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Title: PROPERTIES OF ANTI- *AEROMONAS SALMONICIDA* ANTIBODIES FROM JUVENILE COHO SALMON (*ONCORHYNCHUS KISUTCH*)

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After parenteral immunization with *Aeromonas salmonicida* cells, the agglutination activity in juvenile coho salmon (*Oncorhynchus kisutch*) serum was associated with the macroglobulin serum fraction. Antibody activity was diminished by 30 minutes exposure to 50 C but not 45 C. Reduction with 2-mercaptoethanol and alkylation markedly lowered but did not completely destroy agglutination activity. The residual activity appeared to be maintained by noncovalent forces. The minimum antibody concentration required for agglutination of *A. salmonicida* cells was approximately 0.04 μgm protein/ml. The extreme sensitivity of this bacterial agglutination system is an important consideration in the interpretation of coho salmon anti-*A. salmonicida* titers.

Specific antibodies were isolated from pooled coho salmon serum by dissociation of bacterial cell wall immune complexes with 15 percent NaCl. The procedure resulted in the recovery of about 30 percent of the total anti- A. salmonicida antibody. The purified antibodies had a sedimentation coefficient of approximately 17S. They migrated with the slowest moving serum component in alkaline polyacrylamide gel electrophoresis, and were restricted to the anodic portion of the serum immunoglobulin arc in immunoelectrophoretic separations. In spite of the restriction in electrophoretic mobility, the antibodies were quite heterogeneous and a minimum of 11 electrophoretic subpopulations of light chains were observed. Electron microscopy and sodium dodecyl sulfate-acrylamide gel electrophoresis revealed that the coho salmon anti- A. salmonicida macroglobulin was composed of four IgM-like subunits. No evidence for a low molecular weight antibody was obtained.

Properties of Anti- *Aeromonas salmonicida* Antibodies From Juvenile Coho Salmon (*Oncorhynchus kisutch*)

by

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PROPERTIES OF ANTI- *AEROMONAS SALMONICIDA* ANTIBODIES FROM JUVENILE COHO SALMON (*ONCORHYNCHUS KISUTCH*)

INTRODUCTION

Oral immunization offers an attractive means of preventing furunculosis in populations of hatchery reared salmonids. A distinct advantage of immunization over chemotherapy is the avoidance of problems which result from drug resistant pathogens. In addition, it seems possible that fish, if immunized as juveniles, might be able to cope with furunculosis more effectively in their natural environment. Immune serum has been shown to protect juvenile coho salmon (*Oncorhynchus kisutch*) against furunculosis (Spence, Fryer and Pilcher, 1965). Other work has indicated that protective immunity can be induced by the oral route (Overholser, 1968). In spite of encouraging results, research toward the development of effective immunization procedures has been hampered by a lack of information concerning the immune state in juvenile salmon. Thus, it was necessary to initiate research in this direction.

The purpose of this study was to determine the structural features of coho salmon anti- *Aeromonas salmonicida* antibodies. It was also considered important to examine the biological activity of the molecule. The objective of this work was to provide a basis

for investigations concerning both the location and function of salmon antibodies. Ultimately, it should be possible to obtain an understanding of how immune salmon cope with invading microorganisms.

Studies of anti- *A. salmonicida* activity in immune serum were used to obtain preliminary information concerning the coho antibody molecule. Specific antibodies were purified by an immunoadsorption procedure and structural work was undertaken. Information was obtained concerning: (1) the molecular weights of the heavy and light chains and the immunoglobulin subunit, (2) the polypeptide chain structure of the subunit and (3) the number of subunits in the intact antibody molecule. The antibodies agglutination activity was also examined.

LITERATURE REVIEW

The objective of this review is to summarize recent information and ideas concerning immunoglobulin structure and function in fishes. Other reviews are available on this and closely related subjects (Booke, 1964; Good and Papermaster, 1964; Ridgway, Hodgins and Klontz, 1966; Grey, 1969; Clem and Leslie, 1969; Snieszko, 1970).

Although research reported in this thesis deals with immunoglobulins from coho salmon (class Osteichthyes), it seems important to consider review information from the classes Chondrichthyes (the sharks) and Agnatha (the cyclostomes). The phylogenetic scheme given by Romer (1962) will be used to relate and designate different fishes (Figure 1).

Immunoglobulins From Cyclostomes (Class Agnatha)

Structural studies of lamprey and hagfish immunoglobulins may provide critical information for determining the evolutionary origin of the vertebrate immune mechanism.

Marchalonis and Edelman (1968) reported antigenically identical 6.6 and 14S immunoglobulins from lamprey immune serum. The 6.6S molecule consisted of two μ-like heavy (H) chains[1/] (molecular

[1/] μ is the heavy chain from human IgM.

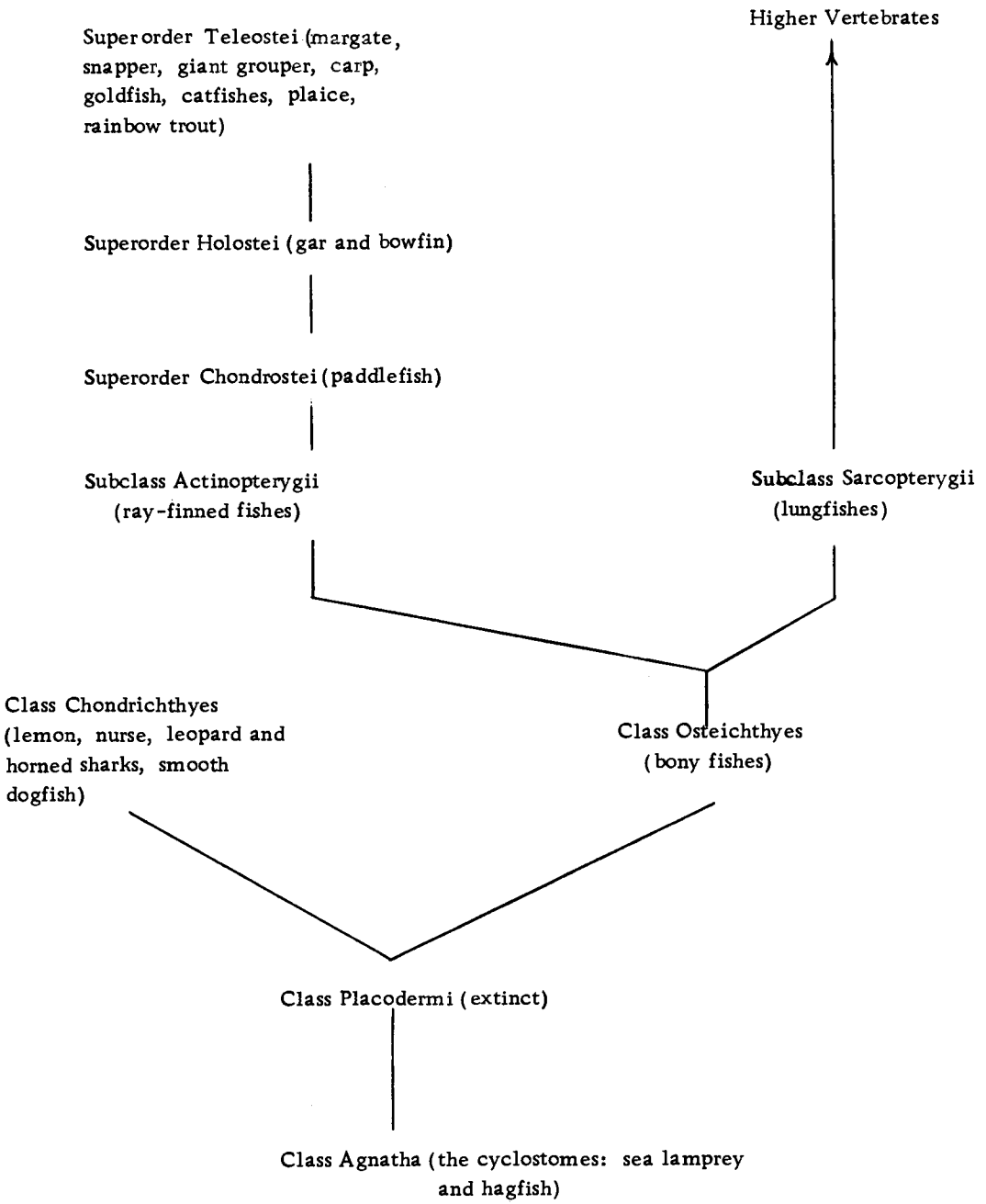

Figure 1. A simplified phylogeny of lower vertebrates (modified from Romer, 1962) showing some of the fishes employed in immunological studies.

weight ~70,000) and two light (L) chains (molecular weight ~25,000). Unlike the usual situation found with higher vertebrate immunoglobulins,[2/] the lamprey chains were reported to be held together by noncovalent attractions. The 14S protein appeared to be composed of 6.6S subunits.

Recent results from another laboratory (Pollara et al. 1970; Litman et al. 1970) seem to be in apparent disagreement with the above findings. This group maintains that lampreys synthesize a 9S antibody (molecular weight ~300,000) composed of four, noncovalently bound, identical subunits. Each subunit was reported to consist of a large and a small fragment presumably joined by disulfide bonding and noncovalent forces. The two fragments did not correspond to any known kind of H or L chain. In addition to its unusual structure, Litman et al. (1971c) concluded that the lamprey immunoglobulin possessed unique conformational properties for an immunoglobulin (a high α-helix content).

A common finding from both laboratories was that the lamprey immunoglobulin was very unstable and tended to spontaneously dissociate. This might account for the differing results from the two laboratories. It would be interesting to see if the architecture of this unusual protein offers a link between typical vertebrate

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[2/] In most vertebrate immunoglobulins disulfide bonds connect H and L chains in the order L-H-H-L.

immunoglobulins and certain invertebrate proteins.

Immunoglobulins From Sharks (Class Chondrichthyes)

Shark immunoglobulins were the first ones from lower vertebrates to be studied, and much of the initial structural work with these proteins has been reviewed (Grey, 1969; Clem and Leslie, 1969). In summary, sharks produce an 18S macroglobulin (molecular weight ~900,000) after initial antigenic stimulation, and a 7S antibody (molecular weight ~180,000) after prolonged immunization. The 7S molecule is composed of two μ-like H chains (molecular weight ~70,000) and two L chains (molecular weight ~22,000) which are linked in the typical arrangement (L-H-H-L). The 18S protein is a pentamer of 7S subunits; both 18 and 7S immunoglobulins are antigenically identical. Based on general characteristics of the H chain, the shark immunoglobulins have been designated as 18S IgM and 7S IgM. The assignment of shark immunoglobulins to the IgM class is also supported by a limited amount of amino acid sequence data (Suran and Papermaster, 1967; Goodman *et al.* 1970).

Proteolytic fragmentation studies (Klapper, Clem and Small, 1971) suggested that shark and human IgM possessed common structural regions. Both ($F(ab)_2\mu$) and ($F(ab)\mu$)-like fragments were detected, however a stable Fc fragment was not found. The authors felt that the Fc region might have been degraded to small peptides

during proteolytic cleavage.

The work of Goodman and Benjamini (1970) suggests that sharks and mammals synthesize antibodies which have similar specificities. This was concluded from the finding that the immunodominant group on the tobacco mosaic virus protein in mammals was also a determinant in the leopard shark.

Valence determinations with shark immunoglobulins are rather confusing. From equilibrium dialysis experiments with the 2, 4-dinitrophenyl (DNP) haptenic group, Voss, Russell and Sigel (1969) concluded that shark 18S and 7S IgM have valences of five and one respectively. In spite of this it seems likely that the 7S IgM molecule has two reactive sites while 18S IgM has ten. Numerous reports have indicated that shark 7S IgM antibody is not monovalent (Clem and Small, 1967; Suran, Tarail and Papermaster, 1967: Klapper *et al*. 1971; Schulkind, Robbins and Clem, 1971). The recent finding that the reductive subunit from horned shark 18S IgM could agglutinate *Brucella* cells also casts doubt on its monovalency (Frommel *et al*. 1971b). Difficulties encountered in valence determinations with mammalian and shark immunoglobulins have been discussed by Metzger (1970) in his review of γ M macroglobulins.

The relationship between shark 7S and 18S IgM appears to be similar to that of mammalian IgG and IgM. Experiments with radioactively labeled immunoglobulins failed to detect any interconversion

of serum 7S and 18S IgM (Small, Klapper and Clem, 1970; Voss and Sigel, 1971). In addition, the body distribution of the two shark proteins was like that of mammalian IgG and IgM (Small et al. 1970). Another similarity is the sequential synthesis of 18S followed by 7S IgM after antigenic stimulation (Fidler, Clem and Small, 1969; Voss and Sigel, 1971; Schulkind et al. 1971).

The relative serological efficiencies of the two shark immunoglobulins are somewhat similar to those observed with human IgG and IgM (Schulkind et al. 1971). Compared to immune 7S IgM, the shark macroglobulin agglutinated bacteria at a much lower antibody concentration. When fetal calf serum was employed as a complement source,[3/] very low concentrations of the immune macroglobulin (0.02-0.1 μgm/ml) were bactericidal for Salmonella typhimurium. In contrast, immune 7S IgM was nonbactericidal at a concentration of 50 μgm/ml. Although functional analogies of shark 7S and 18S IgM to mammalian IgG and IgM exist for the agglutination and bactericidal reactions, the comparison may not hold for the precipitation reaction. In contrast to mammalian IgG (Pike, 1967), certain data suggest that shark 7S IgM is probably a low efficiency precipitin at best (Suran et al. 1967; Voss et al. 1969).

Some rather interesting observations have been made

[3/] As pointed out by the authors, it is questionable whether a mammalian complement system can function with shark immunoglobulins.

concerning concentrations and atypical binding properties of normal immunoglobulins in shark serum. Studies have shown that the immunoglobulin content of shark serum increases from a value of less than 2 percent in the neonatal animal to approximately 50 percent of the total serum protein in the adult (Fidler et al. 1969; Clem and Leslie, 1969). In spite of the increase in immunoglobulin content, the total serum protein remains between 20 and 30 mg/ml in both neonatal and adult sharks (Fidler et al. 1969). Evidence is accumulating which suggests that the normal immunoglobulin functions as more than simply inert serum protein. This is illustrated by the properties of natural shark antibodies to the DNP haptenic group. Specifically purified natural 18S anti-DNP antibody populations bound DNP very weakly and also agglutinated sheep and pigeon erythrocytes[4/] (Voss et al. 1969; Leslie and Clem, 1970; Rudikoff, Voss and Sigel, 1970). Inhibition studies revealed that DNP and erythrocyte binding occurred at different locations on the same molecule. The significance of normal immunoglobulin in sharks is not understood. Its high concentration, broad reactivities and atypical binding behavior are of special interest since these properties appear to exist in many groups of fishes. Some of the evidence for this is presented later.

[4/] Induced populations of anti-DNP antibodies bound DNP normally and did not agglutinate erythrocytes.

Immunoglobulins From Bony Fishes (Class Osteichthyes)

From a phylogenetic standpoint lungfish immunoglobulins are of particular interest since these animals are in the subclass which gave rise to land vertebrates (Figure 1). Marchalonis (1969) isolated antigenically related 19.4S and 5.9S immunoglobulin-like proteins from Australian lungfish serum. The L chains from both proteins had the same molecular weight (~23,000) and were antigenically identical. Heavy chains from the 19.4S and 5.9S proteins were antigenically distinct and had different molecular weights (~70,000 and ~38,000 respectively). Both proteins contained equal numbers of light and heavy chains and it was implied that the 19.4S protein was a pentamer of 7S subunits. Litman et al. (1971a) has obtained similar results from studies with African lungfish immunoglobulins. At present the lungfishes (order Dipnoi) represent the lowest phylogenetic level where distinct immunoglobulin classes exist.

A surge of recent work on members of the Actinopterygii (ray-finned fishes) has contributed to an increased understanding of immunoglobulin structure within this group of animals. The paddlefish (superorder Chondrostei) was shown to synthesize an immune macroglobulin ($S^0_{20,W}$ = 14..2S) with a determined molecular weight of 630,000 (Acton et al. 1971a). The carbohydrate content of the molecule has been reported as 6.8 percent (Acton et al. 1971a) and

9 percent (Frommel et al. 1971a). Electron microscopy revealed that the macroglobulin consisted of only four subunits (Acton et al. 1971a). Molecular weights obtained from gel filtration indicated that the subunit was typical and contained two L chains (~23,000) and two H chains (~70,000) (Acton et al. 1971a; Litman et al. 1971a). From sedimentation equilibrium experiments, it has been suggested that the H chain molecular weight is actually closer to 58,100 ± 1,500 (Acton et al. 1971b). If the centrifugation data proved to be correct, certain ideas concerning immunoglobulin evolution within the class Osteichthyes may have to be reconsidered. Although the paddlefish has generally been thought to synthesize a single macroglobulin type of antibody, a preliminary report from Litman et al. (1971a) indicated that a nonactive low molecular weight form was present. As with shark antibodies, the H chains from low and high molecular weight paddlefish immunoglobulins appeared to be identical.

