evident in both groups. The deaths noted in Tables 2 and 3 occurred because of difficulties encountered in stopping the flow of blood after venipuncture.

3.3 Purification of trout immunoglobulin

3.3.1 Ammonium sulfate precipitation

Aliquots of pooled normal trout serum were subjected to precipitation by different concentrations of ammonium sulfate as described in Section 2.6.1. Precipitates formed in 25 and 35 per cent ammonium sulfate could not be completely redissolved and gave a poor yield of protein; very little protein was brought down by 35 per cent ammonium sulfate as well. However, the 40 to 50 per cent ammonium sulfate-precipitated sera did yield a fair amount of protein; G-200 column chromatography of these redissolved precipitates produced a good amount of first peak components with a minimum of second and third peak components. Based on these observations, 40 per cent ammonium sulfate was selected as a suitable concentration for precipitation of trout immunoglobulin. When trout serum containing antibody to sheep red blood cells was precipitated in 40 per cent ammonium sulfate, and the precipitate redissolved and subjected to G-200 gel filtration, it was found that hemagglutinating activity was not altered by this treatment, and remained confined to the excluded peak (Figure 6).

Table 2. Serum antibody production in 7 rainbow trout kept at 9°C and given weekly intraperitoneal injections of 20% sheep red blood cells. Fractions represent the highest serum dilutions which agglutinate sheep red blood cells. The presence of natural hemolysins interfered with interpretation of hemagglutination at low dilutions, and sera which showed no hemagglutination at a 1/8 dilution were considered to be negative.

Weeks	1	2	3	4	5	6	7	8	9	10	11	13
Fish #1	neg	neg	neg	*								
#2	neg	neg	neg	neg	neg	neg	neg	1/16	1/8	1/16	1/32	1/64
#3	neg	neg	neg	neg	neg	neg	1/32	1/64	1/32	1/64	1/32	1/64
#4	neg	neg	neg	neg	neg	*						
#5	neg	neg	neg	*								
#6	neg	neg	neg	neg	neg	neg	1/16	1/64	1/64	1/32	1/32	neg
#7	neg	neg	neg	neg	neg	neg	1/8	1/32	1/32	1/16	1/16	1/64

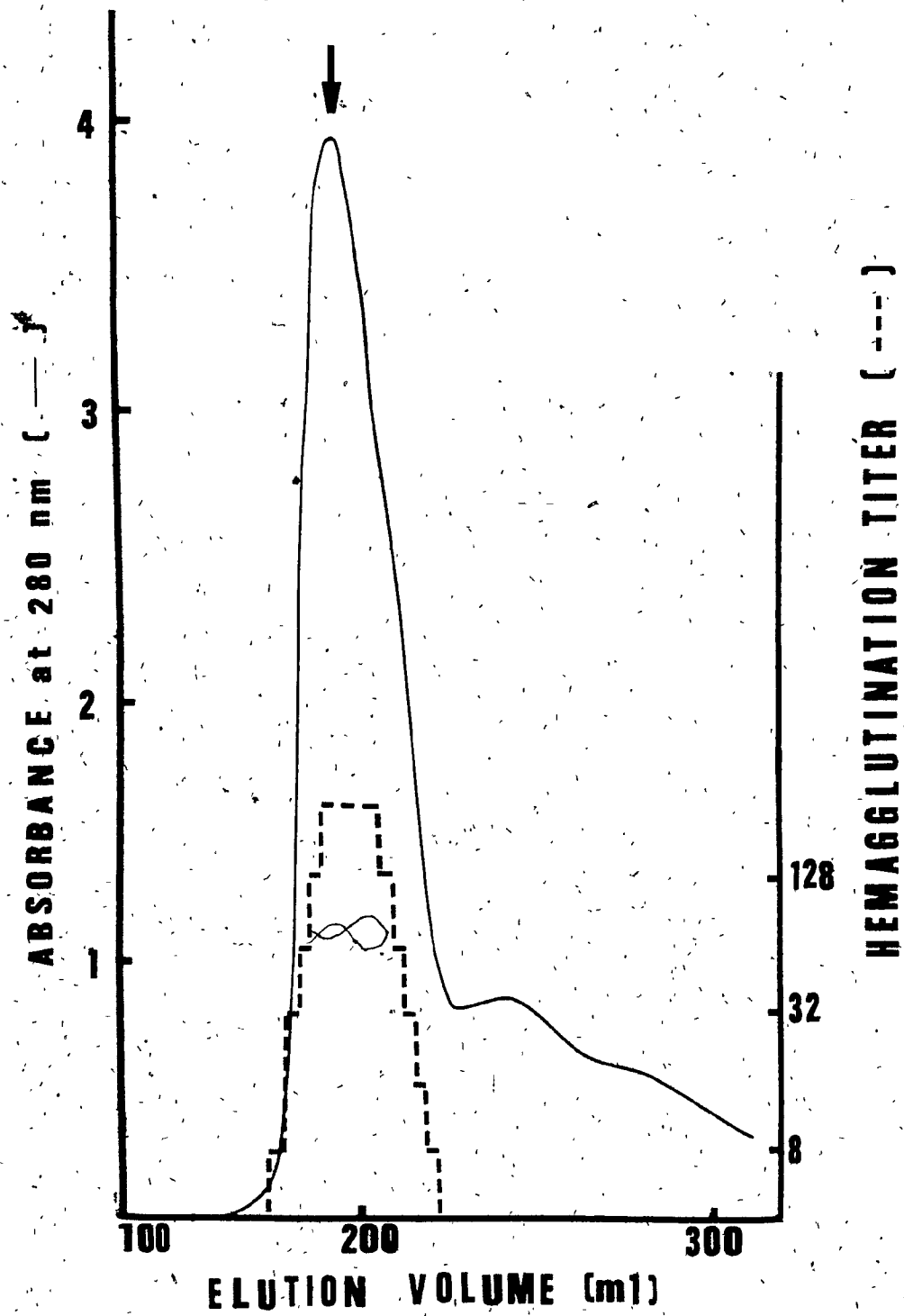
* dead

Table 3. Serum antibody production in 7 rainbow trout kept at 15°C and given weekly intraperitoneal injections of .20% sheep red blood cells. Fractions represent the highest serum dilutions which agglutinate sheep red blood cells. The presence of natural hemolysins interfered with interpretation of hemagglutination at low dilutions, and sera which showed no hemagglutination at a 1/8 dilution were considered to be negative.

Weeks	1	2	3	4	5	6	7	8	9	10	11	13
Fish #1	neg	neg	*									
#2	neg	neg	neg	neg	neg	neg	1/32	1/64	1/32	neg	neg	neg
#3	neg	neg	*									
#4	neg	neg	neg	1/32	neg	neg	1/8	1/16	1/32	1/16	1/16	1/32
#5	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
#6	neg	neg	neg	1/256	1/64	1/32	1/64	1/128	*			
#7	neg	neg	neg	1/64	1/32	1/32	neg	neg	neg	1/32	1/32	1/32

*dead

Figure 6. Sephadex G-200 column chromatography of 40% ammonium sulfate-precipitated immune fish serum, showing presence of antibody in the excluded fraction. The arrow, ↓, indicates the void volume. Sample size, 104 mg protein in 2.5 ml, column dimensions 2.5 x 100 cm, flow rate 13.3 ml per hour at room temperature.



3.3.2 Sephadex G-200 gel filtration

When samples of normal and immune trout serum were chromatographed on Sephadex G-200 (Section 2.6.2), an elution profile of three peaks was seen (Figure 7). Hemagglutinating activity, when present, was always confined to the first peak, which corresponded to the void volume of the column. These results indicated that trout immunoglobulin is a macromolecule with a molecular weight greater than 600,000 daltons.

3.3.3 Sephacryl S-300 gel filtration

Immune trout serum gave an elution profile of four main peaks (Figure 8) when subjected to chromatography on Sephacryl S-300 (Section 2.6.3). Antibody activity was restricted to the second peak, indicating that trout immunoglobulin is smaller than human pentameric IgM and larger than human monomeric IgG.

3.3.4 DEAE anion-exchange chromatography

Trout immunoglobulin eluted between 9 and 14 mmHOs conductance (Figure 9) when chromatographed on DE-52 cellulose (Section 2.6.4). No antibody activity was eluted before the salt gradient was applied.

3.3.5 Preparative zone electrophoresis on agar gel

Trout immunoglobulin migrated in a single band when electrophoresed in an agar gel medium as described in Section 2.6.5. The immunoglobulin found on the cathodic side of the application trough accounted for approximately 85 per cent of the hemagglutinating activity (Figure 10).

Figure 7. Sephadex G-200 column chromatography of immune trout serum. Antibody activity is confined to the first peak. Hemagglutination titer is expressed as the reciprocal of the highest dilution which agglutinates sheep red blood cells. The arrow, ↓, indicates the void volume, determined by previously passing a solution of blue dextran through the column. A volume of 3.8 ml of immune trout serum was applied to a 2.5 x 100 cm column at 4°C.