A unique feature of the paddlefish macroglobulin is its apparent insusceptibility to partial reduction (Fish, Pollara and Good, 1966). Recent findings indicate that this property may be the result of a small peptide which can be removed by papain cleavage (Pollara, Chartrand and Good, 1970).

The work of Legler et al. (1971) revealed that 40 percent of the protein in paddlefish serum was immunoglobulin. Cold agglutinins were detected for S. typhosa "O" antigen, sheep erythrocytes and

human blood group A erythrocytes.

Holostean fish whose immunoglobulins have been studied include the gar and the bowfin. The gar has been shown to synthesize a 14S immunoglobulin which is sensitive to partial reduction (Bradshaw, Clem and Sigel, 1969; 1971). The gar macroglobulin, like that of the paddlefish, appeared to be tetrameric and molecular weight estimates of 610,000 (Acton et al. 1971b) and ~650,000 (Bradshaw, Clem and Sigel, 1971) have been reported. Molecular weight values for the H and L chains from gel filtration are like those of the paddlefish (~70,000 and ~23,000 respectively). Acton et al. (1971b) have determined the total carbohydrate content of gar macroglobulin to be 4.86 percent. In contrast to the gar, bowfin serum was reported to contain both high (13.6S) and low (6.3S) molecular weight immunoglobulins (Litman et al. 1971b). The 13.6S protein is probably like previously described macroglobulins from the paddlefish and gar. A preliminary report (Litman et al. 1971a) indicates that molecular weights for the H chains from bowfin 13.6S and 6.3S immunoglobulins are 70,000 and 52,000 respectively. In spite of the molecular weight differences, both H chains are reported to be antigenically identical.

Various observations concerning gar immunoglobulins are of interest. Acton et al. (1971b) found that 40 percent of gar normal serum protein was immunoglobulin. As with shark serum, Leslie and Clem (1969) found considerable anti-DNP activity in

nonimmunized gar serum. Bradshaw and Sigel (1969) reported substantial cold agglutinin titers for S. typhosa H antigen and sheep erythrocytes; the cold agglutinins were shown to be gar immunoglobulins. Recently, both natural and immune cold hemagglutinins were detected in gar mucus (Bradshaw, Richard and Sigel, 1971). As pointed out by the authors, the location of mucus antibody has obvious survival value.

Although information is available from certain members of the superorder Teleostei, as a group, the immunoglobulins from these animals have not been well studied. An unsolved problem concerns the ability of teleosts to synthesize high and low molecular weight immunoglobulins. Both 16.1S and 6.4S antibodies were isolated from giant grouper anti-DNP serum; however, the possibility was suggested that the 6.4S protein represented a macroglobulin fragment (Clem and Small, 1970; Clem, 1971). The margate, another marine teleost, apparently produces 15 to 16S and 7.2S immunoglobulins (Clem and Leslie, 1969). Initial work failed to detect the margate 7.2S protein since it behaved monovalently and was inactivated by partial reduction. Certain studies indicate that the catfish (Kuhns and Chuba, 1968) and goldfish (Uhr, Finkelstein and Franklin, 1962) also synthesize high and low molecular weight antibodies which are both sensitive to partial reduction. In other work, it appeared that goldfish antibodies were associated with two macroglobulins (~16S)

of slightly different charge and antigenic composition (Trump, 1970). The two macroglobulins were synthesized sequentially during the primary response to bovine serum albumin (Trump and Hildeman, 1970). Marchalonis (1971) was able to detect only macroglobulins in his study of goldfish and carp antibodies, although lower molecular weight material was found which reacted with antiserum against the macroglobulin. Further analysis of the cross reacting material suggested that it was a degradation product of the macroglobulin rather than a low molecular weight immunoglobulin. Smith and Potter (1969) were also unable to detect low molecular weight immunoglobulins in carp anti-DNP antibody preparations. Ambrosius, Richter and König (1967) reported that a fraction of carp antibody activity was resistant to partial reduction; however, the size of this fraction was not indicated. Antibody activity in rainbow trout serum was decreased by partial reduction and was associated with the macroglobulin fraction of immune serum (Hodgins, Weiser and Ridgway, 1967). Although the above observations do not suggest any broad generalizations concerning teleost antibodies, certain points are apparent. Bivalent activity (precipitation, agglutination, etc.) and resistance of activity to partial reduction are not reliable criteria for detecting low molecular weight teleost immunoglobulins. Likewise, macroglobulin degradation must be considered as a possibility when low molecular weight material is found.

Structural studies indicate that teleost antibodies are similar to those from chondrosteans and holosteans. Clem (1971) obtained molecular weight estimates of 700,000 and 120,000 for the giant grouper 16.1S and 6.4S immunoglobulins. The light chains from both proteins were antigenically identical and had the same molecular weight (~22,000). In contrast, the H chain from the 6.4S immunoglobulin was antigenically deficient with respect to the H chain from the 16.1S macroglobulin. The H chains from the 6.4S and 16.1S proteins had estimated molecular weights of 40,000 and 70,000 respectively. From these data and other considerations,[5/] it appeared that the 6.4S protein was a $F(ab'\mu)_2$-like fragment of the macroglobulin. The above information plus the finding that giant grouper 16.1S anti-DNP antibodies have a valence of eight (Clem and Small, 1970) are good evidence that the macroglobulin is composed of four subunits. The carp macroglobulin also appears to be tetrameric (Shelton and Smith, 1970) and is composed of an equal number of H (molecular weight ~71,000) and L (molecular weight ~24,000) chains (Marchalonis, 1971). Acton *et al.* (1971a) have reported that the catfish 14S immunoglobulin is a tetramer which has a molecular weight of 610,000 and a carbohydrate content of 5.5

[5/] Hexose determinations and analysis of peptide maps, suggested that the H chains from the 6.4S protein were lacking $Fc\mu$-like fragments.

percent. Molecular weight estimates for the H and L chains were 70,000 and 23,000 respectively.

Various observations have been made regarding natural antibodies of teleosts. Significant levels of natural anti-DNP antibodies have been found in the margate and snapper (Leslie and Clem, 1969) and the carp (Frenzel and Ambrosius, 1971). DiConza (1970) observed natural macroglobulin hemagglutinins to a variety of mammalian erythrocytes. Hodgins et al. (1967) reported that rainbow trout serum contained specific natural hemagglutinins for rabbit, mouse and human erythroctyes. Physical comparisons revealed differences between natural and immune trout hemagglutinins; however, the lack of fully purified material made antigenic comparisons impossible. Of interest are the observations of Vanstone and Ho (1961) which suggest that the immunoglobulin content of coho salmon serum increases with the age of the fish.

Immunoglobulins have been detected in surface and intestinal mucus of two teleosts. In what appears to be the first documentation of fish secretory antibodies, Fletcher and Grant (1969) reported elevated mucus antibody titers after immunization with various antigens. Physical and chemical studies suggested that serum and mucus antibodies were similar. DiConza (1970) has also found immunoglobulins in the intestinal and surface mucus of a catfish species.

Certain generalizations can be made from recent work with fish immunoglobulins. Based on the properties of H chains from most fish immunoglobulins it appears that IgM-like molecules were the earliest vertebrate immunoglobulins. The finding of two distinct H chains at the phylogenetic level of lungfishes suggests that different immunoglobulin classes arose with the ascendence of land vertebrates. Although the presence of high and low molecular weight immunoglobulins is well established for sharks, this may not prove to be universally true for members of the class Osteichthyes. Any evolutionary considerations regarding tetrameric and pentameric IgM-like macroglobulins must await further results from cyclostome immunoglobulins. The secretory antibody system in fish has received little study, however its role as a defense mechanism seems obvious. Not so apparent is the role of "normal" immunoglobulin in fish serum.

MATERIALS AND METHODS

Commonly Employed Diluents

Three solutions which were commonly employed during this study are defined below.

1. Phosphate Buffered Saline, pH 7.2 (PBS)

	g/100 ml	Molarity
NaCl	4.38	0.075
KH_2PO_4	2.45	0.018
$Na_2HPO_4 \cdot 7H_2O$	15.28	0.057
H_2O	to volume	

2. Dilute PBS (pH 7.2) prepared by diluting PBS 1:10 with saline.

	g/1000 ml	Molarity
NaCl	8.33	0.1425
KH_2PO_4	0.245	0.0018
$Na_2HPO_4 \cdot 7H_2O$	1.528	0.0057
H_2O	to volume	

3. Saline

	g/1000 ml	Molarity
NaCl	8.77	0.15
H_2O	to volume	

Bacterial Antigen Preparations

Preservation of an *Aeromonas salmonicida* Isolate

An isolate of *A. salmonicida* (AS-67) from the laboratory culture collection was employed throughout this research. The organism was originally isolated in 1967 from the kidney of a juvenile coho salmon which had died of furunculosis at the Siletz River Salmon Hatchery. To avoid maintaining stock cultures, the isolate was initially preserved as lyophilized cell preparations. It was felt that this procedure would minimize the possibility of antigenic variation during the study (Society of American Bacteriologists, 1957).

Prior to lyophilization, the isolate was carried through two animal passages in juvenile coho salmon by Mr. W. D. Paterson of this laboratory. After final reisolation from a coho salmon mortality, the bacterium was cultured in furunculosis broth (Overholser, 1968) at room temperature. Several large slants of Furunculosis Agar[6/] were then inoculated with the turbid broth suspension and incubated for 30 hours at room temperature. Bacterial growth from the slants was suspended in 20 percent skim milk, and approximately 200 lyophilized cultures were prepared from the suspension. Lyophilized cells were stored at -20 C.

[6/] Difco Laboratories, Detroit, Mich.

Preparation of *Aeromonas salmonicida* Whole Cells

The following method was employed in preparing stock suspensions of whole *A. salmonicida* cells. Furunculosis broth was inoculated with a lyophilized cell preparation and incubated on a reciprocal shaker for 24 hours at room temperature. An appropriate number of Furunculosis Agar slants (prepared in 32 oz. prescription bottles) were then each inoculated with 5 ml of the turbid broth and incubated for 24 hours at room temperature. The resultant bacterial growth was harvested, checked for purity, and washed three times (50 to 100 ml sterile PBS per wash for each slant of cells) by centrifugation (0 C, 3300 x g). After the final washing the pelleted cells were resuspended and stored at 4 C in 0.3 percent formalin-PBS (50 ml for each slant of cells). Fresh stock cell suspensions were prepared every 2 weeks.

Whole cells employed for immunization purposes with Freund's Complete Adjuvant (FCA),[7/] were prepared as above except that the washed cells were incubated in 0.4 percent formalin-PBS for 1 hour at room temperature and 3 days at 4 C. The sterility of this suspension was established by inoculation of samples into tubes of Fluid

[7/] Baltimore Biological Laboratories, Baltimore, Md.

Thioglycollate Medium.[8/] The cell concentration was estimated with a Petroff-Hauser Bacteria Counter and then adjusted to 3.5 x 10^{10} cells/ml. Equal volumes of this cell suspension and FCA were emulsified in a Waring Blender. By calculation the immunogen contained 1.75 x 10^{10} cells/ml and had a formalin concentration of 0.13 percent.

Antigen for the macroscopic tube agglutination test was prepared by diluting the stock cell suspension with saline until its optical density (OD) at 520 mμ was 0.7 as measured with a Bausch and Lomb Spectronic 20 (0.5 inch diameter cuvette). This generally required about a 1:10 dilution of the stock cell suspension.

Preparation of *Aeromonas salmonicida* Cell Walls

Purified *A. salmonicida* cell walls were prepared by a method similar to that described by Kabat and Mayer (1961). Growth from as many as 24 Furunculosis Agar slants was harvested, then washed twice in 0.1 M NaCl and twice in distilled water. After the final washing, bacterial cells were resuspended in distilled water (5 ml for each slant of cells) and broken by shaking with glass beads (0.25 - 0.3 mm diameter) in a Braun shaker.[9/] Cells were shaken for

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[8/] Difco Laboratories, Detroit, Mich.

[9/] The Braun Company, Melsungen, Germany.

2.5 minutes with continuous cooling. Glass beads were removed from the disrupted cell suspension by filtration through the fritted support of a Millipore filter apparatus. The majority of whole cells were then removed from the filtrate by centrifugation for 10 minutes at 1500 x g, 0 C. The supernatant fluid from this step was collected and adjusted to a 0.1 M NaCl concentration. Cell walls were pelleted (17,300 x g for 15 minutes at 0 C), washed once in distilled water, and incubated overnight at 4 C in 0.1 percent formalin-water.

After formalinization the cell walls were washed, resuspended in a pronase solution[10/] for 12 hours at room temperature, then washed, formalized, and washed again. Following pronase treatment the cell walls were incubated in RNase[11/] for 3 hours at 37 C. The preparation was completed by a series of washings in 0.1 M NaCl and distilled water. Purified cell walls were stored at 4 C in 0.3 percent formalin-water.

10/ Pronase (B Grade, Calbiochem, Los Angeles, Calif.) at a concentration of 0.1 mg/ml in 0.05 M potassium phosphate buffer (pH 7.6). The solution was filtered through a 0.22μ filter to remove insoluble material.

11/ Ribonuclease (5x crystallized, Sigma Chemical Company, St. Louis, Mo.) at a concentration of 0.5 mg/ml in 0.05 M potassium phosphate buffer (pH 7.6).

Serums From Juvenile Coho Salmon

Experimental Animals

During December of 1969, 6,000 juvenile coho salmon (1968 brood) were transported from the Alsea Salmon Hatchery to the Fish Disease Laboratory, Oregon State University. Fish were held in 3- or 4-foot diameter tanks which received constantly flowing, 13 C well water. Animals were fed a diet of Oregon Moist Pellets (Hublou, 1963) once daily.

Collection and Storage of Juvenile Coho Salmon Serum

Blood was obtained by drip from the caudal artery which was severed posterior to the adipose fin. Small culture tubes were used to collect blood from several animals, then incubated at room temperature for 1 hour, and placed at 4 C for clot retraction. Serum was harvested after centrifugation for 10 minutes (600 x g) at room temperature. Each pooled antiserum was filter sterilized (membrane pore size 0.45μ), and kept as small samples in glass screw-cap containers. The major portion of each pooled antiserum was stored at -60 C, although small amounts were kept at 4 C. Due to an equipment failure early in the study, all frozen serum samples thawed to 20 C. Upon discovery the antiserums were immediately

refrozen. No loss of antibody activity was detected as a result of this accident.

Serums for Reduction and Alkylation Studies

In this experiment 1,500 juvenile coho received the previously described emulsion of *A. salmonicida* cells in FCA. An emulsion of FCA and PBS was administered to a second group of 1,500 animals which served as controls. Fish were anesthetized in a dilute solution of methyl pentynol[12/] and injected intra-abdominally with 0.1 ml of the appropriate emulsion. Pooled serums were prepared from both immunized and control groups prior to injection and at biweekly intervals after initiation of the experiment (Table 1). These antiserums were employed in a study of antibody synthesis and the effects of partial reduction and alkylation on antibody activity.

Pooled Coho Salmon Anti- *Aeromonas salmonicida* Serum

A large pool of coho anti- *A. salmonicida* serum was prepared from 2,500 juvenile salmon which were immunized as before. Each of 500 animals was injected with a PBS-FCA emulsion and served as the source of pooled control serum. Immune and control serums were collected approximately 85 days after immunization

[12/] Airco Chemical and Plastics Co., New York, N. Y.

Table 1. Collection of pooled coho salmon antiserums over the course of antibody synthesis.

	Immunized Group			Control Group		
Day	Blood collected from	Average weight (gm/fish)	Average amount of serum obtained (ml/fish)	Blood collected from	Average weight (gm/fish)	Average amount of serum obtained (ml/fish)
0[a]	-	-	-	200 fish[b]	19.9	0.080
14	100 fish	19.9	0.091	100 fish	18.8	0.087
28	100 fish	19.9	0.098	100 fish	19.8	0.095
42	100 fish	21.5	0.105	100 fish	20.4	0.100
56	100 fish	24.6	0.130	100 fish	24.2	0.123
70	100 fish	25.6	0.135	100 fish	27.5	0.130
84	100 fish	28.6	0.135	100 fish	30.2	0.131
98	100 fish	29.4	0.135	100 fish	31.0	0.145
112	100 fish	31.3	0.155	100 fish	32.5	0.167
126	100 fish	34.3	--[c]	100 fish	33.2	--[c]
140	100 fish	34.5	--[c]	100 fish	35.1	--[c]

[a] All animals injected on day zero (12/27/69). [b] Blood was collected from 200 uninjected animals.
[c] Data not collected.

and stored as previously described. At the time of serum collection, the animals had an average weight of 28.1 gm.

Rabbit Antiserums

Three antiserums used in this study were raised in young female, New Zealand white rabbits. Rabbit and salmon blood were processed identically except for initial clotting temperature (37 C for 1 hour with rabbit blood). Each rabbit antiserum is described below.

Rabbit Anti- *Aeromonas salmonicida* Serum

Rabbit anti- *A. salmonicida* serum was obtained from Mr.W.D.Paterson of this laboratory. It was prepared by two subcutaneous inoculations of *A. salmonicida* cells in FCA and a series of intravenous (i.v.) injections of bacterial cells in saline. The serum employed in this study was obtained 5 weeks after initial stimulation. It had a 1:5,120 agglutination titer, and could precipitate *A. salmonicida* endotoxin in agar gel.

Rabbit Antiserum Against Coho Salmon Serum

Pooled normal juvenile coho salmon serum was precipitated on alum by the method of Proom (1943) and injected into two rabbits according to the following schedule:

1st day - 5 ml alum-precipitated serum injected intramuscularly (i. m.) into each buttock.

16th day - 5 ml alum-precipitated serum injected i. m. into each buttock.

26th day - 1 ml of serum injected intraperitoneally (i. p.).

37th day - approximately 50 ml blood collected from each rabbit.

43rd day - approximately 50 ml blood collected from each rabbit.

Both rabbits were given a second set of injections approximately 11 months after the initial series. Immunoelectrophoretic comparisons revealed that antiserums collected after the second series of injections were superior to those obtained from primary stimulations. The more potent antiserum was employed in this study.

Rabbit Antiserum Against the *Aeromonas salmonicida* Immune Complex

Rabbit antiserum was prepared against salmon serum proteins which could bind to *A. salmonicida* cells. A method similar to that described by Dubiski *et al.* (1959) was followed. Stock *A. salmonicida* cells were washed twice in saline and then adjusted to a concentration which when diluted 1:5 had an OD of 0.7 at 520 mμ. Cells from 3 ml of this suspension were pelleted, resuspended in 0.6 ml of coho anti- *A. salmonicida* serum (1:10,240 agglutination titer), and incubated for 1 hour at room temperature. The resulting agglutinate

was washed three times in the cold, resuspended in 2.5 ml of saline, and immediately injected into the rabbit. The immunization schedule consisted of 15 total injections administered on alternate days over a 30 day period (4 i.v., 1 i.p., 4 i.v., 1 i.p., 4 i.v., 1 i.p.). Blood was collected 10 and 16 days after the last injection.

The Determination of Nitrogen and Dry Weight

Nitrogen was determined by the Nessler reaction after Kjeldahl digestion as described by Chase and Williams (1968). Nessler reagent[13/] (Koch-McMeekin Formula) was prepared according to the directions supplied with the purchased compound. The standard nitrogen solution (0.5 mg nitrogen/ml) was obtained from Sigma.[14/] The OD values of all samples were determined at 440 mμ in the same cuvette (0.5 inch diameter) with a Spectronic 20. Prior to any use of the technic, for research purposes, three separate nitrogen determinations were performed with graded amounts of the standard $(NH_4)_2SO_4$ solution (10μgm to 300μgm). An excellent linear relationship was obtained between nitrogen concentration and OD at 440 mμ (correlation coefficient from regression was 0.999). Protein was estimated from nitrogen by the standard conversion factor of 6.25.

[13/] Nessler Compound from Scientific Products, Evenston, Ill.

[14/] Sigma Chemical Co., St. Louis, Mo.

Dry weights were determined after drying samples to constant weight *in vacuo* at 63 C over silica gel.

The Estimation of Anti- *Aeromonas salmonicida* Antibody

Semi-Quantitative Measurement of Agglutinins

The relative concentration of anti- *A. salmonicida* antibody was determined by the macroscopic tube agglutination test (Kolmer, Spaulding and Robinson, 1951). The antigen for this test was described previously. Titrated samples were incubated for 2 hours at room temperature and 16 to 24 hours at 4 C. Agglutination titer was expressed as the dilution of sample at the endpoint of the titration. The endpoint of each titration was determined from macroscopic settling patterns of bacteria which were similar to those of erythrocytes in the passive hemagglutination test (Campbell *et al.* 1964). A reference antiserum was included whenever antibody titrations were performed; in all cases the reference antiserum displayed the expected titer.

It was often more convenient to express the results from this method in terms of agglutinating units rather than agglutination titer. In this study an agglutinating unit was defined as the minimum amount of antibody required to agglutinate the bacterial cells in 1 ml of a 1:2 dilution of the standard antigen suspension. From this definition

the number of agglutinating units in any solution could be calculated by the following formula:

$$\text{agglutinating units} = (\text{mls of solution})\,(\text{agglutination titer}^{-1})$$

For example, in 10 ml of a solution with an anti- A. salmonicida agglutination titer of 1:80 there are 800 agglutinating units. In 1 ml of any solution the number of agglutinating units is equal to the reciprocal of the agglutination titer.

An agglutination inhibition test was employed to detect the presence of nonagglutinating antibody. Bacterial cells in each dilution of the macroscopic tube agglutination test were pelleted at 0 C and the supernatant fluids were carefully removed. The cells in each dilution tube were resuspended in 1 ml of a 1:15,000 dilution of coho anti- A. salmonicida serum[15/] and incubated as in the macroscopic tube agglutination test. In the inhibition test the absence of agglutination was taken as evidence that the cells had been initially coated with nonagglutinating antibody or antibody fragments.

Quantitative Agglutinin Test

The quantitative agglutinin test (Kabat and Mayer, 1961) was employed to estimate the concentration of anti- A. salmonicida antibody in the pooled immune coho serum. Freshly washed bacterial

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15/ This antiserum had an agglutination titer of 1:20,480.

cell walls were employed rather than whole cells. The cell walls were suspended in saline to an OD of 0.5 at 520 mμ (approximately 80 μgm nitrogen/ml). Freshly centrifuged coho antiserum was diluted 1:10 with saline. The cell walls from 2.0 ml of the above suspension were incubated in 1.0 ml of the antiserum dilution for 1 hour at room temperature and 24 hours at 0 C. Agglutinates and blanks were washed three times with cold saline. Nitrogen was determined by the Nessler reaction as previously described. The anti- A. salmonicida antibody concentration was calculated from the nitrogen (N) data as follows:

$$\frac{\text{mg antibody}}{\text{ml}} = (6.25)(10)\ N_{\text{agglutinate}} - N_{\text{cell wall blank}} - N_{\text{serum blank}}$$

The determination was performed in duplicate.

The Effect of Heat on Antibody Activity in Serum

It was of interest to examine the loss of anti- A. salmonicida agglutinating activity after exposure of antiserum to various temperatures for periods of 30 minutes. The temperatures employed were maintained with constant temperature blocks (temperature control ± 0.5 C) and ranged from 35 to 75 C at 5 C increments. All determinations were performed twice in duplicate. Two 13 x 100 mm

Wasserman tubes were equilibrated in each temperature block. An appropriate amount of pooled immune coho serum was diluted 1:10 with dilute PBS. One ml amounts of the serum dilution were simultaneously added to each duplicate set of tubes which were then sealed with parafilm to prevent evaporation. After incubation the tubes were cooled in ice. Antibody activity was determined by the macroscopic tube agglutination test as previously described.

The Effect of Partial Reduction and Alkylation on Antibody Activity

Samples of the previously described coho salmon and rabbit anti- *A. salmonicida* serums were reduced and alkylated by a procedure similar to that of Deutsch and Morton (1957). All serums were diluted 1:10 in PBS prior to treatment. Reduction was performed by dialysis against 33 volumes of 0.1 M 2-mercaptoethanol in PBS (pH 7.2) for 24 hours at room temperature. The reduced sample was alkylated by dialysis against 33 volumes of 0.02 M iodoacetamide in PBS (pH 7.2) at 4 C for 48 hours. Samples were also reduced and alkylated as above and then were dialyzed against 2 M urea in PBS for 12 hours at room temperature. As controls, unreduced samples were dialyzed against both PBS and 2 M urea in PBS. Prior to determination of agglutination titers, all samples were dialyzed against four changes of PBS (12 hours each) at 4 C.

The above procedures were also employed with purified coho anti- *A. salmonicida* antibodies.

Sephadex Column Chromatography

Sephadex G-200 gel filtration medium was prepared for chromatography according to instructions supplied by Pharmacia.[16/] The swollen gel in dilute PBS was poured into a column (2.5 x 45 cm) at 4 C. One ml of freshly centrifuged coho anti- *A. salmonicida* serum was fractionated. The flow rate was approximately 8 ml/hour, and 4 ml fractions were collected with an automatic fraction collector. The OD of each fraction at 280 mμ was determined with the Beckman DU-2 spectrophotometer (sensitivity setting , 3.0). Each fraction was analyzed by an abbreviated agglutination test, and fractions containing activity were titrated by the macroscopic tube agglutination method.

Isolation of Coho Salmon Anti- *Aeromonas salmonicida* Antibodies

Coho anti- *A. salmonicida* antibodies were isolated by a batch procedure from whole cell and cell wall immunoadsorbents. In the end analysis, the work with whole cells was only of importance in selecting an effective antigen-antibody dissociating agent. All

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16/ Pharmacia Fine Chemicals, Piscataway, N. J.

purified antibody solutions were prepared from cell wall adsorbents.

Selection of an Effective Antibody-Antigen Dissociating Agent

Four solutions were tested for their ability to reverse the coho antibody-antigen complex. These solutions were: (1) 3 M NaSCN in PBS, (2) 3 M urea in PBS, (3) 15 percent NaCl dissolved in 0.075 M phosphate buffer, pH 7.2 and, (4) a pH 3.0 glycine-HCl buffer (0.445 M glycine).

Freshly prepared *A. salmonicida* cells were repeatedly washed and resuspended to a concentration which when diluted 1:20 had an OD of 0.7 at 520 mμ. This suspension was divided into five aliquots. Cells from each of four aliquots were repeatedly washed in one of the four antigen-antibody dissociating solutions. Cells in the fifth aliquot were untreated and served as a rough control for evaluating the ability of treated cell preparations to adsorb antibody from immune serum. Prior to reaction with antibody, each aliquot of cells was washed in PBS and resuspended to its original volume.

Cells from 5 ml of each suspension were pelleted then resuspended in 0.5 ml of coho anti- *A. salmonicida* serum. After incubation for 2 hours at room temperature, the resulting agglutinates were pelleted, and each supernatant fluid was titrated for antibody activity. Agglutinates were repeatedly washed in cold dilute PBS until the OD of supernatant fluids was less than 0.05 at 280 mμ.

After final washing each agglutinate was resuspended in 5 ml of the appropriate antibody-antigen dissociating agent and incubated for 90 minutes at room temperature. The mixtures were then centrifuged and the supernatant fluids collected. After exhaustive dialysis against PBS, the four solutions were tested for antibody activity, OD at 280 mμ and 260 mμ, and electrophoretic properties in alkaline polyacrylamide gels.

Antibody Isolation From Cell Wall Immune Complexes

The basic approach of the following method is similar to that employed by other workers for isolation of specific antibodies against insoluble particulate antigens (Kabat and Mayer, 1961). The details of the procedure were the end result of minor modifications throughout this study. Unless otherwise stated all centrifugations were performed at 0 C.

Prior to antibody isolation antiserum was repeatedly centrifuged for 30 minute periods (12,100 x g) as in the quantitative agglutinin test. This procedure removed lipid and sediment from the serum.

Cell walls were freshly washed in saline (12,100 x g for 15 minutes) and then extracted for four 1 hour periods at 37 C in the 15 percent NaCl solution. As an aid in pelleting the cell walls after each extraction period, it was helpful to dilute the salt concentration to 7.5 percent with distilled water. Cell walls were pelleted by 20 minutes centrifugation at 17,300 x g. After extraction, the cell walls were washed twice in distilled water, once in saline and then resuspended in saline to an

OD of 0.5 at 520 mμ (0.5 inch diameter cuvette).

Prior to reusing a given batch of cell walls for a second antibody isolation, bound antibody was removed by incubation in the previously described pronase solution. The cell walls were then repeatedly washed and formalized. To prepare the "repurified" cell walls for a second antibody isolation, they were treated as above; however, only a single extraction with 15 percent NaCl was performed.

As a rule cell walls from 20 ml of suspension were reacted with each ml of coho antiserum. The mixture was incubated for 1 hour at 24 C with constant agitation, then diluted 10-fold with cold saline, and incubated an additional hour in ice.

The cell wall antibody complex could be completely pelleted by centrifugation at 3,020 x g for 10 minutes. Greater centrifugal forces resulted in pellets which were difficult to resuspend. The agglutinate was washed with cold saline until supernatant fluids gave OD values of about 0.01 at 280 mμ; this generally required three to four washings.

The washed agglutinate from each ml of antiserum was suspended in 10 ml of the 15 percent NaCl solution, incubated at 37 C for 1 hour with occasional mixing, and centrifuged at 30 to 37 C for 15 minutes, 17,300 x g. Supernatant fluids containing released antibody were harvested and pooled.

Salt was removed from the antibody solution by dialysis[17/] in the cold, against saline containing a drop of toluene (Kabat and Mayer, 1961). Ultrafiltration in the cold with a Diaflow XM-50 membrane[18/] was employed for concentration purposes and as a rapid means of removing any low molecular weight contaminants. This was done by repeatedly (three to four times) concentrating the antibody solution then diluting it to 50 ml with saline. The final antibody solution (generally about 0.2 mg/ml) was filter sterilized (0.22μ Millipore filter) and stored at 4 C. In experiments where higher protein concentrations were required, negative pressure ultrafiltration through 1/4 inch dialysis tubing, or dialysis against 10 percent Ficol[19/] in 0.15 M NaCl was employed.

Analytical Ultracentrifugation

Sedimentation velocity measurements were performed in a model E analytical ultracentrifuge[20/] equipped with a photoelectric scanner. During the experiment the temperature was held at 20 C

17/ Prior to use, dialysis tubing was extracted twice with boiling distilled water.

18/ The Amicon Corporation, Cambridge, Mass.

19/ Pharmacia Fine Chemicals, Piscataway, N. J.

20/ Spinco Division, Beckman Instruments Inc.

and the calculated rotor speed was 30,266 RPM. Each cell was scanned (280 mμ) at 6 minute intervals. Sedimentation coefficients of immunoglobulins in 0.15 M NaCl were calculated on a Control Data Corporation 3300 computer, then corrected for viscosity and density to give $S_{20,W}$ values.

Immunodiffusion and Immunoelectrophoresis

Immunodiffusion was carried out on microscope slides with the materials described by Campbell *et al.* (1964). A thin layer of molten 2 percent agar was spread on each slide and allowed to dry. Slides were then covered with 2.5 ml of the molten 1 percent buffered gel mixture. Wells (2 mm diameter) were cut in the agar with a capillary tube according to a symmetrically designed pattern. The centers of adjacent wells were 6 mm apart. The *A. salmonicida* endotoxin (TCA extract of whole cells) employed in immunodiffusion was obtained from Mr. W. D. Paterson of this laboratory. This material (10 mg/ml) was allowed to diffuse for 5 hours before the other reagents were added to their respective wells.

Immunoelectrophoresis was performed by the microtechnic (Scheidegger, 1955). The detailed procedure supplied with the commercial immunoelectrophoresis outfit[21/] was slightly modified to meet the needs of the problem. Initial trials indicated that separation

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21/ Shandon Scientific Co. Inc., Sewickley, Pa.

of coho serum at pH 8.2 (barbital buffer, ionic strength 0.05) resulted in migration of all components toward the anode. The desired anodic and cathodic migration of the coho serum proteins was obtained in gels buffered with a pH 7.5 phosphate buffer (ionic strength 0.05).[22/]

This buffer was prepared in 1X concentration by adding 7.3 ml of 0.5 M KH_2PO_4 to 30.9 ml of 0.5 M K_2HPO_4, then bringing the solution to 1,000 ml total volume with distilled deionized water. The gel was prepared by mixing equal volumes of molten 2 percent Ionagar[23/] in water with hot 2X potassium phosphate buffer then adding merthiolate to a final concentration of 0.01 percent. The mixture was filtered by suction through Whatman No. 1 filter paper, cooled to 60 C, then poured and spread over microscope slides to form the agar gel layer. A pattern cutter, supplied with the immunoelectrophoretic apparatus, was employed to cut the necessary wells and troughs in the agar.

Prior to electrophoresis the small wells were filled twice with coho serum samples. Electrophoresis was conducted for 3 hours at 4 C with a constant potential of 125 volts across the electrodes. Undiluted antiserums were employed for development of precipitin

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22/ Mr. D. Anderson, Immunologist, Western Fish Disease Laboratory, Seattle, Wash., suggested the use of phosphate buffers.