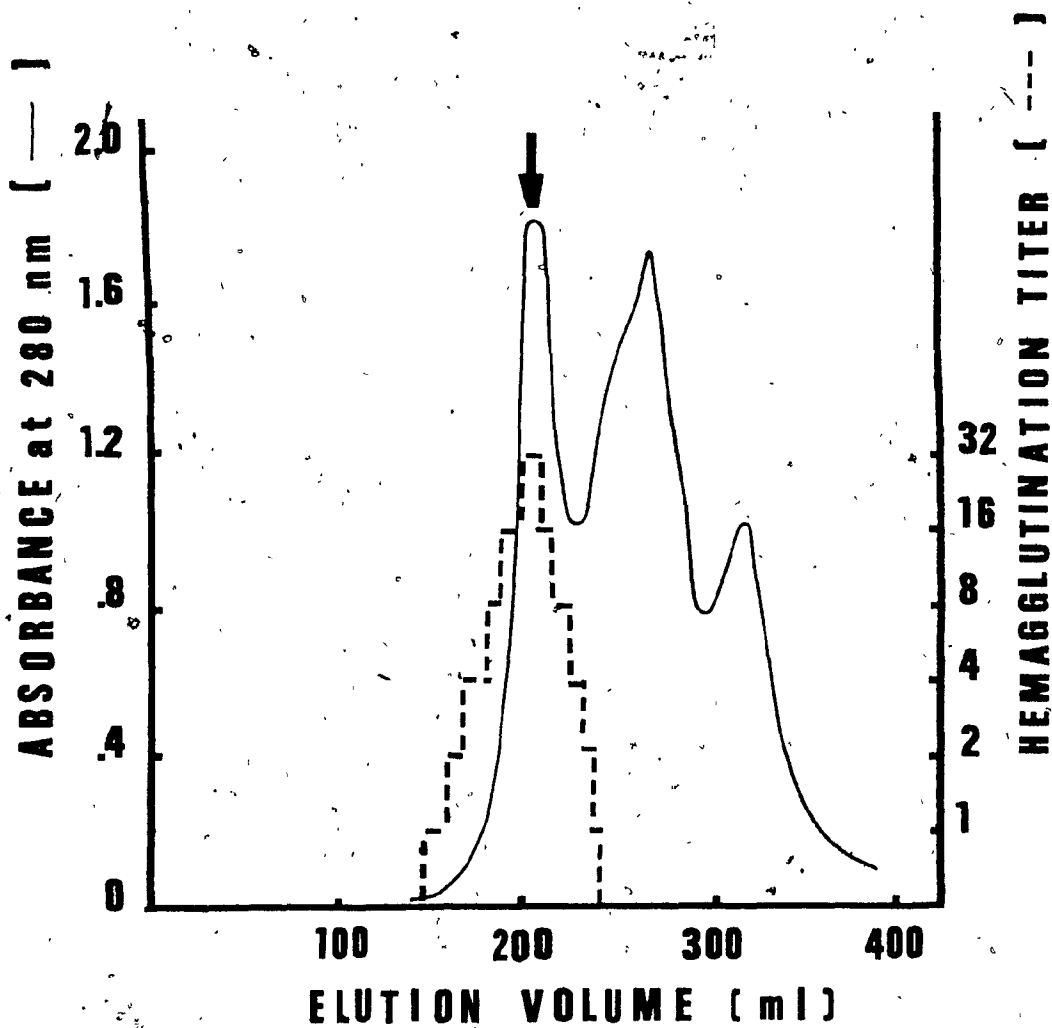


Figure 8 . . Elution profile of immune trout serum on Sephacryl S-300. Positions of elution of blue dextran (a), human pentameric IgM (b) and human monomeric IgG (c), which were previously eluted through the same column, are indicated by arrows. Total column volume 484 ml, sample size 4 ml, flow rate 19.5 ml per hour.

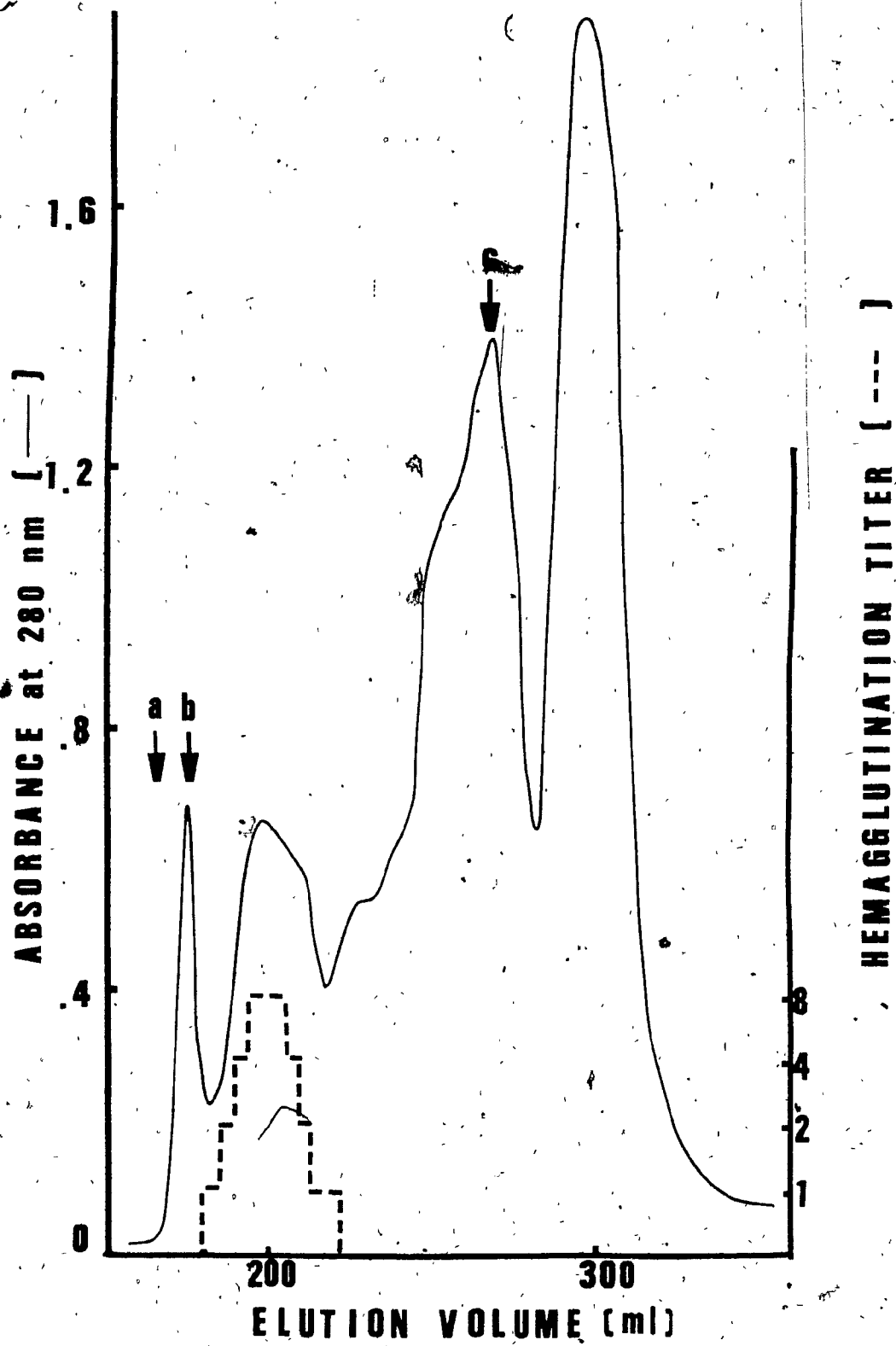


Figure 9. DEAE-anion exchange column chromatography of immune trout serum. A 2.5 ml sample of concentrated antibody-containing fractions from 40 per cent ammonium sulfate precipitation and subsequent G-200 column chromatography was applied to a .9 x 30 cm column. Elution conditions are as described in materials and methods (Section 2.6.4). Trout antibody to sheep red blood cells eluted between 9 and 14 mMHos conductivity. Total column volume 18 ml, flow rate 40 ml per hour.