23/ Ionagar No. 2, Consolidated Laboratories Inc., Chicago Heights Ill.

arcs except for the rabbit anti- A. salmonicida immune complex serum which was diluted 1:2 with saline. Precipitin arcs were allowed to develop for 20 to 24 hours at room temperature. The slides were then washed, dried, and stained with amido black as described by Shang (1966). During the drying procedure the agar gel was covered with wet Whatman No. 1 filter paper to prevent distortion.

Ultraviolet Absorption Spectroscopy

Ultraviolet (UV) absorption spectroscopy was employed for the evaluation of antibody purity and reactivity. All work was performed with a Beckman DU-2 spectrophotometer. The sensitivity control of the instrument was maintained at a setting of 3.0. Extinction coefficients and UV absorption spectra were determined in standard 10 mm path length rectangular cells. A matched set of 100 μl cells (10 mm path length) was employed for measuring the amount of protein removed from antibody solutions after adsorption with A. salmonicida cell walls.

Extinction coefficients ($E_{1cm}^{1\%}$) at 280 mμ were determined for two coho antibody solutions and a human IgG solution (all in 0.15 M NaCl). After OD measurements at 280 mμ, the protein concentration of each solution was determined from duplicate nitrogen analyses as previously described.

Absorption characteristics of antibody solutions were an

important test of purity since bacterial nucleic acids were a source of contamination. The OD values of freshly isolated antibody solutions were measured at certain critical wavelengths (251, 260, 276, 278, 280, and 320 mμ). In addition the detailed absorption spectrum of one purified antibody solution was determined between 240 mμ (slit width, 0.28 mm) and 330 mμ (slit width , 0.21 mm) at 2 mμ increments.

In purified solutions the amount of active antibodies was determined by measuring the OD loss at 280 mμ after adsorption of samples with A. salmonicida cell walls. A solution of human IgG was included as a control for nonspecific adsorption. Prior to adsorption with cell walls, the OD of each immunoglobulin solution was measured in an assigned microcell. One ml of each protein solution and 1 ml of saline were then adsorbed with the cell walls from 4 ml of a washed suspension (OD 0.4 at 520 mμ). The reaction mixtures were incubated for 1 hour at room temperature and 1 hour in ice. Cell walls were pelleted at 0 C and the OD of each solution was measured at 280 mμ. To determine the amount of antibody activity removed, the agglutination titer of each solution was determined before and after adsorption.

Electrophoresis in Polyacrylamide Gels

An electrophoresis unit made especially for slab gel

electrophoresis was employed in these studies.[24/] The design of the unit made it possible to continuously cool the acrylamide slab during electrophoresis.

Acrylamide, N, N'-methylenebisacrylamide (BIS), N, N, N', N'-tetramethylethylenediamine (TEMED) and riboflavin were obtained from Eastman.[25/] Tris (hydroxymethyl) aminomethane (Tris) was obtained from Eastman and Sigma.[26/] Reagent grade ammonium persulfate and urea were from Matheson Coleman and Bell.[27/] Sodium dodecyl sulfate (SDS) was obtained from Nutritional Biochemicals.[28/] Both NaH_2PO_4 and $Na_2HPO_4 \cdot 7H_2O$ were reagent grade.

Alkaline Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis at pH 8.9 was employed as a criterion of antibody purity and for examining various coho serums.

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

25/ Eastman Organic Chemicals, Division of Eastman Kodak Co., Rochester, New York.

26/ Sigma Chemical Co., St. Louis, Mo.

27/ Matheson Coleman and Bell, Division of Matheson Co., Norwood, Ohio.

28/ Nutritional Biochemicals Corporation, Cleveland, Ohio.

The original method of Davis (1964) was followed in preparing stock solutions, working solutions, and gels. Coho serum diluted 1:20 or other samples were layered under the upper buffer in a 10 percent sucrose solution. Electrophoresis was conducted with a constant potential of 200 volts across the electrodes of the unit (approximately 28 volts/cm of gel). The separation was stopped when the marker dye (Bromphenol blue) reached the bottom of the gel (approximately 2 hours). During electrophoresis the unit and gel slab were cooled with constantly flowing water from an ice bath. Gels were stained with Coomassie brilliant blue[29/] in 12.5 percent trichloroacetic acid (TCA) (Chrambach *et al.* 1967). After destaining in 10 percent TCA the gels were soaked in distilled water and stored in 7 percent acetic acid.[30/]

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis

The SDS-polyacrylamide gel electrophoresis technic was employed for estimating molecular weights. In this study the method was performed as described by Dunker and Rueckert (1969) with some modifications. Specifications for the 5 percent acrylamide-SDS

29/ Colab Laboratories, Inc., Chicago Heights, Illinois.

30/ To insure that this was the actual acetic acid concentration, gels were soaked in one or two changes of 7 percent acetic acid prior to storage. In some cases when this was not done, mold-like growth appeared on the gel surface.

gel and the reservoir buffers were employed to formulate the solutions shown in Table 2. The ammonium persulfate solution was freshly prepared for each determination. Gel solutions 2 and 3 were stored at 4 C and used within 4 days of preparation.

The following proteins were employed in the standardization procedure:

1. Lysozyme (3x crystalline) Nutritional Biochemicals Corporation, Cleveland, Ohio.

2. Trypsin (2x crystallized)Type I, Sigma Chemical Co., St. Louis, Mo.

3. α-Chymotrypsin (3x crystallized) from bovine pancreas, Sigma Chemical Co., St. Louis, Mo.

4. Pepsin (2x crystallized) Nutritional Biochemicals Corporation, Cleveland, Ohio.

5. Bovine Albumin (crystallized) Research Products Division, Miles Laboratories, Inc., Kankakee, Ill.

6. Human Immunoglobulin G (DEAE ion exchange fraction) Research Products Division, Miles Laboratories, Inc., Kankakee, Ill.

Modifications of two procedures (Edelman and Marchalonis, 1967) were employed to reductively cleave human IgG into its polypeptide chains. Soluble chain preparations were obtained by partial reduction and alkylation in saline. Human IgG (~2 mg/ml) was reduced for 4 hours at room temperature in a solution of 0.1 M 2-mercaptoethanol and 0.15 M NaCl (pH adjusted to 8.0 with 1 M Tris). Alkylation was performed by addition of solid iodoacetamide

Table 2. Solutions for preparing 0.1 percent SDS, 5.0 percent polyacrylamide gels.

Gel Solution Number	Number of Volumes	Substance	Amount
1	0.05	Ammonium Persulfate	0.25 g
		H_2O	to 25 ml
2	0.45	Acrylamide	11.11 g
		Bis	0.384 g
		H_2O	to 100 ml
3	0.50	SDS	0.2 g
		TEMED	0.2 ml
		NaH_2PO_4	0.672 g
		$Na_2HPO_4 \cdot 7H_2O$	3.86 g
		H_2O	to 100 ml[a]
Reservoir buffer (2X):		SDS[b]	2.0 g
		NaH_2PO_4	6.7 g
		$Na_2HPO_4 \cdot 7H_2O$	38.6 g
		H_2O	to 1000 ml[a]

[a] Adjust pH to 7.2 before bring to volume.

[b] SDS omitted from the lower reservoir buffer.

to a concentration of 0.2 M (pH held at 8.0 with 1 M Tris). After 10 minutes at room temperature the solution was exhaustively dialyzed against 0.15 M NaCl in the cold, then filter sterilized and stored at 4 C. Extensively reduced and alkylated IgG chains were also prepared. Human IgG in saline (~2 mg/ml) was dialyzed into 8 M urea then reduced against 50 volumes of 0.1 M 2-mercapto-ethanol in 8 M urea (pH 8.0 with 1 M Tris) for 24 hours at room temperature. Alkylation was by dialysis against 50 volumes of 0.05 M iodoacetamide in 8 M urea (pH 8.0 with 1 M Tris) for 24 hours at 4 C. The solution was then exhaustively dialyzed against 8 M urea at 4 C with continuous stirring of the dialyzate.

Proteins and polypeptide chain preparations were prepared for electrophoresis as described by Dunker and Rueckert (1969). Lysozyme, trypsin, pepsin, and bovine serum albumin (BSA), all at a concentration of approximately 0.1 mg/ml, were incubated for 45 minutes at 45 C in a solution containing 1 percent 2-mercapto-ethanol (v/v), 4 M urea, and 1 percent SDS. Human IgG, the reduced and alkylated IgG chain preparations, and α-chymotrypsin were treated as above, except that the solution contained 0.002 M iodoacetamide instead of 2-mercaptoethanol.

In a typical determination 0.2 ml of each sample was layered between the upper buffer and an assigned gel space. A potential of 30 volts (approximately 4 volts/cm of gel) was applied across the

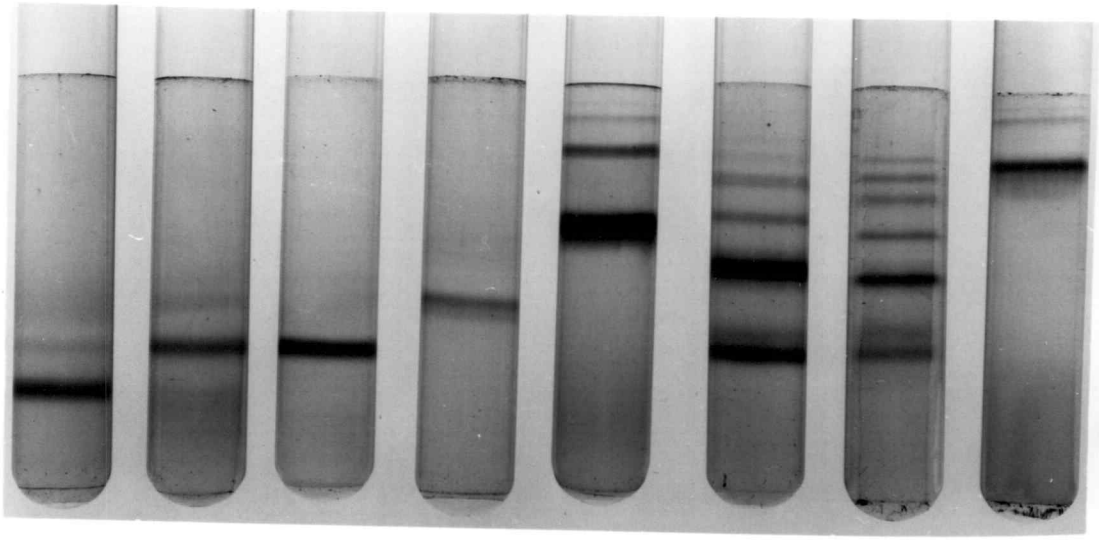
electrodes for 4.5 hours. Throughout the separation the unit was kept cool with flowing tap water (~20 C). After electrophoresis the gel slab was cut appropriately and processed through two changes (12 hours each) of 10 percent TCA to fix proteins and leach out SDS. Gels were stained for 10 to 12 hours with 0.02 percent Coomassie brilliant blue in 12.5 percent TCA, and then destained for 2 to 4 hours in 10 percent TCA. After destaining, migration distances were measured from the top of the gel to the center of each band.

The internal calibration technic (Dunker and Rueckert, 1969) was employed for determining the relative mobility of each marker α-Chymotrypsin was chosen as the standard and thus assigned a relative mobility of 1.00. To obtain the relative mobility of a protein, its migration distance was divided by the migration distance of an immediately adjacent sample of α-Chymotrypsin. For convenience, relative mobilities were taken to three places past the decimal, and molecular weights were computed to the nearest 100 daltons. This degree of precision is certainly beyond that of the method.

All information obtained from constructing the molecular weight standard curves is presented below. This is done so that the results obtained from salmon antibodies can be presented later in an uninterrupted manner.

Figure 2 shows the different markers considered in preparing standard curves. Lysozyme, trypsin, α-Chymotrypsin, and pepsin

Figure 2. Stained SDS-acrylamide gels showing the proteins employed as molecular weight markers: A, lysozyme; B, trypsin; C, α-chymotrypsin; D, pepsin; E, bovine serum albumin; F, human IgG (extensively reduced and alkylated); G, human IgG (partially reduced and alkylated); H, human IgG. Each protein's actual and apparent molecular weights are given in Table 3.



A B C D E F G H

preparations (A to D respectively) each contributed a single point to the final standard curve. Reduced bovine albumin showed the monomer, dimer, and trimer forms of this protein. These seven nonimmunoglobulin markers displayed a good linear relationship between the logarithms of their known molecular weights and their relative mobilities.

The observation that partially reduced human IgG samples contained six components was unexpected (Figure 2, G). The mobilities of the two fastest moving bands identified them as the heavy (H) and light (L) chains of the molecule. Further analysis of the six bands led to the conclusion that during the reduction step of the procedure not all the interchain disulfide bonds had been cleaved. As a result, both chains (H and L), the three possible chain combinations (H-L, H-H, and H-H-L) and the intact molecule (L-H-H-L) were present. This interpretation was strongly supported by the linearity between the six bands when each one's measured relative mobility was graphed against its predicted molecular weight as expressed by a semilogarithmic plot (Figure 3). The curve obtained from the six IgG bands was quite similar to that from the seven nonimmunoglobulin proteins and a single standard curve was prepared from all 13 markers (Figure 4, Standard Curve A). The regression equation for the 13 markers was designated as Standard Equation A and is given below (M = molecular weight, m_r = relative mobility).

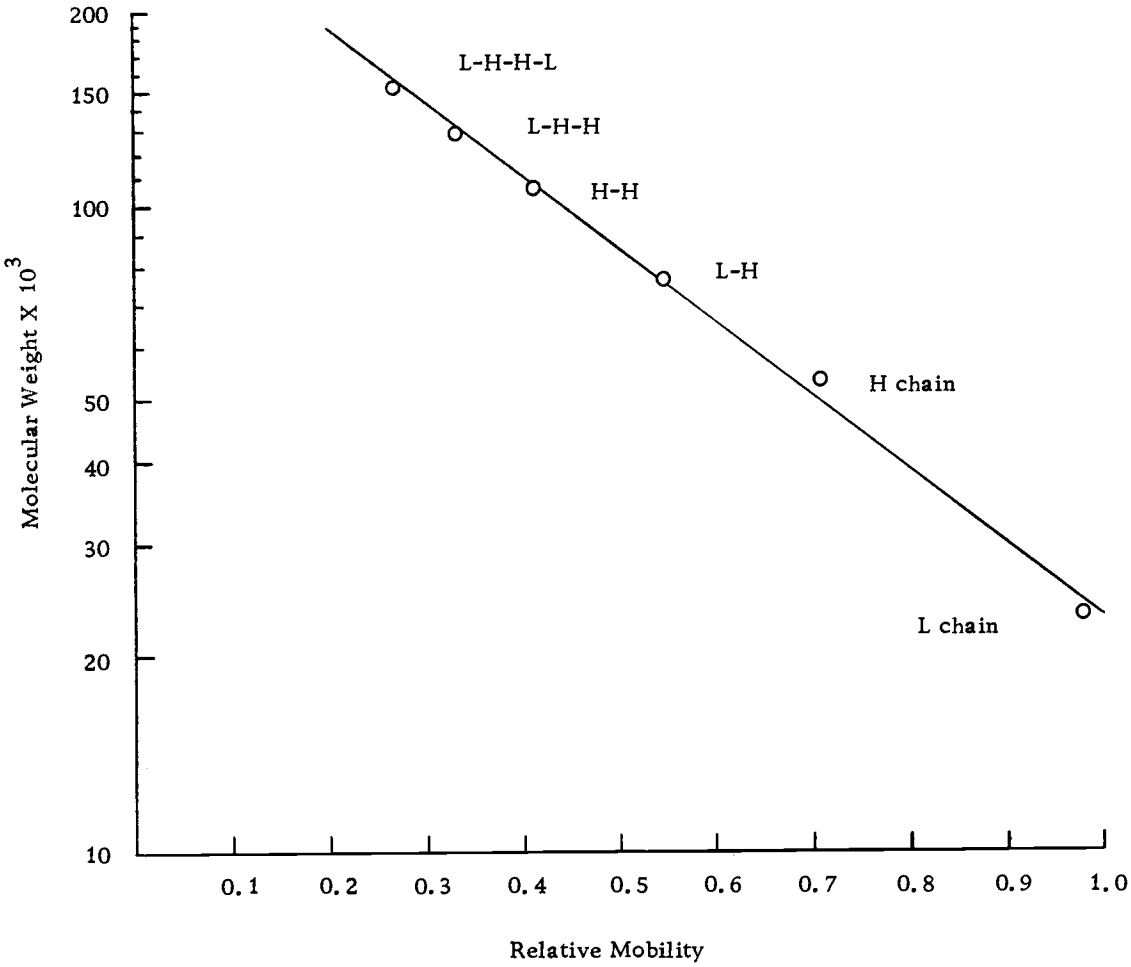


Figure 3. Semilogarithmic plot of molecular weight vs. relative mobility showing the relationship of the six components in samples of partially reduced and alkylated human IgG (G, Figure 2).

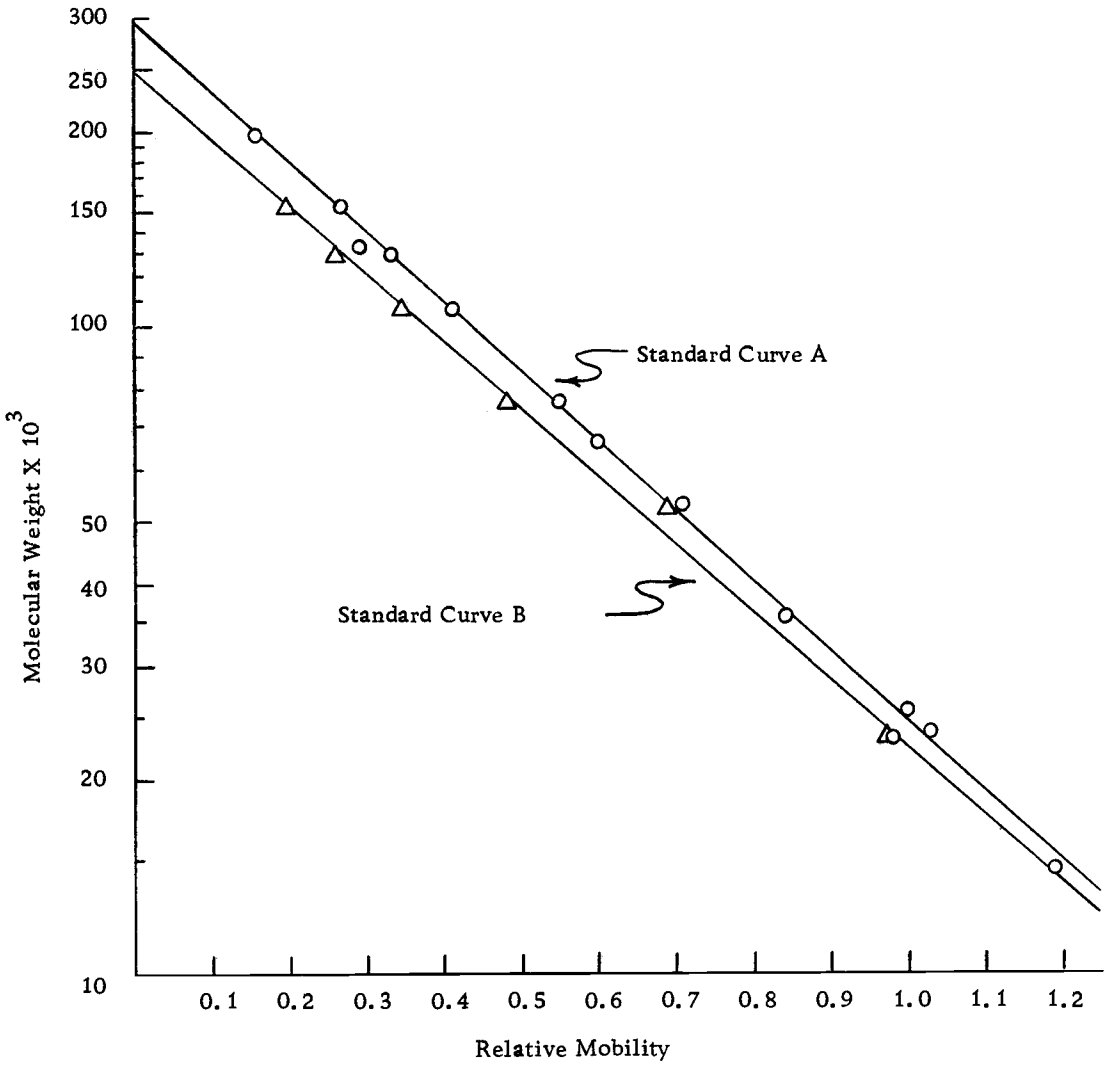


Figure 4. Semilogarithmic plots of Standard Equation A and Standard Equation B. The coordinates of each point are given in Table 3.

Standard Equation A: $\log M = -1.08852\ m_r + 5.46993$

Samples of extensively reduced and alkylated human IgG also displayed six components in freshly stained gels. The relative mobilities for each of these bands were less than those obtained from the corresponding bands in the partially reduced IgG preparation. In spite of the mobility differences, the six bands from the extensively reduced preparation also appeared to represent the two polypeptide chains, the three chain combinations, and the intact molecule. Using the same type of analysis as above, the six bands displayed an excellent linear relationship to each other when each band's measured mobility was plotted against the logarithm of its predicted molecular weight (see Standard Curve B, Figure 4). The six bands from the extensively reduced and alkylated IgG preparation were considered as markers for a second standard curve when it was found that the salmon antibody chains displayed the same sort of preparation dependent mobilities. The equation for this curve was designated as Standard Equation B.

Standard Equation B: $\log M = -1.04676\ m_r + 5.39088$

The explanation for the mobility differences between corresponding bands in the two IgG preparations most likely rests on the effects of reductive cleavage in saline and in a denaturing solvent (Edelman and Marchalonis, 1967). Reduction in saline results in the cleavage

of mainly interchain disulfide bonds. In contrast both interchain and intrachain disulfides are cleaved by reduction in a denaturing solvent. The cleavage of intrachain disulfides would allow the SDS-protein complex to assume a more extended shape (Reynolds and Tanford, 1970b). This would increase the frictional resistance on the particle as it moved through the porous gel, and therefore decrease the relative mobility.

Table 3 summarizes the data employed for calculating both standard equations. Apparent molecular weights were calculated for each marker by substituting the marker's average relative mobility into each standard equation.

From Table 3 it can be seen that both equations were about equally effective in predicting the molecular weights of their own markers. In contrast large errors occurred, especially at higher molecular weights, when the mobilities from one set of markers were substituted into the other standard equation. Analysis of the data from the IgG samples indicated that on a percent-error basis the molecular weight estimates for the different IgG chain combinations were better than the estimates obtained from individual chains. This is at least partially the result of obtaining the standard equation by linear regression. Since this statistical method tended to minimize the average difference between apparent and actual molecular weights, one would expect higher percent errors in the lower

Table 3. A summary of the molecular weight data obtained from the marker proteins.

Protein or polypeptide chain(s)	Number of independent determinations	Ave. relative mobility m_r	Actual molecular weight M	Standard equation A		Standard equation B	
				Apparent M	Error Δ M	Apparent M	Error Δ M
For Standard Equation A							
lysozyme	2	1.188	14,400[b]	15,200	800	14,000	400
L chain (IgG)	3	0.979	23,000[c]	25,400	2,400	23,200	200
trypsin	2	1.025	23,800[b]	22,600	1,200	20,800	3,000
α-chymotrypsin	-[a]	1.000	25,200[b]	24,100	1,100	22,100	3,100
Pepsin	2	0.843	35,500[b]	35,700	200	32,200	3,300
H chain (IgG)	3	0.710	53,000[c]	49,800	3,200	44,400	8,600
BSA-monomer	2	0.603	66,000[b]	65,100	900	57,500	8,500
L-H chains (IgG)	3	0.548	76,000[d]	74,700	1,300	65,700	10,300
H-H chains (IgG)	3	0.414	106,000[d]	104,500	1,500	90,700	15,300
H-H-L chains (IgG)	3	0.334	129,000[d]	127,800	1,200	110,000	19,000
BSA dimer	2	0.291	132,000[b]	142,300	10,300	122,000	10,000
IgG(L-H-H-L chains)	3	0.269	152,000[d]	150,400	1,600	128,600	23,400
BSA trimer	2	0.157	198,000[b]	199,100	1,100	168,500	29,500
Average					2,100		10,400
For Standard Equation B							
L chain (IgG)	3	0.967	23,000[c]	26,100	3,100	23,900	900
H chain (IgG)	3	0.669	53,000[c]	55,200	2,200	49,100	3,900
L-H chains (IgG)	3	0.481	76,000[d]	88,400	12,400	77,200	1,200
H-H chains (IgG)	3	0.345	106,000[d]	124,300	18,300	107,100	1,100
L-H-H chains (IgG)	3	0.263	129,000[d]	152,600	23,600	130,500	1,500
IgG (L-H-H-L chains)	3	0.199	152,000[d]	179,200	27,200	152,300	300
Average					14,500		1,500

[a] Since α-chymotrypsin was used as the internal standard, the number of determinations is meaningless.

[b] From Dunker and Rueckert (1969), value for α-chymotrypsin was calculated from the molecular weight of chymotrypsinogen A.

[c] From Cohen and Milstein (1967).

[d] Predicted from values for L and H chains.

molecular weight markers. This suggested that the molecular weight of an immunoglobulin H chain could be effectively estimated from the H-H dimer. The same holds true for estimating the molecular weight of the H-L chain combination from L-H-H-L. These points were considered in analysis of data from the salmon immunoglobulin.

Salmon immunoglobulin was prepared for electrophoresis by various procedures. Samples (0.1 to 0.2 mg/ml) were reduced or alkylated in the presence of 4 M urea and SDS by incubation with the previously mentioned solutions (Dunker and Rueckert, 1969). Partially reduced and alkylated immunoglobulin was prepared in 0.15 M NaCl (pH 8.0) as previously described and also by the dialysis method employed for reduction and alkylation of antiserum (Deutsch and Morton, 1957). Samples were extensively reduced and alkylated in 8 M urea or 7 M guanidine HCl by the previously mentioned dialysis procedure.

The proportion of H to L chains in the intact immunoglobulin was estimated from their respective band densities and molecular weight ratios (Marchalonis, 1969). Stained gels were scanned with a Beckman DU spectrophotometer attached to a Gilford linear transport system and recorder.[31/] Results were obtained from gels

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[31/] Mr. E. Albin, Dept. of Biochemistry and Biophysics, Oregon State University, provided invaluable assistance in these determinations.

stained with amido black (Davis, 1964) after SDS removal by prolonged leaching in 7 percent acetic acid. From limited attempts it seemed that this staining method resulted in a more uniform background than that obtained with Coomassie brilliant blue in TCA.

Polyacrylamide Gel Electrophoresis in the Presence of Urea

Electrophoresis in the presence of urea was performed by the method of Reisfeld and Small (1966). Samples of human IgG and coho antibody were extensively reduced and alkylated in 8 M urea as previously described. Electrophoresis was for 2.75 hours with a constant potential of 200 volts (approximately 28 volts/cm across the gel). During the electrophoretic separation, the unit was cooled with flowing tap water (~20 C). After electrophoresis, proteins were fixed in 10 percent TCA. Gels from this technic were stained with Coomassie brilliant blue by the same procedure employed with SDS-gels.

Electron Microscopy

Purified coho antibodies were fixed and negatively stained[32/] for electron microscopy as described by Shelton and Smith (1970) except that the glutaraldehyde solution was buffered with 0.001 M

32/ Phosphotungstic Acid, J. T. Baker Chemical Co., Phillipsburg, N. J.

ammonium acetate (pH 7.0) rather than 0.02 M potassium phosphate (pH 7.2). Antibody preparations were examined with a Phillips 300 electron microscope and 60 kv accelerating voltage. Photomicrographs were taken at an initial magnification of 80,150 X.

Bacterial cell walls were shadowed with chromium and examined in the electron microscope at various magnifications.

RESULTS

Properties of Coho Salmon Anti- *Aeromonas salmonicida* Antibodies in Immune Serum

The Effect of Heat on Antibody Activity

It was of interest to determine the heat sensitivity of coho antibody activity since this property apparently varies among different lower vertebrates. Clem and Sigel (1963) reported heat stable activity (56 C for 30 minutes) in shark and margate antiserums. Antibody activity in rainbow trout antiserum was destroyed by heating at 59 C for 30 minutes (Hodgins *et al.* 1967).

Figure 5 shows the results of heating pooled coho anti- *A. salmonicida* serum (1:10 dilution) at various temperatures for 30 minute intervals. Antibody activity was unaffected at temperatures up to 45 C. After heating at 50 C, 50 percent of the activity was abolished (drop in titer from 1:20, 480 to 1:10, 240) and, at 55 C, 75 percent of the activity was lost. Temperatures of 60 C and above resulted in a loss of more than 95 percent of the original antibody activity. At 55 and 60 C prozone reactions[33/] were observed in lower dilutions of antiserum. This suggested that a portion of the inactivated

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33/ The absence of visible agglutination in the presence of sufficient antibody.

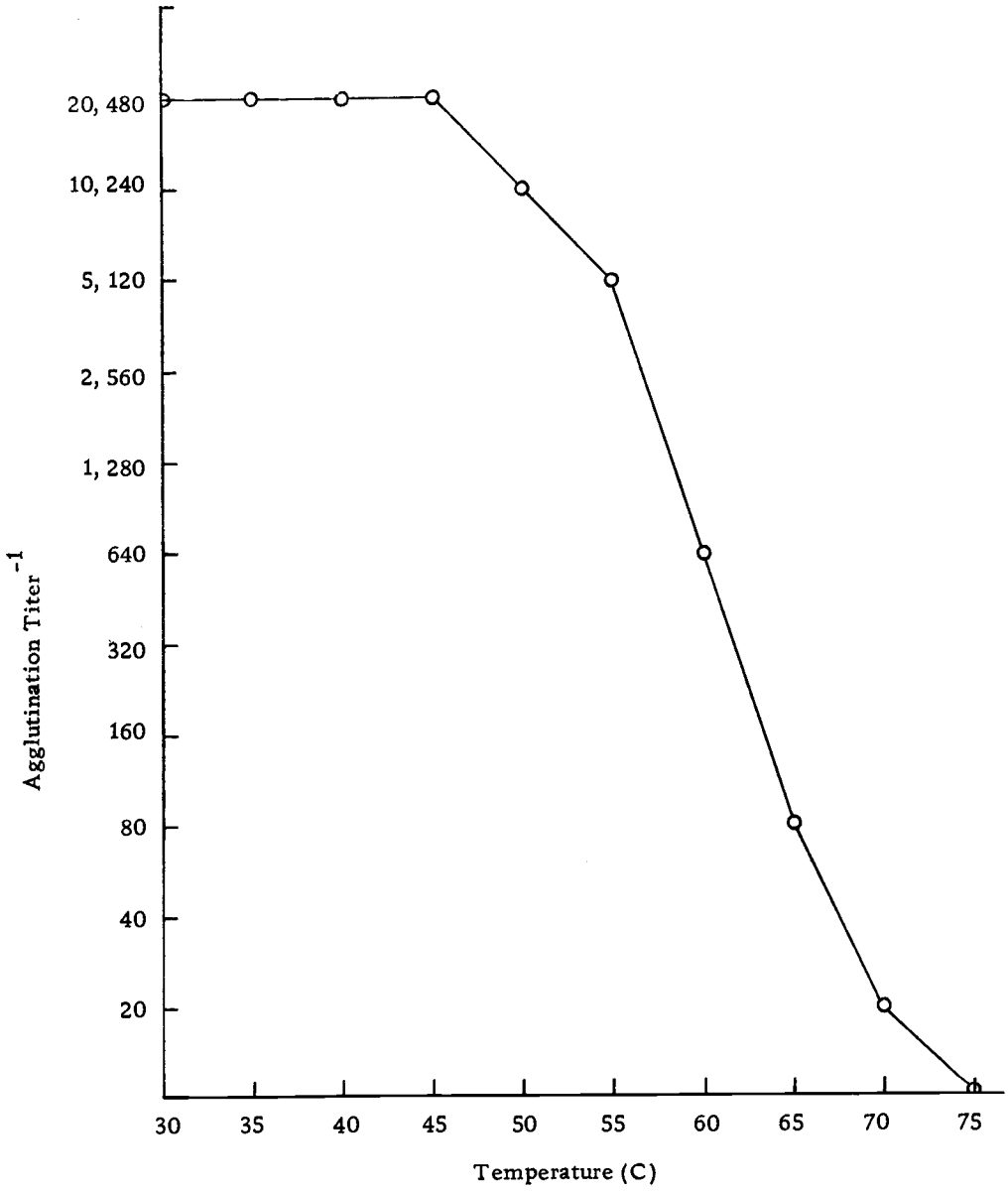

Figure 5. The effect of temperature (30 minute exposure period) on agglutination activity in coho anti- A. salmonicida serum (initial titer , 1:20, 480).

antibodies were only partially denatured and could still bind to A. salmonicida cells.

Coho anti- A. salmonicida antibody activity appeared to be considerably more heat sensitive than activity in mammalian antiserums. Thirty minutes exposure to 56 C has no measurable effect on mammalian antibody activity[34/] but would destroy approximately 75 percent of the activity in coho anti- A. salmonicida serum. Pike and Schulze (1965) have examined the heat sensitivity (30 minute exposure period) of rabbit IgM and IgG antibody fractions against various antigens. These workers found that a detectable loss of IgM activity generally occurred between 60 and 65 C while IgG activity began to decrease between 70 and 75 C. As shown by Figure 5, the initial loss of coho activity occurred between 45 and 50 C.