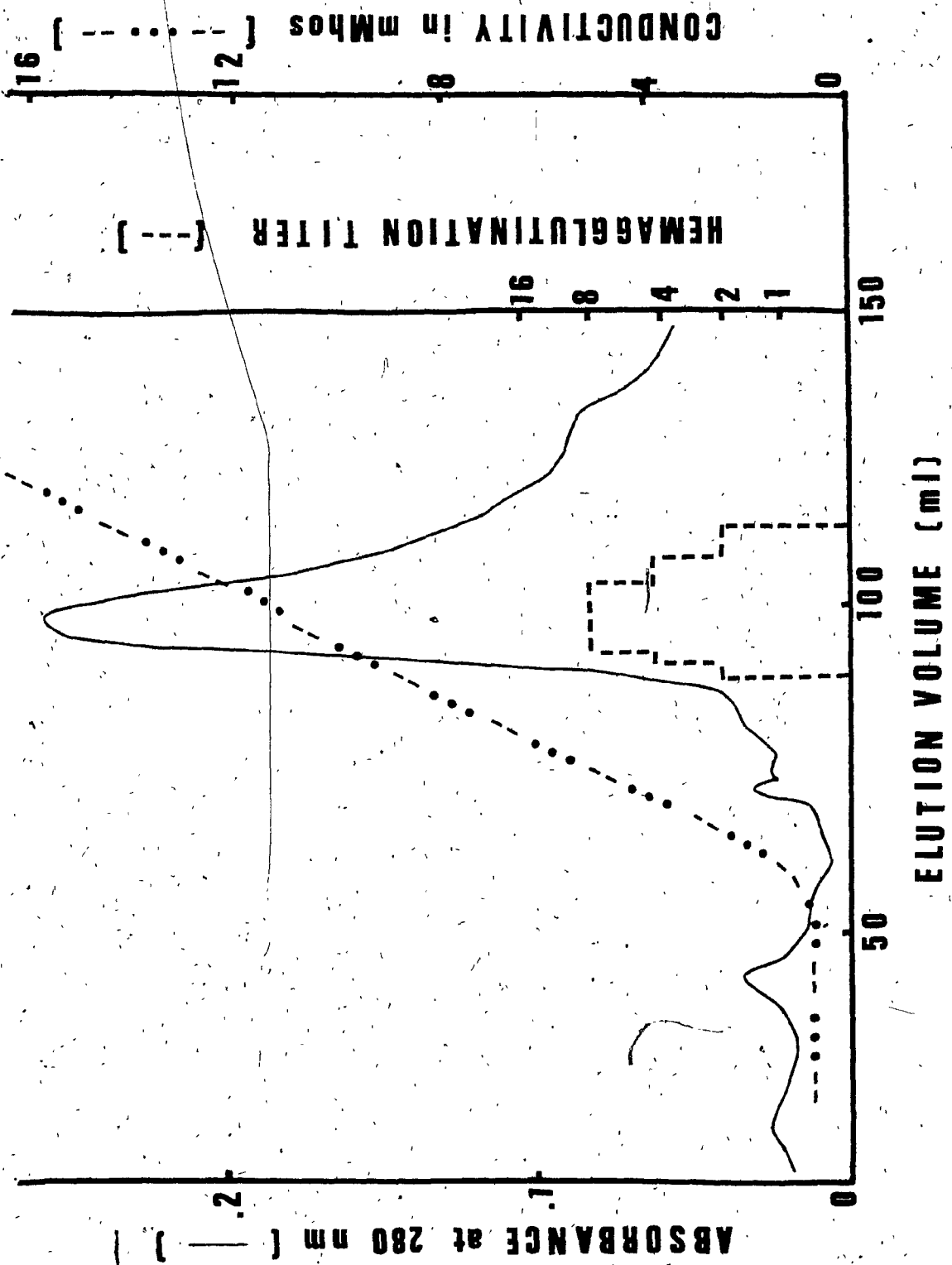
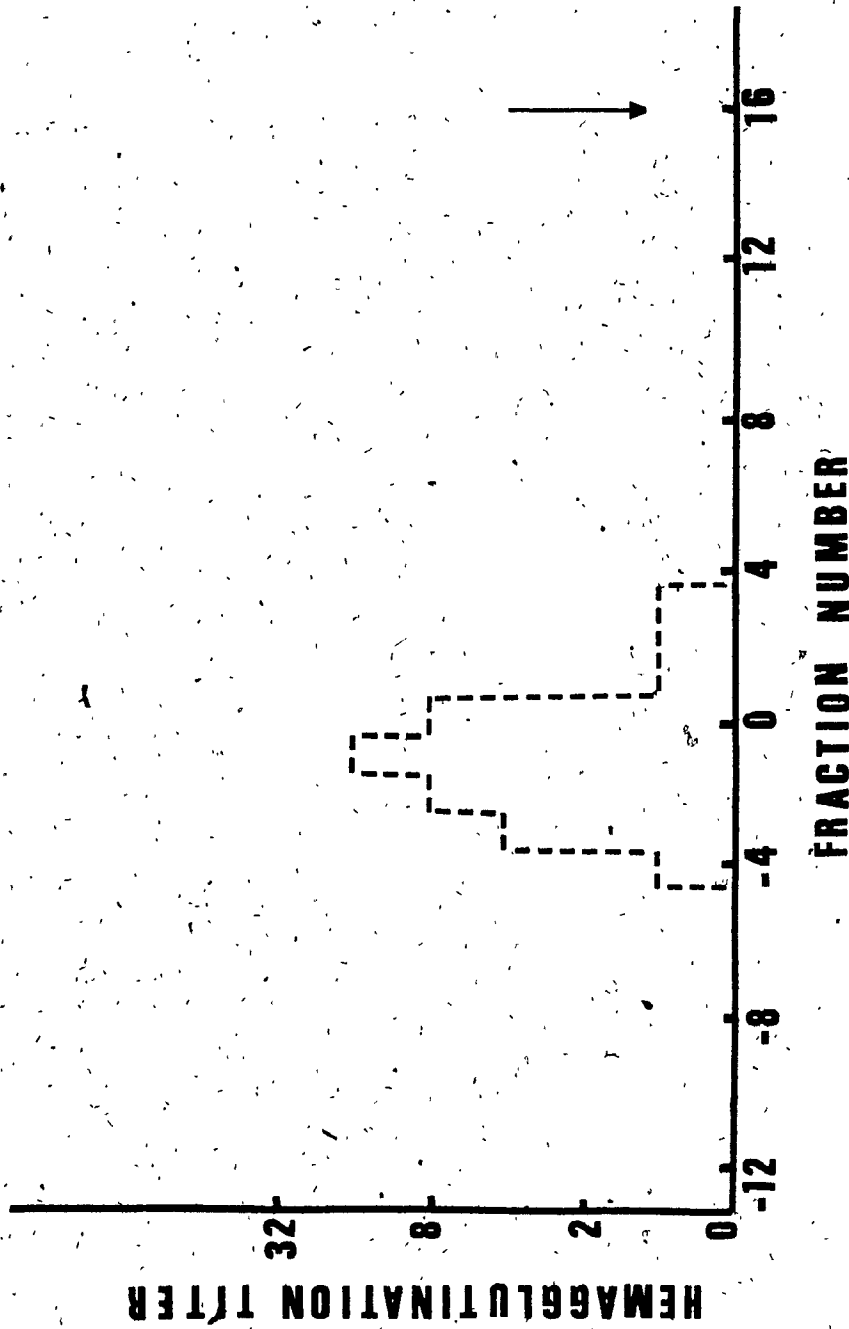


Figure 10. Agar gel electrophoresis of immune trout serum at pH 8.6.

Most of the hemagglutinating activity was contained in the cathodic fractions. Each fraction represents the undiluted eluate from a 7.25 mm section of the gel bed. "0" marks the application trough; negative fractions are those which have migrated cathodally and positive fractions have migrated anodally from the application trough. The arrow, ↓, represents the anodic front of the tracking dye bromophenol blue.



3.4 Characterization of trout immunoglobulin

3.4.1 Attempts to identify 7S immunoglobulin in trout

Direct hemagglutination tests carried out during purification procedures showed that antibody activity was confined to high molecular weight material only (Sections 3.3.1-3.3.3). The possibility remained, however, that the direct hemagglutination technique used could not detect low molecular weight monomeric antibody. An indirect hemagglutination technique, developed specifically for detection of monomeric antibody, was set up as described in Materials and Methods (Section 2.5.2.2). Sera from trout which had been immunized with sheep red blood cells were chromatographed on Sephacryl S-300 (Section 2.6.3), and individual fractions were tested for antibody activity, using both the direct and indirect antiglobulin tests. Antibody activity was confined to high molecular weight fractions only; none was found in any other fraction, including those fractions where a monomeric 7S immunoglobulin would be expected to elute. These results suggested that trout have only high molecular weight, multimeric, antibody.

3.4.2 Determination of molecular weight of trout immunoglobulin

As mentioned previously, trout immunoglobulin appears to be a high molecular weight multimeric molecule (Section 3.4.1), larger than human IgG and smaller than human IgM (Section 3.3.3). To obtain a more precise estimation of the molecular weight, a Sephacryl S-300 column was calibrated with several proteins, as described in Section 2.7. The K_{av} values were determined for each of the standard proteins (Table 4) and a selectivity curve was constructed from the K_{av} values and the log of the molecular weights (Figure 11). The average K_{av} value for

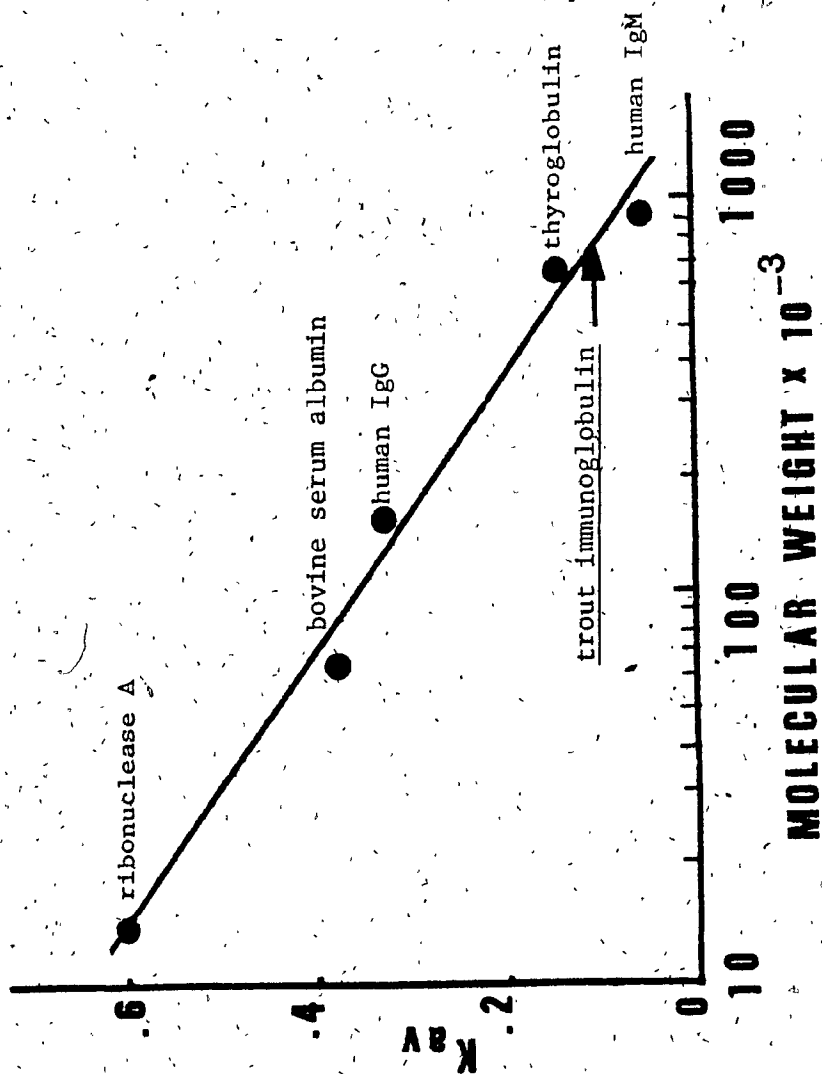
Table 4. The molecular weights, elution volumes (V_e) and K_{av} values of the 5 proteins used to calibrate the Sephacryl S-300 column which was used to determine the molecular weight of trout immunoglobulin. The gel bed volume (V_t) was 476 ml, and the eluting volume of blue dextran (V_0) was 164 ml.