The Effects of Partial Reduction and Alkylation on the Activity of Antiserums

The objectives of this experiment were threefold: (1) to follow antibody synthesis, (2) to determine if a shift from 2-mercaptoethanol (2-ME) sensitive to 2-ME resistant antibodies occurred during the immune response, and (3) to evaluate the dependence of antibody activity on covalent and noncovalent forces. The results of the experiment are presented in Figure 6.

34/ An exception to this is the heat labile IgE class of antibodies.

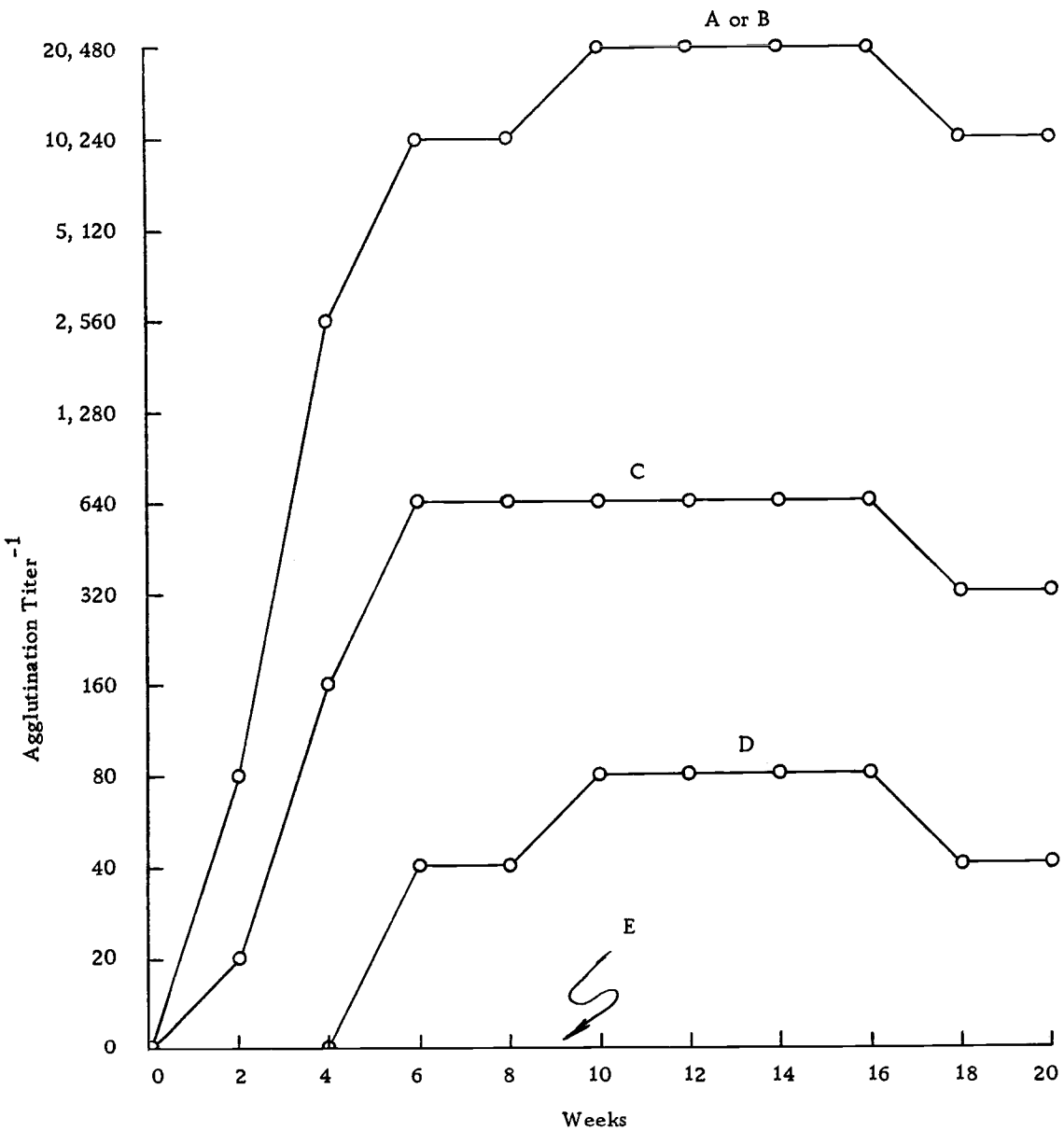

Figure 6. The effects of partial reduction and alkylation on the activity of antiserums collected over the course of antibody synthesis: A, untreated antiserum; B, antiserum exposed to 2 M urea; C, reduced and alkylated antiserum; D, highest dilutions of reduced and alkylated samples which displayed prozones; E, reduction and alkylation followed by exposure to 2 M urea (titers less than 1:20).

The agglutination titer of untreated antiserum (curve A, Figure 6) reached its maximum value (1:20, 480) 10 weeks after immunization and then remained constant through the 16th week of the experiment. The titers of serums from unimmunized fish (not shown in Figure 6) increased from less than 1:20 to a maximum value of 1:160 at the end of the experiment. This may have resulted from either an increase in natural agglutinins or the ubiquitous nature of *A. salmonicida*.

When a sample of each antiserum was partially reduced and alkylated (curve C), antibody activity was significantly lowered but not completely destroyed. Maximum titers in reduced and alkylated samples were 1:640 (approximately 3 percent of the original activity). As expected, the data indicated the critical contribution which disulfide bonds make toward antibody activity. There was no indication that the ratio of 2-ME resistant activity (curve C) to total activity (curve A) increased with time. This suggested that a 2-ME resistant population of agglutinins was not synthesized during the experiment. It did not rule out the possibility that a low molecular weight antibody was produced. As a control, it was found that the partial reduction treatment had almost no effect on the activity of a "late" rabbit anti- *A. salmonicida* serum.[35/]

[35/] Presumably this antiserum had a high content of IgG antibodies which display 2-ME resistant activity.

Prozones appeared following reduction and alkylation of high titer coho antiserums.[36/] The previously described agglutination inhibition test revealed that bacterial cells from dilutions which displayed prozones could not be agglutinated with unreduced antiserum. The results also indicated that noncovalent bonding was responsible for the activity which remained in reduced and alkylated samples (curve C). This conclusion was reached from the finding that exposure of unreduced antiserum to 2 M urea did not result in a detectable loss of activity (curve B). In contrast, parallel treatment of reduced and alkylated samples abolished their activities (curve E).

In summary, the data indicated that maximum antibody titers were reached between 10 and 16 weeks after immunization. This agrees with the finding that agglutination titers reached a maximum value three months after immunization of rainbow trout with *A. salmonicida* (Krantz, Reddecliff and Heist, 1963). The data also suggested that the small amount of antibody activity which remained after partial reduction was maintained by noncovalent forces. The cleavage of macroglobulin antibodies into monovalent fragments seemed to be an attractive explanation for the appearance of prozones

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36/ All antiserum dilutions on and below curve D (Figure 6) failed to agglutinate bacterial cells. Higher antiserum dilutions (up to and including those on curve C) displayed visible agglutination. Antiserum dilutions greater than those on curve C showed agglutination due to the absence of sufficient antibody.

in partially reduced samples.

Sephadex Column Chromatography of Antiserum

Fractionation of pooled coho anti- *A. salmonicida* serum with G-200 Sephadex was employed as a means of correlating antibody activity with molecular size (Figure 7). The serum proteins were separated into three distinct fractions. Titrations indicated the presence of a single symmetrical activity peak which was directly associated with the macroglobulin fraction of the serum (molecular weight > 200, 000). No activity was detected in lower molecular weight fractions. Figure 8 shows an electrophoretic comparison between whole antiserum and macroglobulin fractions from the Sephadex column. It appeared that the large molecule fraction of the serum contained only two major components although a few other faint bands were visible in the freshly stained gel.

The above findings are similar to those reported by Hodgins *et al.* (1967) for rainbow trout antiserum and indicate that the coho agglutinin is associated with a high molecular weight serum protein. Results presented later showed that the slowest migrating serum protein in alkaline acrylamide gels was the coho immunoglobulin.

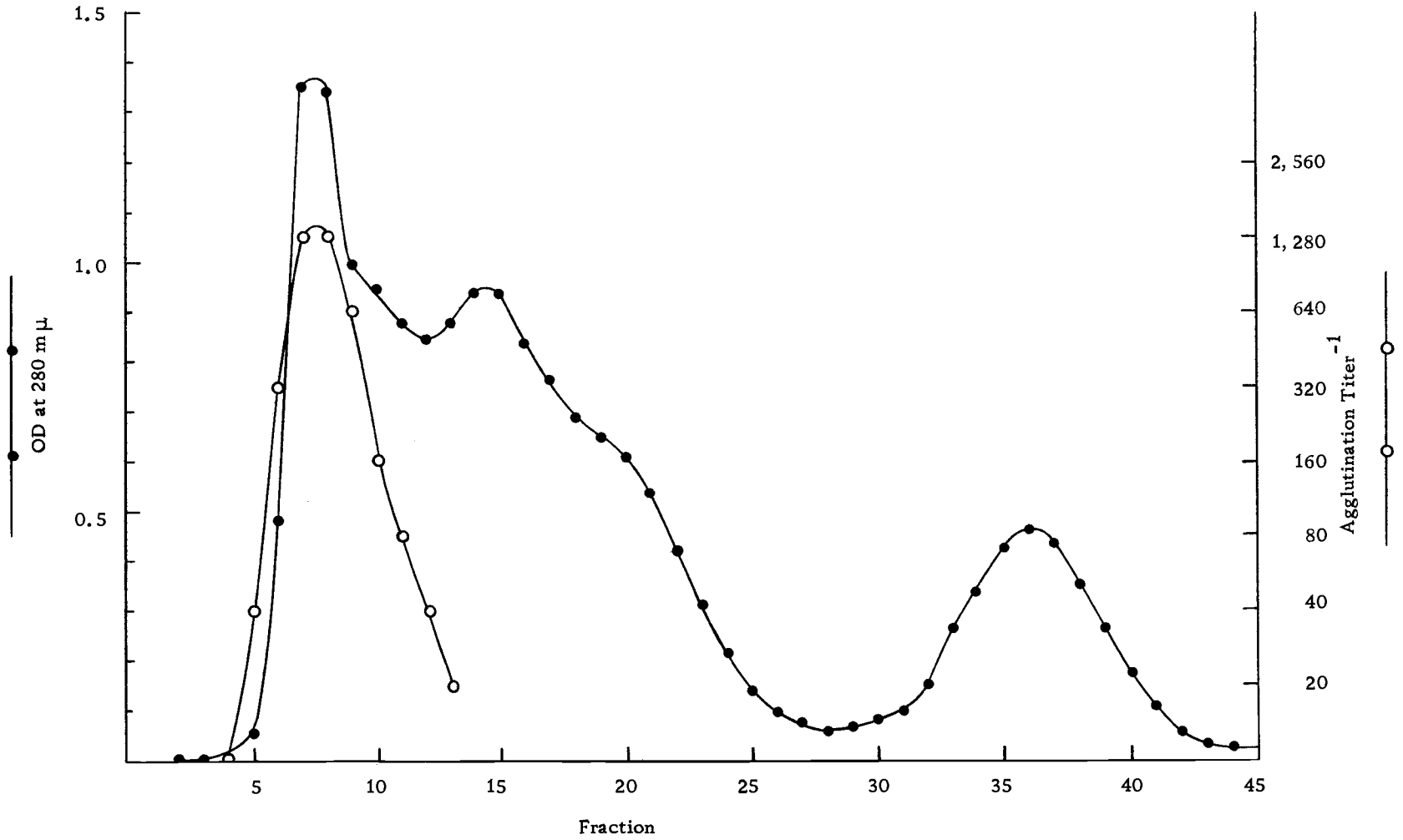
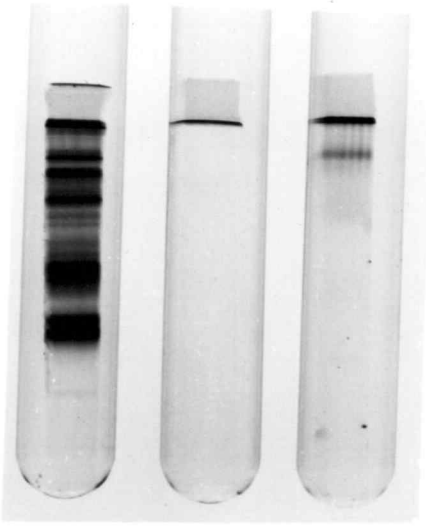


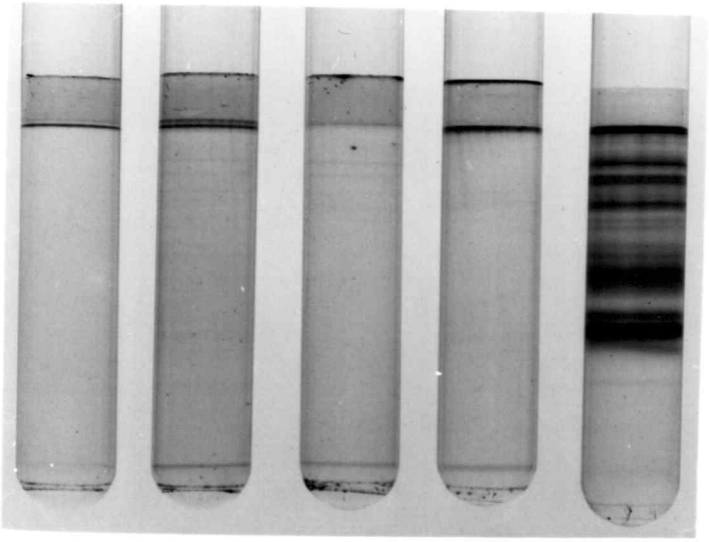
Figure 7. Fractionation of coho anti- A. salmonicida serum with G-200 Sephadex showing the distribution of protein (OD at 280 mμ) and the location of activity (agglutination titer) within the excluded protein fraction.

Figure 8. Alkaline polyacrylamide gel electrophoresis: A, coho anti- *A. salmonicida* serum; B, purified coho anti- *A. salmonicida* antibodies; C, antibody containing fraction (excluded fraction) from G-200 Sephadex column chromatography.

Figure 9. Alkaline polyacrylamide gel electrophoresis of coho anti- *A. salmonicida* antibody solutions obtained from dissociation of whole cell immune complexes with: A, 15 percent NaCl; B, 3 M urea; C, pH 3.0 glycine-HCl buffer; D, 3 M NaSCN. Coho anti- *A. salmonicida* serum (E) is included for comparison.



A B C



A B C D E

Purification of Coho Salmon Anti-*Aeromonas salmonicida* Antibodies

Studies based on antibody activity provided only preliminary information regarding antibody structure. As a step toward more definitive work, immunoadsorption procedures were examined for purifying specific coho antibodies against *A. salmonicida*.

Selection of an Antibody-Antigen Dissociating Agent

The ability of different dissociating agents to reverse the coho antibody *A. salmonicida* immune complex was determined. The chaotropic ion SCN^-, was considered because of its effect on protein conformation and its ability to dissociate certain antigen-antibody complexes (Dandliker and de Saussure, 1968). Urea was also selected for its ability to unfold polypeptide chains. A concentrated NaCl solution was included because of its effectiveness in reversing carbohydrate-anticarbohydrate systems (Kabat and Mayer, 1961). Numerous investigators have employed low pH as a means of separating antibody-antigen complexes (Campbell and Weliky, 1967).

The results of this experiment are summarized in Table 4. It appeared that none of the antigen-antibody dissociating agents had a profound effect on the antigenic character of *A. salmonicida* cells. This was suggested by the fact that cells which were treated with the

Table 4. The evaluation of four antibody-antigen dissociating solutions: a summary of activity data.

Description	Control	Antibody-Antigen Dissociating Solutions			
	PBS pH 7.2	3 M NaSCN pH 7.2	3 M Urea pH 7.2	15% NaCl pH 7.2	Glycine-HCl Buffer pH 3.0
Initial number of agglutinating units available	10, 240	10, 240	10, 240	10, 240	10, 240
Number of agglutinating units adsorbed by "pretreated"[a] bacterial cells	9, 920	9, 600	9, 920	9, 600	9, 600
Percent available activity which was adsorbed	~97	~94	~97	~94	~94
Number of agglutinating units recovered in final "purified" antibody solution	--	10	1, 280-2, 560[b]	2, 560	<10
Percent activity recovered	--	~0.1	~12.5-25	~25	<0.1

[a] Prior to adsorption of antibody, bacterial cells were repeatedly washed in the appropriate dissociating solution.

[b] Uncertainty due to ± reaction at end point of the titration.

different solutions could still adsorb about the same amount of antibody activity as untreated cells. Each of the immune complexes were washed and then dissociated with the appropriate solution. Titrations of the final antibody preparations indicated that 3 M urea and 15 percent NaCl were the only solutions tested which released active antibody from the immune complex.