Standard proteins	Molecular Weight	V_e	K_{av}^*
Human IgM	950,000	183 ml	.061
Thyroglobulin	630,000	204 ml	.128
Human IgG	150,000	262 ml	.314
Bovine serum albumin	66,000	283 ml	.381
Ribonuclease A	13,700	350 ml	.596

* K_{av} values were determined from the formula;

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

Figure 11. Selectivity curve of a Sephacryl S-300 column calibrated with five protein standards (Table 4). The V_e value for trout immunoglobulin was found to be 195 ml. The K_{av} value (.099) is indicated by the arrow, \longrightarrow . The molecular weight of trout immunoglobulin was estimated to be approximately 760,000 daltons.



trout immunoglobulin was determined to be .099, and, when plotted on the selectivity curve, gave a molecular weight estimate of approximately 760,000 daltons for trout immunoglobulin.

3.4.3. Purified trout immunoglobulins: two populations with different electrophoretic mobilities

Trout immunoglobulin was isolated as described in Materials and Methods (Section 2.6). Isolated immunoglobulin fractions were analyzed for purity by immunoelectrophoresis (Section 2.8.2) against rabbit anti-whole-trout-serum. Two populations of proteins with antibody activity were isolated. One protein had hemagglutinating activity and migrated as a single cathodic arc in immunoelectrophoresis (Figure 12). The other had very weak hemagglutinating properties and migrated as a single anodic arc in immunoelectrophoresis (Figure 13).

In earlier purification procedures, antibody-containing fractions were stored at -20°C between each step; agar gel electrophoresis was used as the final purification step. In later experiments, antibody-containing fractions were subjected to interim storage at 4°C , and DEAE anion-exchange chromatography was substituted for gel electrophoresis as the final step. Purification techniques employing the -20°C agar-gel-electrophoresis procedure yielded only the cathodic immunoglobulin, whereas both anodic and cathodic immunoglobulins were isolated in the 4°C -DEAE procedure.

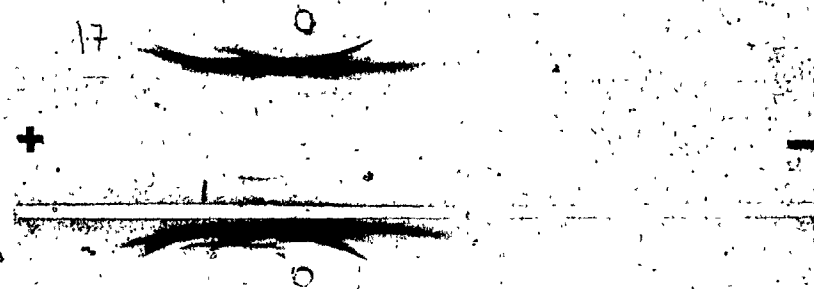
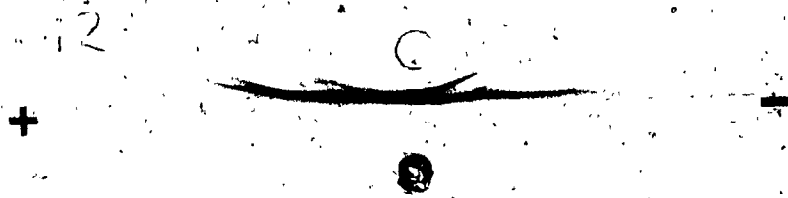
Attempts were made to determine whether the differences in storage conditions could have been responsible for the differences seen in the final immunoglobulin preparations. Samples of anodic and

Figure 12. Immunelectrophoresis of purified trout immunoglobulin with cathodic mobility. Samples were added in this and in subsequent figures as indicated in the accompanying illustrations.

○ whole trout serum
————— rabbit anti-whole-trout-serum
○ purified immunoglobulin fraction

Figure 13. Immunelectrophoresis of purified trout immunoglobulin with anodic mobility.

○ whole trout serum #1
————— rabbit anti-whole-trout-serum
○ purified immunoglobulin fraction
————— rabbit anti-whole-trout-serum
○ whole trout serum #2



cathodic immunoglobulins were kept at 4°C for a period of three months, following which they were subjected to repeat immunoelectrophoresis. The electrophoretic patterns had not undergone any change; neither the anodic nor the cathodic proteins showed a shift in electrophoretic mobility with time. These results suggested that the differences seen in the electrophoretic behaviour of the final immunoglobulin preparations were not the result of 4°C-storage-induced degradation of either component, but presumably resulted from the different techniques used in the final stages of the purification procedures.

3.5 Specificity of rabbit antisera raised against trout anodic and cathodic immunoglobulins

3.5.1 Absorption studies

Rabbit antisera were raised against the purified anodic and cathodic trout immunoglobulins as described in Section 2.9.2, and absorption studies were carried out to determine whether or not the rabbit anti-trout-immunoglobulin antisera were specific for trout immunoglobulin. A direct hemagglutination test was set up as described in Materials and Methods (Section 2.5.2.1), except that dilutions of immune trout serum were made in rabbit anti-trout-immunoglobulin sera or normal rabbit serum instead of saline. Hemagglutinating activity was removed by rabbit antisera against both trout immunoglobulin fractions (Table 5). Absorption with normal rabbit serum had no effect on antibody activity, indicating that the loss of activity was due specifically to interaction of trout immunoglobulin and anti-trout-immunoglobulin rather than to some non-specific component found in the rabbit serum itself. The antisera raised against both anodic and

Table 5. Effects of absorption of immune trout serum with rabbit antisera against trout immunoglobulin. Tube hemagglutination tests were read using a scale of 1+ to 4+, where 1+ represents weak agglutination and 4+ represents complete clumping of sheep red blood cells.

Final dilution of immune trout serum	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Untreated	4+	4+	4+	3+	2+	1+	0
Absorbed with normal rabbit serum	4+	4+	4+	3+	2+	1+	0
Absorbed with rabbit antiserum to trout anodic immunoglobulin	1+	0	0	0	0	0	0
Absorbed with rabbit antiserum to trout cathodic immunoglobulin	2+	0	0	0	0	0	0

cathodic immunoglobulin fractions, then, appeared to be specific for trout immunoglobulin.

3.5.2 Double diffusion and immunoelectrophoresis studies

Double diffusion experiments (Section 2.8.1) showed that both anti-anodic-trout-immunoglobulin and anti-cathodic-trout-immunoglobulin antisera appeared to recognize similar components from whole trout serum: the precipitin lines formed by the reaction of each antiserum with its individual antigen fused completely without spur formation, suggesting that both antisera precipitated the same serum protein (Figure 14). Whole trout serum was then subjected to immunoelectrophoresis (Section 2.8.2) against the two rabbit anti-trout-immunoglobulin antisera. Again, both the anti-cathodic- and the anti-anodic-immunoglobulin antisera recognized the same component from normal trout serum (Figure 15); the mobility of the component recognized by the antisera was mainly cathodic, but also extended into the anodic side of the application well.

3.6 Studies on mucus from rainbow trout

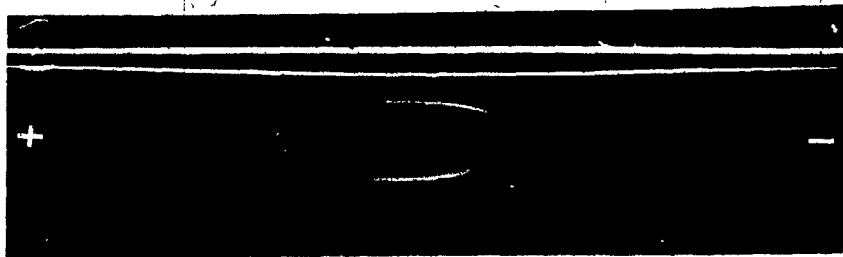
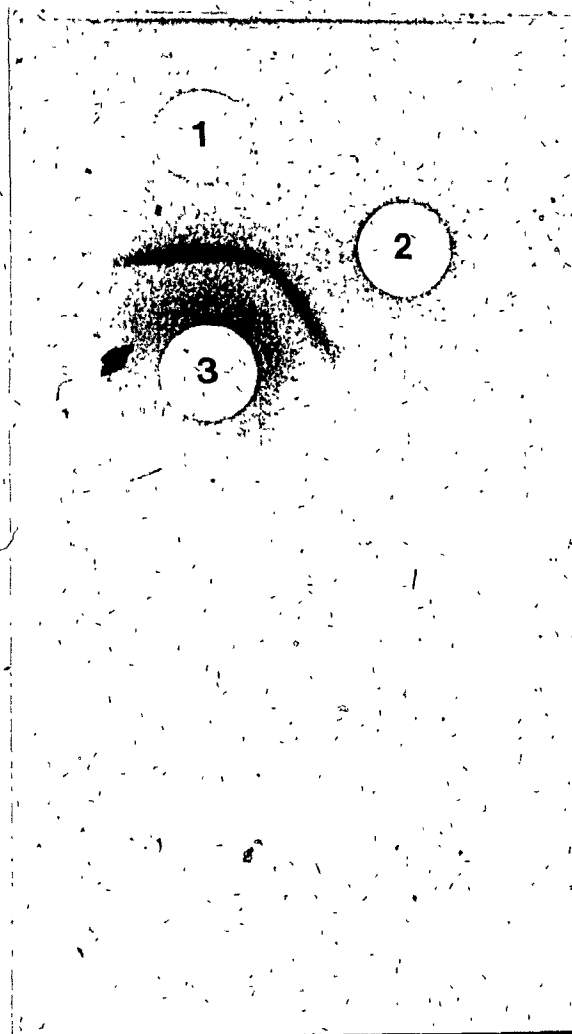
3.6.1 Induction of mucus antibodies to sheep red blood cells

Mucus from non-immunized trout did not contain natural hemolysins or hemagglutinins against sheep red blood cells. Low amounts of antibody to sheep red blood cells were detected in mucus samples taken from fish which were immunized with sheep red blood cells; unconcentrated mucus extracts did not agglutinate sheep red blood cells at dilutions greater than 1/16. Mucus antibodies were not found unless serum antibodies were also present; it was not possible, however, to

Figure 14. A double diffusion study of rabbit anti-anodic- and anti-cathodic-immunoglobulins, developed against whole trout serum. Fusion of precipitin lines suggests that both antisera recognize the same serum component. Well #1 contains anti-anodic-immunoglobulin; well #2, anti-cathodic-immunoglobulin; well #3, whole trout serum.

Figure 15. Immuno-electrophoresis of normal trout serum, developed against rabbit anti-anodic- and anti-cathodic-immunoglobulins. Both antisera recognize the same serum protein. The gel was photographed wet and unstained.

_____ rabbit anti-anodic-immunoglobulin
○ normal trout serum
_____ rabbit anti-cathodic-immunoglobulin



correlate levels of mucus antibodies with those from serum because of the variable dilution factors which were introduced into the mucus samples during their collection (Section 2.3.2).

3.6.2 Relation of mucus antibodies to serum antibodies

Immunoelectrophoretic studies (Section 2.8.2) indicated that trout mucus and serum have a number of components in common; rabbit anti-whole-trout-serum recognized several mucus components with anodic and cathodic mobilities (Figure 16). Rabbit anti-trout-immunoglobulin was reacted against trout serum and mucus in double diffusion experiments (Section 2.8.1); the anti-trout-immunoglobulin antiserum formed precipitin lines against the immunoglobulin components of both serum and mucus (Figure 17). These specific lines of precipitation fused at the edges, indicating that mucus and serum antibodies are antigenically similar. Further evidence of the antigenic similarities between serum and mucus antibodies was seen by the abrogation of specific hemagglutinating activity of mucus extracts following absorption of the extracts with rabbit anti-trout-immunoglobulin antisera (Table 6).

3.7 Microscopic studies on trout mucus and skin

3.7.1 Mucus smears

3.7.1.1 Wright's stain

A smear of mucus cells was stained with Wright's stain (Section 2.3.3) for identification of cell types in parallel with the immunofluorescent studies. Among the cell types identified were the epithelial cells and lymphocytes which have already been described by Ourth⁸³ in the skin mucus of the channel catfish. Occasional lymphoid cells were seen which

○ trout serum

○ trout mucus

rabbit anti-whole-trout-serum

1

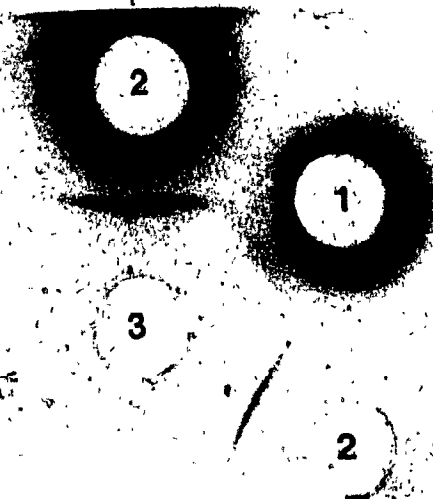
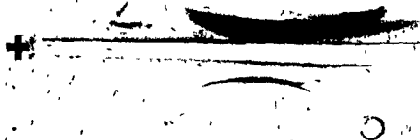


Table 6. Effects of absorption of mucus extract with rabbit antisera against trout immunoglobulin. Tube hemagglutination tests were read using a scale of 1+ to 4+, where 1+ represents weak agglutination and 4+ represents complete clumping of sheep red blood cells.

Final dilution of mucus extract	1/2	1/4	1/8	1/16
Untreated	2+	1+	0	0
Absorbed with normal rabbit serum	3+	1+	0	0
Absorbed with rabbit antiserum to trout anodic immunoglobulin	0	0	0	0
Absorbed with rabbit antiserum to trout cathodic immunoglobulin	0	0	0	0

resembled classical mammalian plasma cells,^{103,107} the end-product of antigen-driven differentiation of mature B-lymphocytes. These plasmacytoid cells were larger than the small lymphocytes and had an oval shape, abundant blue cytoplasm and a dense, reticular eccentrically-placed round nucleus (Figure 18). Some of these cells also showed the typical clear zone adjacent to the nucleus.

3.7.1.2 Immunofluorescent studies,

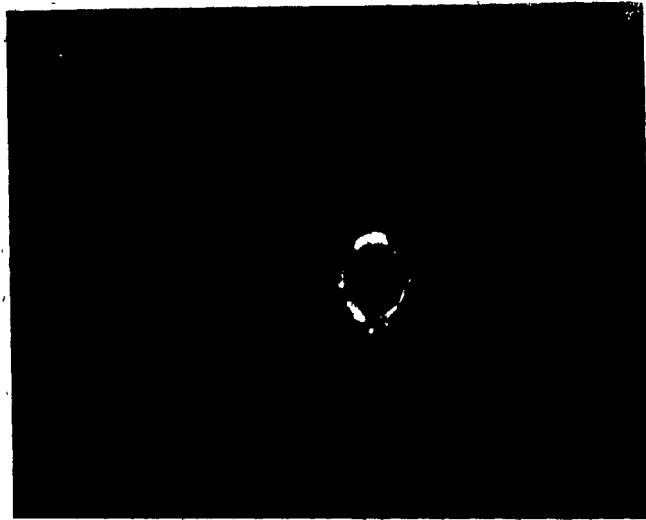
Smears were processed for immunofluorescence as described in Section 2.11. A few cells were identified as antibody-containing cells by their prominent dark nuclei and brightly-fluorescent cytoplasm (Figures 19 - 22). Cell types representing intermediate stages between the B-lymphocytes and the end-stage plasma cells have been described in immunofluorescence studies of mammalian tissues¹⁰⁴⁻¹⁰⁷ and were also identified among the various cells in trout mucus smears. These cell types consisted of large plasmablasts, with their large nuclei and rim of fluorescent cytoplasm (Figures 19,20), smaller proplasmacytes (immature plasma cells), and the small irregular or oval plasma cells with small eccentrically-placed round nuclei and abundant cytoplasm (Figures 21, 22). This staining was not seen in negative control slides, where normal rabbit serum was substituted for rabbit anti-trout-immunoglobulin serum (Section 2.11). The presence of antibody-containing cells in the mucus suggested that mucus antibody can be synthesized locally.

Figure 18. Photomicrograph of a Wright's-stained smear of trout mucus cells. A plasmacytoid cell is indicated by the arrow, ←. (Approximate magnification X 720).



Figure 19. Photomicrograph of a trout mucus smear, showing a plasma-blast-like cell. This specimen and those which are depicted in subsequent photographs, except for Figure 23, were stained by the indirect fluorescent-antibody technique (Section 2.11). (Approximate magnification X 720).

Figure 20. Photomicrograph of a trout mucus smear. An arrow indicates a late plasmablast-like or proplasmacytoid cell. (Approximate magnification X 720).



NOT SUITABLE FOR MICROFILMING
NE SE PRETE PAS AU MICROFILMAGE


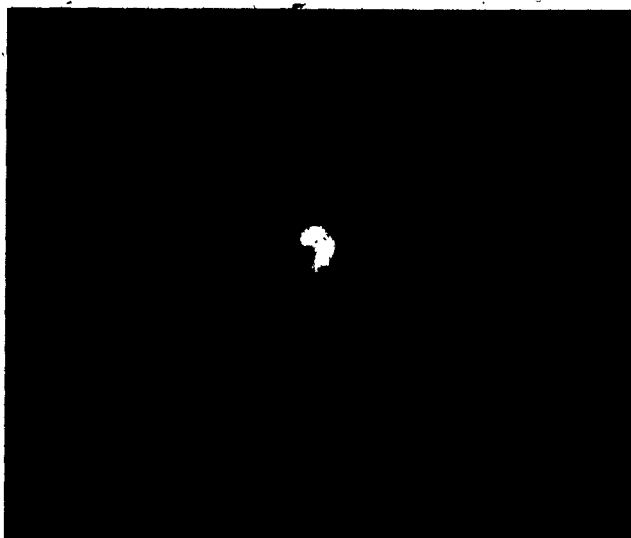


Figure 21. Photomicrograph of a trout mucus smear. The arrow indicates a proplasmacytoid cell. (Approximate magnification X 720).