Electrophoretic analysis of each final product in alkaline polyacrylamide gel suggested a rather high degree of purity and indicated that the coho antibody was the slowest migrating serum protein (Figure 9). The antibody solution obtained with 15 percent NaCl showed a single sharp band and no indication of any other components. Although the antibody solution obtained from dissociation with 3 M urea contained a prominent immunoglobulin band, numerous faint bands were also present. The sample which had been prepared by 3 M NaSCN dissociation showed a rather prominent but diffuse immunoglobulin band. No immunoglobulin component was noted in the sample obtained with the glycine buffer (pH 3.0). Based primarily on electrophoretic patterns, 15 percent NaCl was selected as the best dissociating agent tested.

The UV absorption properties of the different solutions indicated that nonprotein contaminants were present in all samples (OD_{280}/OD_{260} ratios were about 1.0). This and other UV data collected throughout the experiment suggested the need for an

immunoadsorbent which was more stable than formalinized bacterial cells. Satisfactory results were obtained when purified bacterial cell walls were employed.

Antibody Isolation From Cell Wall Immune Complexes

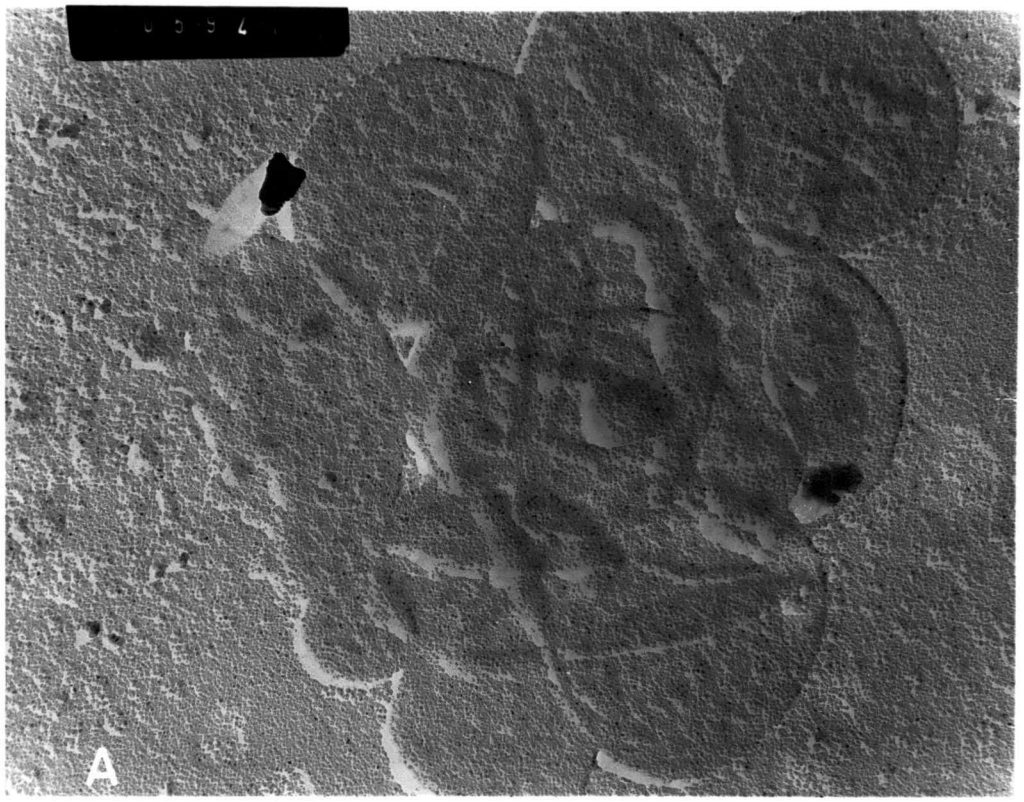
Prior to considering the quantitative aspects of two preparative antibody isolations, it seems appropriate to summarize certain information concerning *A. salmonicida* cell walls. Results from the quantitative agglutinin test are also presented.

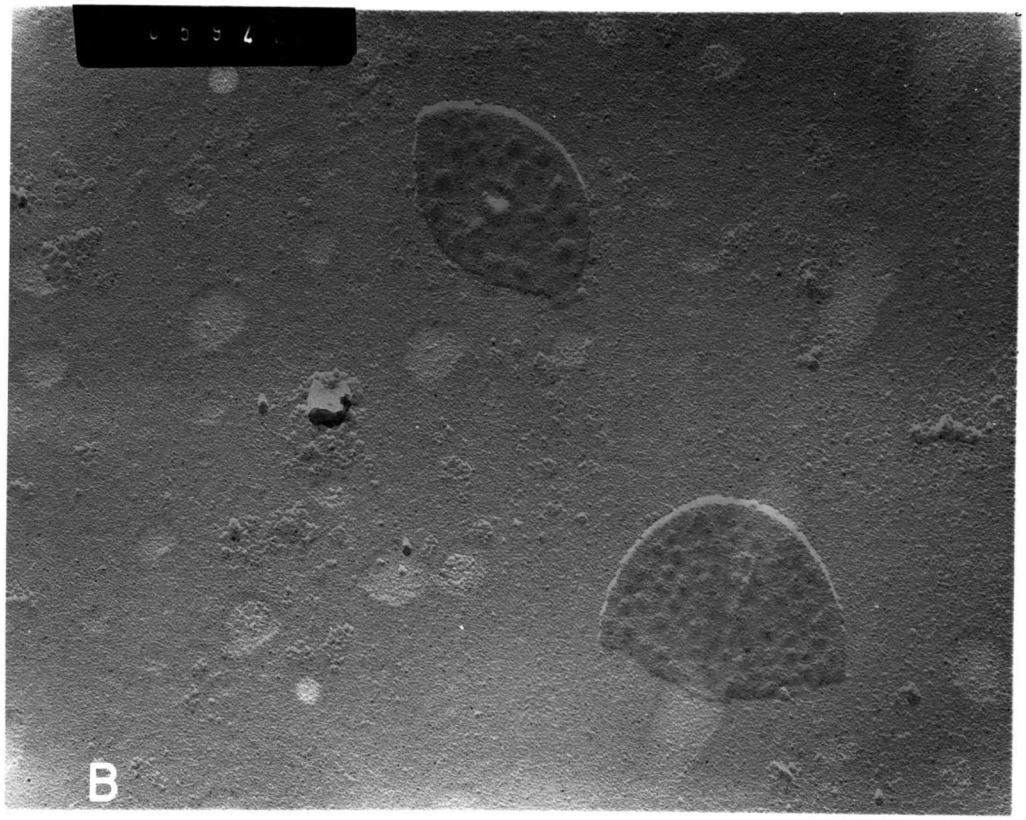
Electron microscopy was employed to confirm the presence of cell walls in purified preparations (Figure 10). The method of preparing cell walls appeared to result in a substantial percentage of cell wall fragments in addition to "envelope"-like, intact cell walls (individual and in aggregates). Whole bacterial cells were rarely found.

From the limited studies which were conducted it appeared that the composition of different cell wall preparations was similar. Duplicate determinations of two separate preparations indicated that cell wall suspensions with an OD at 520 mμ of 0.5 (0.5 inch diameter cuvette) contain approximately 1,100 μgm dry weight/ml (1,075 and 1,065 μgm dry weight/ml). The nitrogen content of both preparations was about 7.6 percent (7.54 and 7.65 percent nitrogen).

An important consideration in this study concerned the possible

Figure 10. Electron photomicrographs of chromium shadowed *A. salmonicida* cell walls: A, aggregated cell walls (37,800 X); B, individual cell walls (28,800 X).


A


B

loss of A. salmonicida somatic antigens during cell wall purification. The observation that the agglutination titer of pooled coho antiserum was decreased from 1:20,480 to 1:40 (> 99.9 percent of the activity) by a single adsorption with cell walls strongly suggested that A. salmonicida whole cells and cell walls contained the same surface antigens. Because of this it seemed valid to employ cell walls for both the quantitative agglutinin test and as an immunoadsorbent for purification of A. salmonicida agglutinins.

The pool of coho anti- A. salmonicida serum had an agglutination titer of 1:20,480. Results from the quantitative agglutination test indicated that the antiserum had an agglutinin content of 0.8 ± 0.4 mg antibody/ml. Antibody titrations revealed that approximately 2 percent of the total agglutinating activity was lost in supernatant fluids prior to nitrogen analysis. The estimate of the antibody content suffered from certain factors (Kabat and Mayer, 1961). As shown below, the amount of antibody nitrogen was small in comparison to the antigen nitrogen.

a.	Total nitrogen in agglutinate/0.1 ml antiserum	= 165 μgm ± 3.3 μgm
b.	Nitrogen in cell wall blank	= 152 μgm ± 3.0 μgm
c.	Nitrogen in serum blank	= none detected
	Antibody nitrogen (a - b - c)/ 0.1 ml antiserum	= 13 μgm ± 6.3 μgm

The fact that the final antibody nitrogen value had to be multiplied by 10 to obtain mg antibody/ml of antiserum also increased any error. In spite of the uncertainties in the determination, 0.8 mg/ml was taken as the best estimate for the anti- A. salmonicida antibody content of the pooled coho antiserum. From these data it appeared that 0.04 μgm/ml was the minimum antibody concentration required for bacterial agglutination. Additional work supported this estimate.

Specific anti- A. salmonicida antibodies were isolated by dissociation of cell wall immune complexes with 15 percent NaCl. The quantitative aspects from two antibody isolations are presented in Table 5. Calculations were based on data from the quantitative agglutinin test, macroscopic tube agglutination tests, and nitrogen determinations of purified antibody solutions. The same batch of cell walls was employed for both antibody isolations. After their initial use in antibody isolation 6, they were repurified as previously described (pronase treatment).

Since greater than 99 percent of the available activity was adsorbed from antiserum in both isolations, it appeared that the amounts of cell walls employed were in excess. When the immune complex was washed to remove nonantibody serum proteins, approximately 1 percent of the bound activity was lost. No detectable loss of antibody occurred during ultrafiltration (Diaflow XM-50 membrane). This step was important in the removal of nonantibody, UV absorbing

Table 5. Quantitative aspects of two preparative antibody isolations employing 15 percent NaCl as the antibody-antigen dissociating agent.

Letter Designation	Description	Antibody Isolation 6	Antibody Isolation 7
A	OD of cell wall suspension (each ml of antiserum was adsorbed with the cell walls from 20 ml of suspension).	0.5[a]	0.3
B	Volume (ml) of coho anti- *A. salmonicida* antiserum employed	8	8
C	Total mg of anti- *A. salmonicida* antibody available (in 8 ml antiserum)	6.4[b]	6.4[b]
D	Total *A. salmonicida* agglutinating units available (in 8 ml antiserum)	163,840	163,840
E	Number of agglutinating units which were not adsorbed to cell walls	1,280	1,280
F	Percent activity adsorbed to cell walls $\left(\frac{D-E}{D} \quad 100\right)$	> 99	> 99
G	Number of agglutinating units lost from the immune complex during three washings with cold saline	1,600	1,820
H	Percent of adsorbed activity lost during washings $\left(\frac{G}{D-E-G} \quad 100\right)$	~1	~1
I	Number of agglutinating units lost during "washing" and concentration of dilute antibody solution with Diaflow XM-50 membrane	<400	<400
J	Number of agglutinating units recovered in purified antibody solution	56,320	29,950 to 59,900[c]
K	Percent of total activity recovered in the purified antibody solution	34	14 to 37[c]
L	Total mg of antibody recovered in the purified antibody solution	2.12[d]	1.87[d]
M	Percent of total antibody recovered $\left(\frac{L}{C} \quad 100\right)$	33	29

a/ Approximately 1,100 μgm dry weight/ml.

b/ From quantitative agglutinin test.

c/ Uncertainty due to ± reaction at end point of macroscopic tube agglutination test.

d/ From nitrogen determinations of purified antibody solutions.

material. Approximately 30 percent of the initial antibody activity was present in the purified antibody solutions.

Quantitative information also indicated that 30 percent recoveries were achieved[37/]. This seemed reasonable since the salt dissociation method resulted in 15 to 30 percent yields when applied to certain other antigen-antibody systems (Kabat and Mayer, 1961). In this study approximately 2 mg of antibody were obtained from 8 ml of antiserum.

On several occasions the immune complex was extracted a second time with 15 percent NaCl. This practice was ineffective since it routinely yielded only 1 to 2 percent of the initial agglutinating activity.

Properties of Purified Coho Salmon Anti-*Aeromonas salmonicida* Antibodies

Analytical Ultracentrifugation

Sedimentation velocity determinations were performed with coho antibody solution 7 at three protein concentrations (0.05, 0.08 and 0.18 mg/ml). Ultraviolet scans (Figure 11) of the antibody solution (0.18 mg/ml) failed to detect any evidence of gross size heterogeneity. This is shown by the complete removal of UV

37/ This assumes that all the nitrogen in the final product was from coho anti- *A. salmonicida* antibodies.

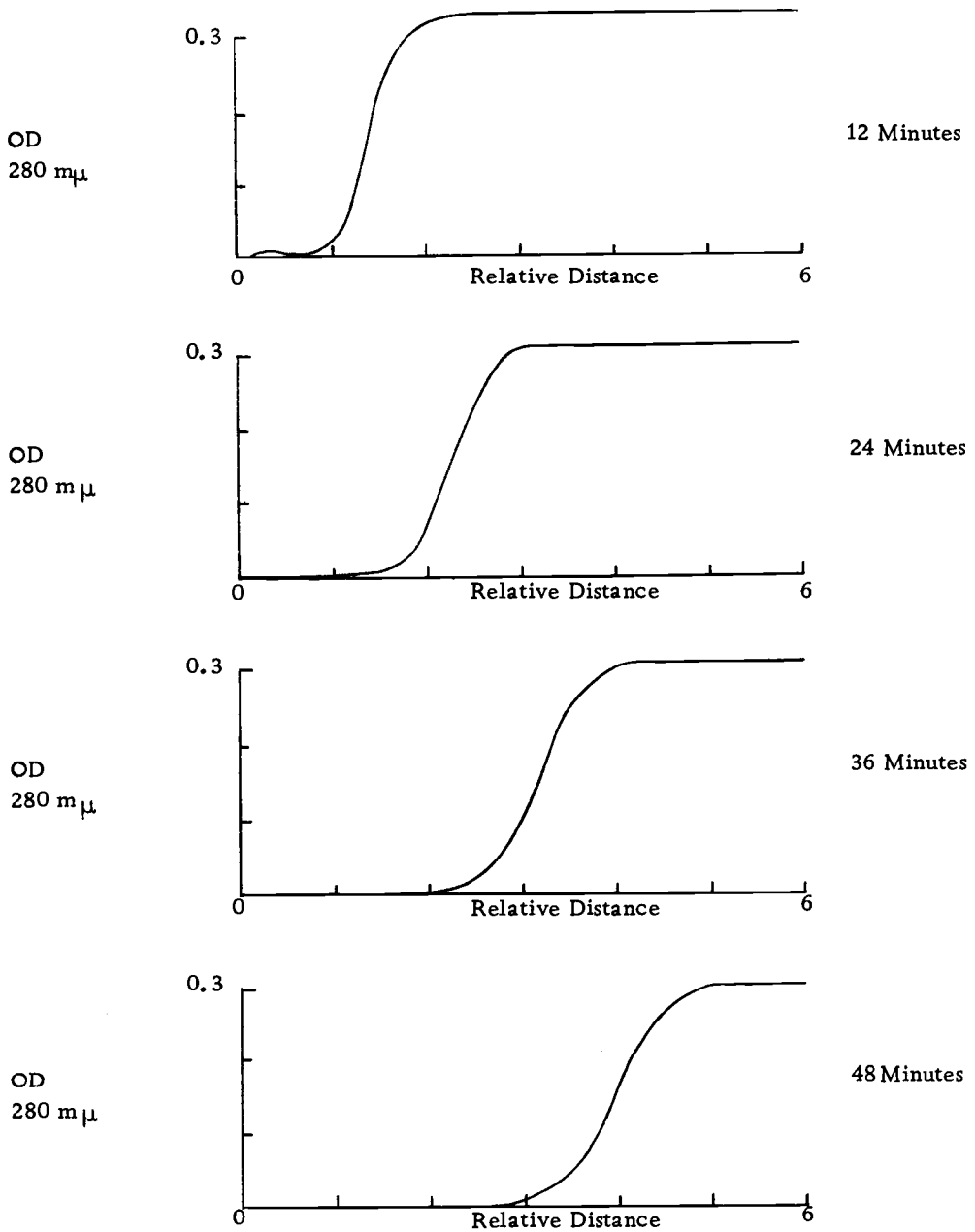


Figure 11. Ultraviolet scans of coho antibody solution 7 (0.18 mg/ml in 0.15 M NaCl) obtained during analytical ultracentrifugation (30,266 RPM, 20 C). The presence of a symmetrically migrating UV boundary throughout the experiment indicated the absence of gross size heterogeneity in the antibody preparation.