Figure 22. Photomicrograph of a trout mucus smear, showing a plasma-
cytoid cell. (Approximate magnification X 720).



NOT SUITABLE FOR MICROFILMING
NE SE PRETE PAS AU MICROFILMAGE

3.7.2 Skin sections

3.7.2.1 Hematoxylin- and eosin-stained frozen sections

The hematoxylin- and eosin-stained sections of trout skin demonstrated the presence of layers of epidermis, loosely-arranged dermis and underlying muscle (Figure 23). Hematoxylin- and eosin-stained frozen sections typically give poor morphologic detail,^{105,106} and it was not possible to determine whether or not lymphocytes or their end-product plasma cells were present in any of the skin layers.

3.7.2.2 Immunofluorescent studies

Immunofluorescent studies showed a brilliant fluorescence of the blanketing mucus layer, and less bright staining between the cells of the epithelial layer (Figure 24); this staining pattern was not seen in negative control slides (Figure 25). Occasional individual cells (Figure 26a, b) and clusters of cells (Figure 27) which exhibited cytoplasmic staining were seen in the dermis, but their identification as plasma cells was tenuous, and they were not present in the large numbers which would be expected to accompany a local system of antibody secretion.

Figure 23. Photomicrograph of hematoxylin- and eosin-stained frozen section of trout skin. Stratum #1, epidermis; #2, dermis; #3, muscle. (Approximate magnification X 180).

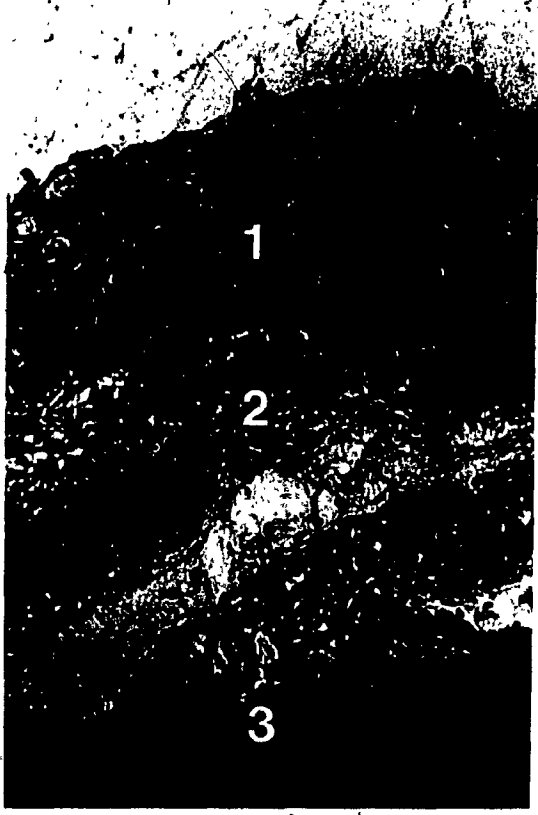
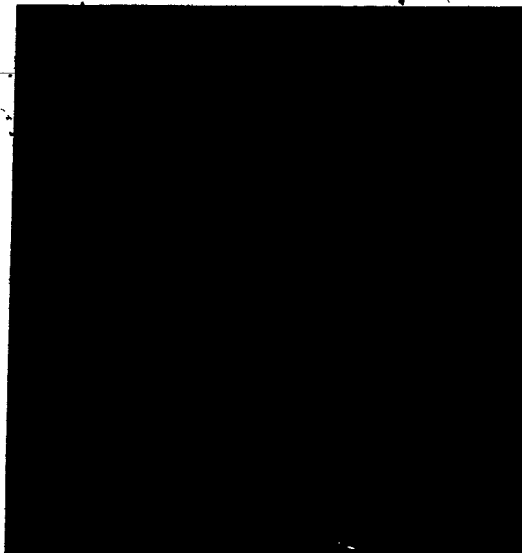
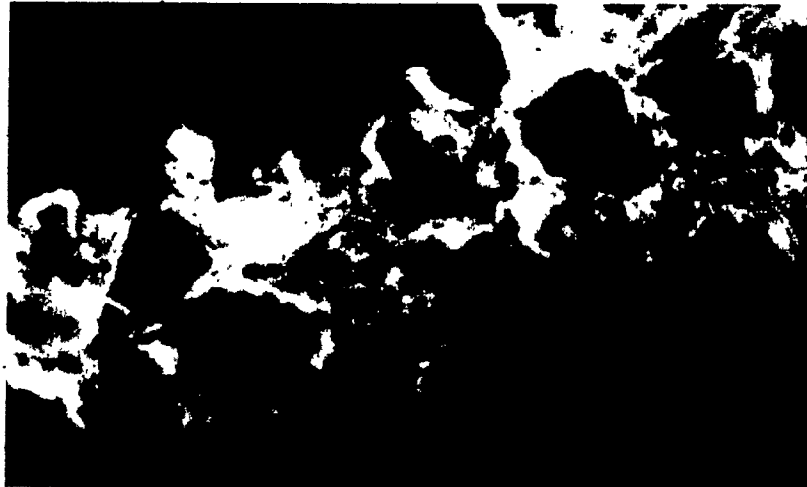


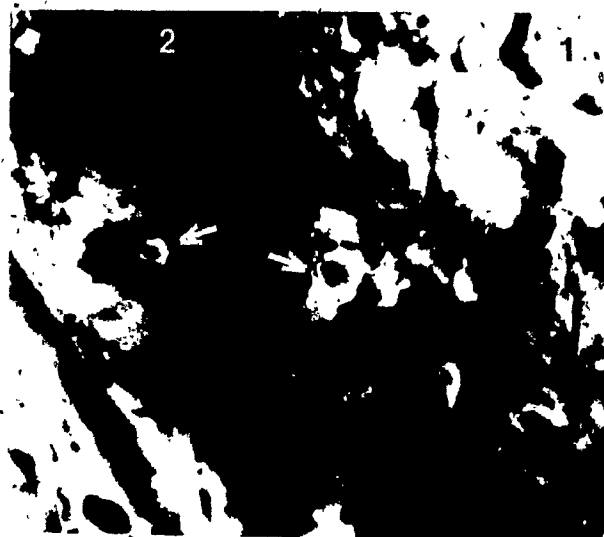
Figure 24. Photomicrograph of a frozen section of trout skin, showing localization of antibody in the mucus blanket and between the cells of the epithelium. (Approximate magnification X 450).

Figure 25. Photomicrograph of a frozen section of trout skin; negative control, showing lack of fluorescent staining. (Approximate magnification X 285).



NOT SUITABLE FOR MICROFILMING
NE SE PRÊTE PAS AU MICROFILMAGE

Figure 26 a (upper photo) and b (lower photo). Photomicrographs of frozen sections of trout skin. Individual dermis cells which contain cytoplasmic antibody are indicated by arrows. (Approximate magnification X 720).



NOT SUITABLE FOR MICROFILMING
NE SE PRÊTE PAS AU MICROFILMAGE

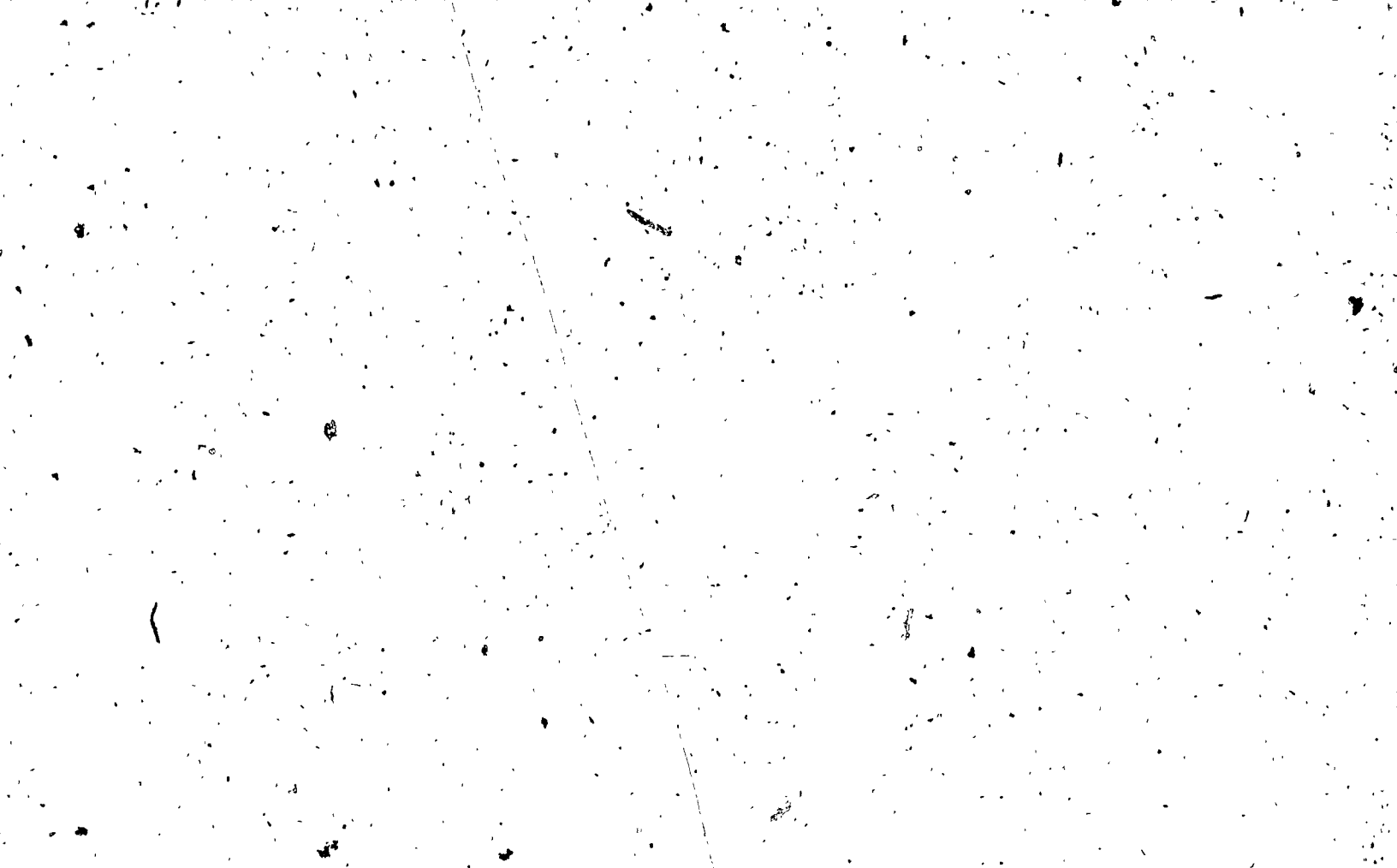
The image is a photomicrograph of a frozen section of trout skin. It shows a cluster of cells with cytoplasmic fluorescence. The background is dark, and the cells are lighter, with some internal structure visible. The text is located in the lower right quadrant of the image.

Figure 27. Photomicrograph of a frozen section of trout skin, showing a cluster of cells with cytoplasmic fluorescence. (Approximate magnification X 720).



DISCUSSION

4.0 Discussion: general comments

The present studies on rainbow trout (Salmo gairdneri), provided insight into the nature of the humoral immune response following antigenic stimulation and the effects of temperature on production of antibodies. Information was gathered on the size, molecular weight, and electrophoretic characteristics of serum antibodies. Evidence was obtained which supports the antigenic similarity of serum and mucus antibodies, and indicates local synthesis of body surface mucus antibodies.

4.1 Induced tolerance to human gamma globulin in trout

The phenomenon of immunologic tolerance or immune unresponsiveness involves the loss of ability of an organism to express immunity to an antigen to which, under different conditions, it would otherwise respond. The specifically tolerized individual maintains its capacity to respond to other antigens. The failure of rainbow trout in these studies to respond to injections of human gamma globulin, while maintaining the ability to respond to sheep red blood cells, suggests that under the conditions described in Section 2.4.1

specific tolerance to human gamma globulin was induced in these fish.

The establishment of tolerance has been well-documented in mammals

and has been observed in fish as well.

69,79,113

Without the phenomenon of tolerance, many harmless antigens would be recognized as foreign, including one's own tissue antigens. This characteristic of the immune response presumably plays as important a part in the survival of fish as does the production of antibodies.

Factors contributing to the experimental induction of tolerance

in fish include low ambient temperature, dosage, route of immunization, use of adjuvants, physicochemical nature of the antigen, and time schedule of immunization.^{69,79,113}

Immunization of an animal with large doses or with repeated low doses of antigen has been reported to produce immunological tolerance^{69,112} and it is possible that the

repeated weekly injections of a low dose (1 mg) of human gamma globulin used in the present study brought about a state of tolerance.

The trout used in this study were kept at a fairly low temperature

(9°C) throughout the course of immunization; another possible explanation for the loss of immune potential lies in the low dose-low ambient temperature induction of partial or complete tolerance described by

Avtalion et al in 1973,⁶⁹ following immunization of carp with a soluble protein antigen, bovine serum albumin. In the present study the

tolerance, once induced, could not be broken by raising the ambient water temperature to 14°C. Still another possibility is indicated by

studies on rabbits,¹¹⁴ where the presentation of human IgG in its native form led to an induction of tolerance, while denaturation of the gamma globulin by heat-aggregation prior to its injection into the animals led to a good antibody response. It may be, then, that the same mechanisms operate in rainbow trout, and that injection of human gamma globulin in its native form resulted in specific immunosuppression.

The final possibility is that the choice of the intramuscular route was inappropriate for this antigen. It has been reported that intramuscular injections of 20 mg of native bovine gamma globulin did not result in production of either hemagglutinating or precipitating antibodies in rainbow trout over a five-month period⁶⁰, but that

intraperitoneal injection of 25 mg of native human IgG produced specific serum antibodies in carp and goldfish.⁵⁴ Further investigations were not carried out with human gamma globulin in the present study and would be required to determine the cause of the loss of the specific immune potential in rainbow trout following intramuscular immunization with native human gamma globulin. In practical situations, procedures involving immunization of fish against pathogens would necessitate determination of, and control of, the factors which could evoke tolerance rather than immunity.

4.2 Natural "antibodies" in trout

Natural antibodies may be non-specific humoral factors, or they may be a reflection of previous exposure to the same antigen. Whatever their origin, the ever-ready presence of natural antibodies would be highly valuable as a first line of defense in fish exposed to infection with a pathogenic organism.

4.2.1 Natural agglutinins

Naturally-occurring agglutinins, in low and high titers, against a variety of vertebrate red blood cells and bacteria have been noted in representatives from all classes of fish, from cyclostomes to

teleosts.^{22,23,25,29,34,38,42,60,69,106-111} There is some evidence

that these natural agglutinins are immunoglobulins,^{34,42,108} with

specificities which differ from induced antibodies. No natural hemagglutinating antibodies were detected in non-immunized rainbow trout in the present study, but their presence may have been masked by hemolysins (Section 4.2.2).

4.2.2 Natural hemolysins

In the present study, low titers (up to 16) of heat-labile hemolysins for sheep red blood cells were detected in serum from non-immunized rainbow trout; injections of sheep red blood cells did not appreciably increase the titers of hemolysin. Naturally occurring hemolysins for a variety of vertebrate red blood cells have been described in fish,^{29,33,60,89,109,115,116} and several reports have suggested that this natural hemolytic activity involves interaction of antibody and complement factors.^{60,109,115-118} The natural hemolysins of rainbow trout have been reported to be heat-labile and to elute ^{60,118} the high molecular weight fraction in G-200 column chromatography, and, alternatively, in the low molecular weight fraction.⁸¹ Chiller et al in 1969⁸⁹ noted that hemolysis occurred only after a 24- to 48-hour incubation period and postulated that the hemolysin was not an antibody, but an enzyme. There have been reports of significant increases in titers of specific hemolysins following immunization of fish with red blood cells.^{115,118} In the present study, hemolysis occurred within five minutes following addition of trout serum to washed sheep red blood cells (Section 3.1.2.2); the titer did not increase appreciably following a 24-hour incubation period, and hemolysis was abolished if the serum was first heat-inactivated for 30 minutes at 56°C. The levels of this natural hemolysin could not be increased by immunization of trout with sheep red blood cells. No attempts were made in the present study to determine the nature of these hemolysins.

4.3 Production of specific anti-sheep-red-blood-cell antibody in trout, and effects of temperature

Despite the failure of rainbow trout to respond to injections of human gamma globulin, good antibody responses were elicited in most trout following antigenic stimulation with sheep red blood cells. The responses were characterized by tremendous individual variation; antibody production was transient in some trout, prolonged in others. Some trout produced antibody in low titers, others produced antibody in high titers, the highest titer obtained being 4096. It is not inconceivable that the individual differences in the magnitude of responses might reflect differences in genetic constitution. If the ability to respond to certain pathogens were found to be genetically-controlled, high-responders could be selectively bred on a commercial scale to produce disease-resistant strains of fish.

Serum antibodies were detected in both 9°C- and 15°C- acclimated fish; production of antibodies showed marked individual variation at both temperatures. The latent period prior to appearance of antibodies in the serum was found to be affected by temperature, and was shorter by three to four weeks in the 15°C fish. It is not known which mechanism in the immune response is sensitive to temperature. In 1948, Bisset⁷⁴ carried out a series of experiments on frogs and fish, and concluded that the stages of antibody-production and its release into the blood were affected by temperature. Bisset's results were widely accepted until more recently, when Avtalion et al, in 1973⁶⁹, were unable to reproduce Bisset's results and found evidence indicating that the temperature-sensitive stage was located somewhere between the process of phagocytosis and antigen-metabolism and the stage of

antibody-synthesis and release.

The critical temperature below which a poikilotherm fails to produce antibodies in response to antigenic stimulation is species-specific,⁶⁹ and while a warm-water fish like the carp may not produce antibodies at 9°C, antibody-production will occur at this temperature in a cold-water fish like the rainbow trout. A much lower temperature may be required to abolish antibody-production in rainbow trout. The effect of temperature on antibody-production is undoubtedly a vital consideration in the practical application of immunizing fish against pathogens. There is evidence that carp are able to acquire long-term immunity at temperatures below the critical level if they are first kept at optimal temperatures for several days after immunization.⁶⁹ Raising the water temperature to a predetermined optimal temperature during, and for a short initial period following, immunization could induce long-term resistance to pathogens in hatchery-raised fish prior to their release. If immunization on a large scale basis were found to be impractical, changing the water temperature according to the disease might enhance survival. Depending on its etiology, the course of an infection can be altered by upward or downward shifts in ambient water temperature.⁶⁹ Fish which have been experimentally infected with Aeromonas hydrophila have been found to actively seek out higher temperatures, bringing about behavioural fever and subsidence of the infection.⁷¹

4.4 Characterization of trout immunoglobulin

4.4.1 Two populations of high molecular weight immunoglobulin with different electrophoretic mobilities

A review of the literature indicates tremendous discrepancies in characterization of the nature of the antibody response and immunoglobulins of fish.¹⁹⁻⁶⁶ While some of these differences may be ascribed to properties inherent in the various immune systems themselves, others may be due to variations in antigens and in their modes of presentation; still others may result from the different technical procedures used in isolation of the antibodies. The possibility that some of the unexpected characteristics may be due to in vitro degradation of immunoglobulin preparations should also be considered. Prolonged storage at 4°C. has been found to alter the antigenic and physical structure of human immunoglobulin,¹¹⁹ resulting in alterations in immunoelectrophoretic patterns, and there is some evidence that the low molecular weight form of antibody reported in carp and goldfish was an in vitro enzymatic digestion product of the high molecular weight form, brought about by storage at 4°C.⁵⁴ However, there have also been reports that, in sharks at least, immunoglobulin is not altered by prolonged storage.^{41,42} In the present study, long-term storage was found not to have an effect on the immunoelectrophoretic mobility of either the anodic or the cathodic immunoglobulin preparations from rainbow trout. Neither protein seemed to be a degradation product of the other.

In the present study, only the cathodic immunoglobulin was isolated when separation procedures used gel electrophoresis as the final step, while both anodic and cathodic immunoglobulins were purified when DEAE was used as the final step (Section 3.4.3). While the separation characteristics of the DEAE and agar gel electrophoresis procedures themselves, undoubtedly differ, the elution of only

a cathodic protein following gel electrophoresis presumably reflects a technical bias, which was introduced into the collection of the eluates from the agar gel. Small amounts of hemagglutinating activity did migrate toward the anode (Section 3.3.5), but these fractions did not yield a single precipitin arc in immunoelectrophoresis. Antibody-containing fractions eluted from the cathodic side of the application trough did yield a single arc in immunoelectrophoresis, and these are the ones which were collected for the final purified immunoglobulin preparation.

The isolation of two populations of high molecular weight antibodies with different charge properties in rainbow trout is presumably a reflection of the electrophoretic heterogeneity which has been noted in salmonid fish.¹²⁰⁻¹²² It is not inconceivable that these two populations of antibody represent different subclasses of antibody, with enough similarities between them such that antisera produced against either would react in immunoelectrophoresis against the entire spectrum of polymorphic antibodies. The different subclasses of human IgG differ in their electrophoretic mobilities, half-lives, synthetic and catabolic rates, susceptibilities to enzymatic digestion, abilities to activate the complement factors which lead to lysis, and responses to antigens: the existence in fish of different subclasses with different biologic characteristics and capabilities would better guarantee them increased chances of survival against a wide range of infectious organisms.

4.4.2 Attempts to detect 7S immunoglobulin

While there is evidence that the 7S immunoglobulin seen in fish can be an in vitro degradation product of multimeric high molecular weight immunoglobulin,⁵⁴ it has also been suggested that the failure to detect monomeric 7S antibodies may be due to the fact that the test systems used to assay for antibody activity were not capable of detecting 7S antibodies.^{37, 57} The high molecular weight antibodies of groupers, for example, were found to behave approximately thirty times more efficiently than low molecular weight antibodies in a phage neutralization test,³⁷ and the 16S high molecular weight antibodies of the margate have been found to be at least 20,000 times more efficient than 7S forms of antibody in hemagglutination assays.⁵⁷

In the present study, a modification of the Coombs indirect antiglobulin test,⁹³ which was developed specifically for the detection of monomeric antibodies in agglutination testing systems, was set up and used (Section 2.5.2.2) for detection of 7S antibodies in the serum of rainbow trout. Agglutinating antibody was found only in the high molecular weight fraction from S-300 gel filtration of sera from early and late antibody responses. Serum antibody of the 7S type was not found in any of the low molecular weight fractions for up to 133 days following the first immunization, indicating that there is no shift in production from high molecular weight to low molecular weight antibody in rainbow trout.

4.5 Body surface mucus, and its involvement in defense against infection

A number of functions have been ascribed to the mucus layer which coats the body surface of fish. The thick mucus substance may play important roles in locomotion,⁸⁰ osmoregulation⁸² and precipitation of suspended mud particles.⁸² The continuous replacement and sloughing-off of mucus is thought to prevent microbial colonization.⁸¹ In addition to the protective physical barrier provided by the mucus substance itself, there is evidence of secretion into the mucus layer of components which may be involved in non-specific defense systems against the microorganisms which pervade the external environment. For example, lysozyme is able to bring about microbial destruction and has been described in the skin mucus of catfish⁸³ and plaice.¹²³ Natural factors, which may¹²⁴ or may not be antibody,⁸³ with hemagglutinating and bacteriolytic properties have also been found in the mucus of fish.^{48,83,124}

There is a growing body of evidence which suggests that an inducible antibody-dependent defense system is present in the external surface of fish. Harrell et al,¹²⁵ in 1976, demonstrated the presence of complement in mucus of rainbow trout, and found that in vitro anti-microbial activity required the interaction of antibody-like and complement-like components for its expression. Several authors have been able to induce specific mucus antibodies against a variety of bacterial and erythrocyte antigens.^{48,83,85,123,125,126} There is evidence that the induced antibodies of mucus have antigenic properties in common with serum antibodies.^{48,124-126} It has been suggested that a specific secretory system is involved in the production

of mucus antibodies,^{48,85,125,126} and, alternatively, that skin mucus⁸³ may be derived from the serum.

The rainbow trout used in the present study showed no detectable natural hemolytic or agglutinating antibodies against sheep red blood cells prior to antigenic stimulation. Specific anti-sheep-red-blood-cell antibodies were induced in the mucus by intraperitoneal injections of sheep red blood cells; immunodiffusion and absorption studies (Section 3.6.2) showed that these mucus antibodies shared antigenic components with serum antibodies. Immunofluorescence procedures demonstrated the presence of antibody-containing plasmacytoid cells in the mucus. Plasma cells are the end-product of antigen-driven differentiation of mature B lymphocytes and are responsible for the production of antibody. They have been described in immunocompetent tissues from representatives of all classes of fish,^{86,115,127,128} and their morphology and cell lineage are identical to those of mammals.^{86,128} The identification of these cells in the mucus suggested that local synthesis of antibody occurs at the skin level in rainbow trout, but the origin of these cells could not be determined. Immunofluorescence microscopy showed that antibody was present between the epithelial cells (Figure 24), suggesting that it diffused into the mucus from the underlying tissues. However, plasmacytoid cells could not be definitely identified in either the epithelial or dermal layers (Figures 24,26,27). It should be noted that the skin sections were taken from a non-immunized fish which appeared to be in good health. A few cells or occasional clumps of possible antibody-containing cells were seen in the dermis (Figures 26, 27) but they were not present in the large numbers which would be expected in an active antibody-secreting system.

Since plasma cells are the end-result of a cellular response to antigenic stimulation, it is possible that mucus and skin sections taken from a fish which had been exposed to infection or antigenic stimulation would have exhibited a more intense response as a result of lymphoid cellular proliferation, facilitating identification of antibody-secreting cells.

While non-specific lysozymal and anti-microbial activity in the body mucus would be of value to a fish as a first line of defense, a system for local synthesis of antibody would greatly enhance chances of survival. The thick mucus could entrap micro-organisms, which, if not destroyed by the natural antibodies and lysozymes, would activate the mechanisms of local synthesis and secretion of antibody.

The finding of evidence for a local synthesis of mucus antibody does not rule out the possibility of simple diffusion of serum antibody into the mucus, and it is conceivable that both mechanisms could operate simultaneously. Identification in fish mucus antibody of a secretory piece similar to that found in mammalian secretory antibody would confirm the existence of a secretory immunoglobulin system in fish.

4.6 Final comments

Natural factors and inducible antibodies have been described in the serum and body mucus of fish. Through the information gathered and the questions raised, the present study invites further investigations into the immunological defense mechanisms of fish. A more thorough characterization of their immune response would be useful in attempts to successfully develop disease-resistant fish, in the

assessment of the effects of pollutants on resistance to disease, and for the determination of the phylogenetic emergence of the immunological mechanisms which operate in man.

SUMMARY

Rainbow trout (Salmo gairdneri) did not respond to intramuscular challenge with human gamma globulin; these same fish produced serum antibodies following immunization with sheep red blood cells, suggesting that they had become tolerized by the gamma globulin. Antibody production in response to stimulation with sheep red blood cells occurred at both 9°C and 15°C, but the latent period prior to detection of serum antibodies was shorter at the higher temperature. Trout immunoglobulin was found to be of high molecular weight (approximately 760,000 daltons) only, and was isolated as two populations with different electrophoretic mobilities. No low molecular weight antibodies were detected in rainbow trout, even after prolonged stimulation. Mucus antibodies were found to be antigenically related to serum antibodies, and immunofluorescence microscopy suggested a local system for production of mucus antibodies.

The present studies indicated that rainbow trout are immunologically competent, and that a variety of factors can influence their immune responsiveness. It was suggested that further attempts be made toward a more complete understanding of the factors which might influence the immune response in fish, and that this additional knowledge would be useful in providing them with increased capacities in resisting infection by environmental pathogens.

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