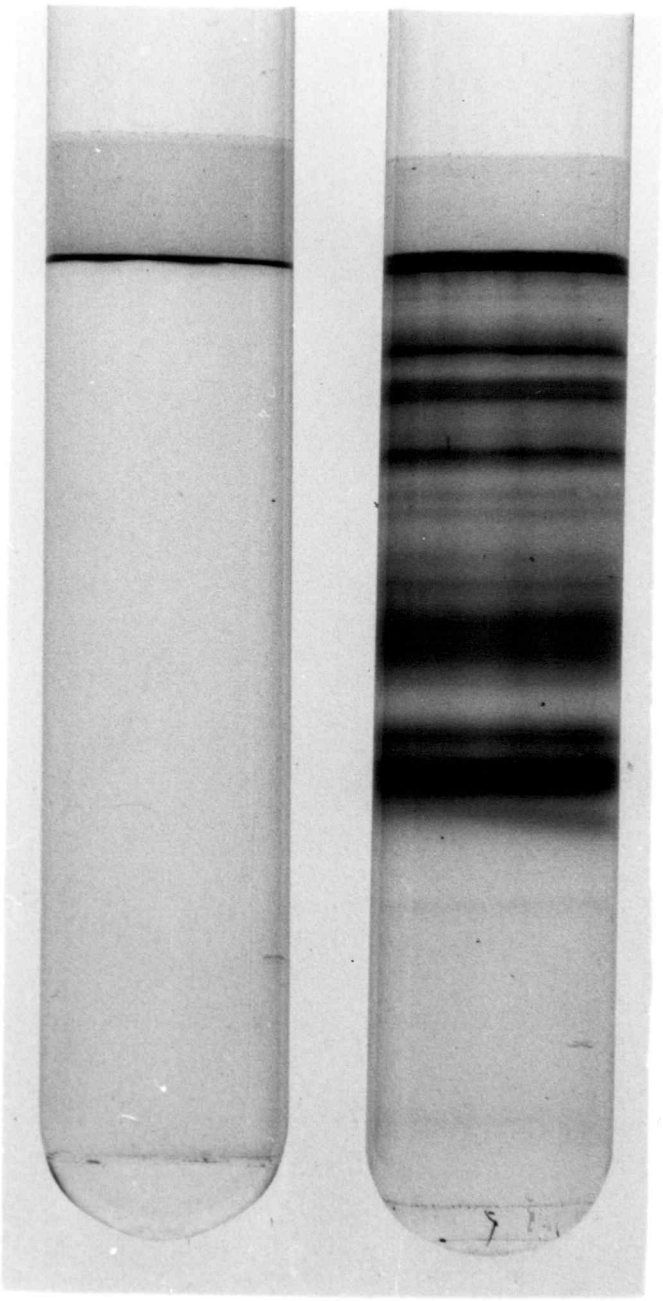
absorbing material behind the symmetrically sedimenting boundary. At the protein concentrations employed, the $S_{20,W}$ values displayed no apparent concentration dependence: 0.18 mg/ml, $S_{20,W}$ = 16.9S; 0.08 mg/ml, $S_{20,W}$ = 17.3S; 0.05 mg/ml, $S_{20,W}$ = 17.1S. The $S^0_{20,W}$ for the coho antibody was estimated at 17.1S from the average of the three $S_{20,W}$ values.

The estimated $S^0_{20,W}$ for the coho antibody was slightly higher but similar to values reported for immunoglobulins from other bony fishes. Acton et al. (1971a) found that macroglobulins from catfish, gar and paddlefish were all about 14S. The giant grouper (Clem, 1971) and the carp (Trump, 1970) both appear to synthesize 16S macroglobulins. Hodgins et al. (1967) reported an uncorrected sedimentation coefficient of 13.5S for a macroglobulin component in rainbow trout serum.

Electrophoresis in Alkaline Polyacrylamide Gel

In alkaline polyacrylamide gels the coho immunoglobulin was found to be the slowest migrating serum protein (Figure 12). The very sharp appearance and slow mobility of this band was like that observed with purified grey snapper antibodies (Russell, Voss and Sigel, 1970). No evidence for a lower molecular weight component (7S immunoglobulin) was found when samples of coho antibody were incubated then applied to the gel in a 4 M urea solution rather

Figure 12. Alkaline polyacrylamide gel electrophoresis: A, purified coho anti- *A. salmonicida* antibodies (approximately 25 μgm); B, coho anti- *A. salmonicida* serum.



A B

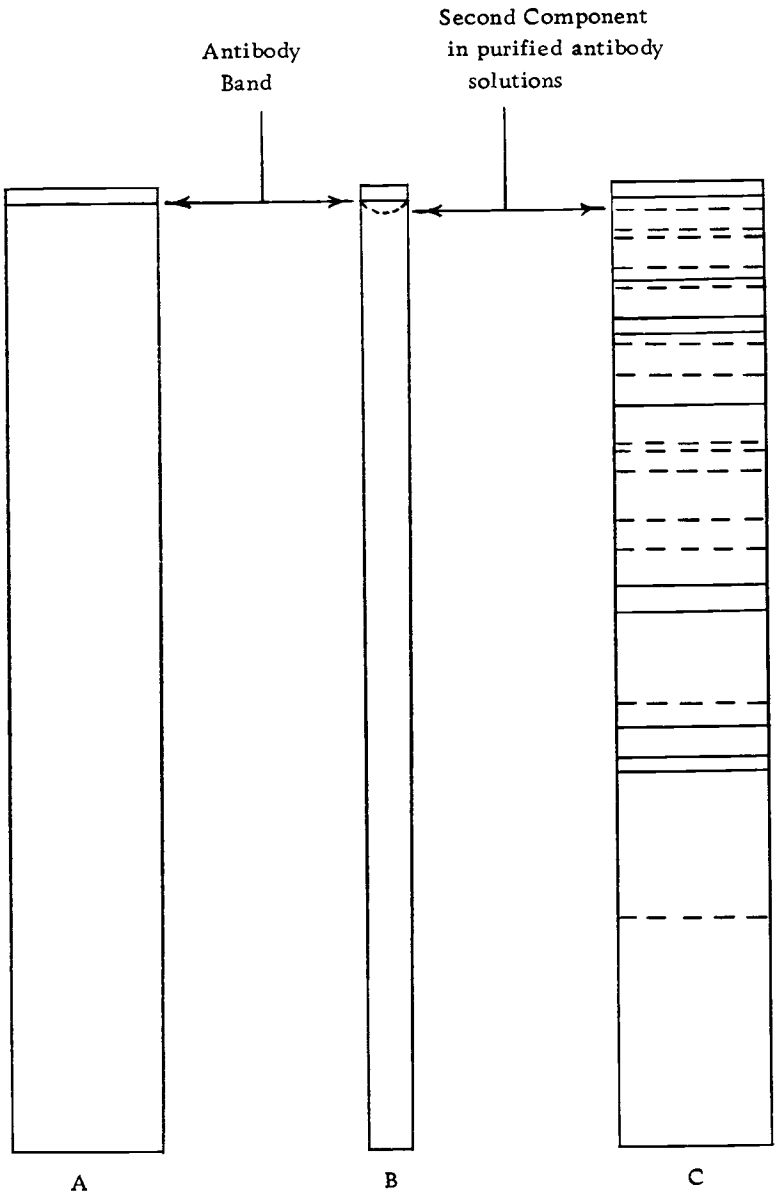


Figure 13. Diagrammatic representations (~2 X) of alkaline polyacrylamide gels (large pore gel omitted): A, frontal view of purified coho antibody solution showing only the antibody band; B, lengthwise view of the same acrylamide slab revealing a trace amount of a second component in the antibody solution; C, whole serum pattern showing the approximate location of major (————) and minor (----------) bands.

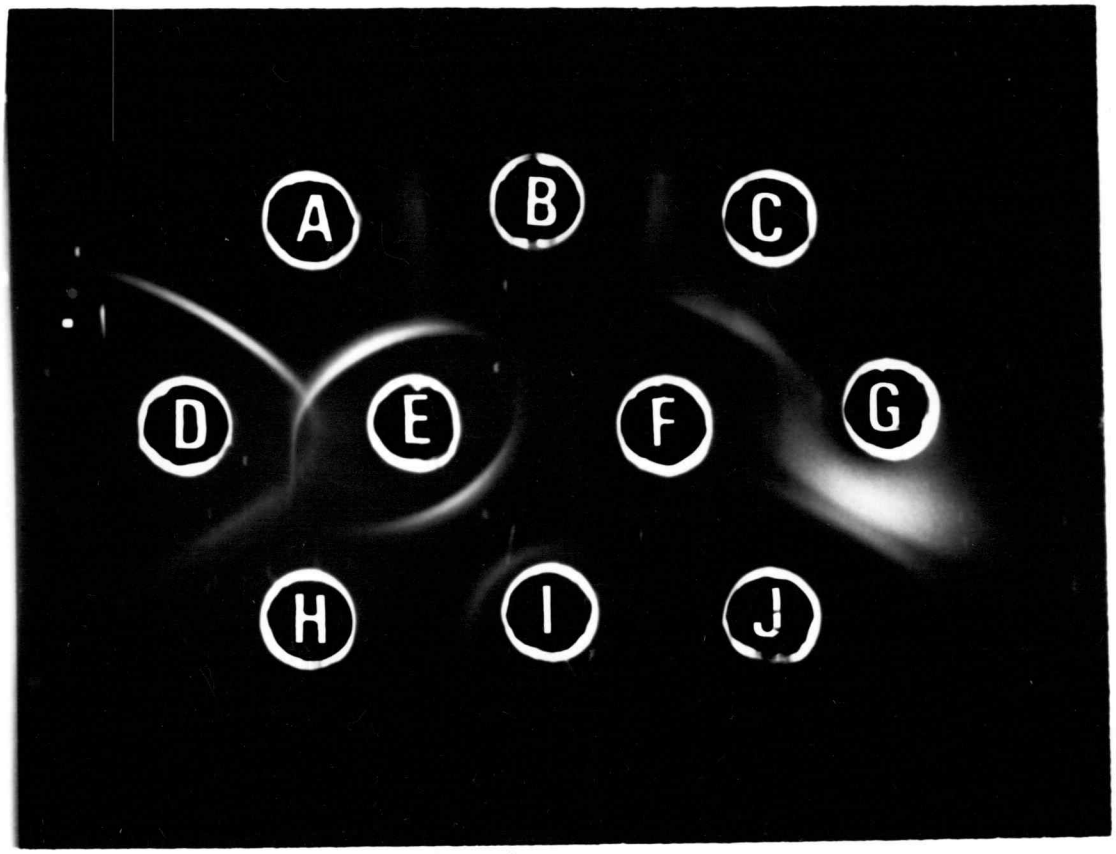
than 10 percent sucrose.

In spite of the apparent purity indicated by Figure 12, acrylamide gel electrophoresis revealed a trace amount of a second component in concentrated antibody solutions (0.2 mg/ml). The faintness of this band and its tendency to arc downward from the prominent immunoglobulin band, made it impossible to see when acrylamide slabs were viewed frontally (as in Figure 12 and Figure 13, A). However, when the slab was viewed lengthwise (Figure 13, B), the second component was visible. Careful examination of well resolved whole serum patterns revealed 24 bands (Figure 3, C), however, many were very faint and others could not be seen by frontal inspection of the gel slab. The faint band that was detected in antibody solutions also seemed to exist in whole serum patterns although it was not visible in all separations. Further information concerning this minor component was not obtained. In view of its very low concentration, it was thought to be inconsequential to the results of this study.

Immunodiffusion and Immunoelectrophoretic Analyses

Immunodiffusion (Figure 14) failed to detect any bacterial or serum contaminants in the purified coho antibodies. It also served as a convenient means of illustrating the relationship between different reagents employed in this study.

Figure 14. Immunodiffusion: (A and C), rabbit antiserum to A. salmonicida immune complex; (B and J), coho antiserum to A. salmonicida; (D and F), coho anti- A. salmonicida antibodies (approximately 0.8 mg/ml); (E), A. salmonicida endotoxin (10 mg/ml); (H and G), rabbit antiserum to coho serum; (I) rabbit antiserum to A. salmonicida.


A
B
C
D
E
F
G
H
I
J

Single precipitin arcs developed between rabbit antiserum against coho serum (wells H or G) and the purified coho antibodies (0.8 mg/ml, wells D and F respectively). The latter example (reaction between wells G and F) was partially masked by the complex precipitate which formed between the anti-whole serum reagent (well G) and coho serum (well J). Rabbit antiserum against the A. salmonicida immune complex (wells A and C) also precipitated a minimum of one component from the purified coho antibody solution (wells D or F respectively). The spur between wells C, G and F may have resulted from an unbalanced system.

Reactions involving A. salmonicida endotoxin are also shown in Figure 14. Rabbit antiserum against A. salmonicida (well I) precipitated at least two components in the endotoxin preparation (well E) and none in the purified antibody solution (well F). Considering the method of preparation, it was not surprising that the anti-immune complex reagent (well A) was also quite effective in precipitating an endotoxin component (well E). At least a portion of the purified coho antibodies reacted with the same substance. This is best seen by the vertical precipitin arc between wells D (coho antibodies) and E (endotoxin).

Immunoelectrophoresis was employed to analyze certain of the reactions shown by gel diffusion. No coho proteins with γ-like mobilities were observed in serum after electrophoretic separation

in agar gel buffered to a pH value of 8.2. The absence of "γ-globulin" has been widely observed in lower vertebrate serums. The presence of γ-globulin-like arcs in Figure 15 resulted from buffering the gel to a pH of 7.5.

Figure 15 (A) shows the reactions of coho serum with the rabbit anti-whole serum and anti-immune complex reagents. Unfortunately, certain conclusions which were reached from these patterns are not totally apparent. With the anti-whole serum reagent it was possible to distinguish approximately 23 coho serum proteins. At least three of these were bound by A. salmonicida cells as shown by the reaction between the anti-immune complex reagent and coho serum. Other investigations (Krauel and Ridgway, 1963; Ambrosius, 1966; Anderson and Klontz, 1970) have also revealed multiple precipitin arcs from reactions of fish serums with anti-immune complex reagents. In this study it seemed likely that only the major arc represented coho immunoglobulins. In work with rainbow trout (Hodgins, Ridgway and Utter; 1965; Anderson and Klontz, 1970), the asymmetrical shape of the immunoglobulin arc suggested electrophoretic and possibly size heterogeneity. These same conclusions seemed to apply to coho immunoglobulins. In certain patterns developed with the anti-whole serum reagent, the immunoglobulin arc appeared to result from two molecular populations which differed in electrophoretic mobility but were antigenically identical.

Figure 15. Immunoelectrophoretic analyses of purified coho anti-*A. salmonicida* antibodies (approximately 0.8 mg/ml). Patterns obtained with whole coho serum are included for comparison (anode to the right).

A.

Upper trough:	rabbit antiserum to coho serum
Center well:	coho antiserum
Lower trough:	rabbit antiserum to *A. salmonicida* immune complex

B.

Upper well:	coho antibodies
Center trough:	rabbit antiserum to coho serum
Lower well	coho antiserum

C.

Upper trough:	rabbit antiserum to *A. salmonicida* immune complex
Center well:	coho antibodies
Lower trough:	rabbit antiserum to coho serum

D.

Upper trough:	rabbit antiserum to *A. salmonicida*
Center well:	coho antibodies
Lower trough:	rabbit antiserum to *A. salmonicida* immune complex

A

B

C

D

The immunoglobulin arc which resulted from precipitation with the potent anti-immune complex reagent often displayed a diffuse spur. In Figure 15 (A) the spur can be seen projecting from near the center of the arc in a cathodic direction.

Analysis of purified antibodies (0.8 mg/ml) revealed a single component against either the anti-whole serum reagent or the anti-immune complex reagent (Figure 15, B and C). It appeared that the electrophoretic mobilities of the purified antibodies were restricted to the anodic portion of the serum immunoglobulin arc. As was the case in immunodiffusion, no A. salmonicida antigens were detected in the purified coho antibodies (Figure 15, D).

In summary, the antibody solution (0.8 mg/ml) appeared to be immunologically pure and consisted of a single immunoglobulin class. At least a portion of the anti- A. salmonicida antibodies reacted with a high molecular weight endotoxin component. The purified antibodies seemed to have restricted electrophoretic mobilities when compared to the entire immunoglobulin arc in immunoelectrophoretic separations of coho serum.

Ultraviolet Absorption Properties

It appeared that nucleic acids were a contaminant in antibody solutions obtained from whole cell agglutinates. For this reason, the UV absorption properties of antibodies from cell wall immune

complexes were an important test of purity.

Ultraviolet absorbance data from a variety of higher vertebrate immunoglobulins suggests certain common features (Little and Donahue, 1968; Chase and Williams, 1968). Purified immunoglobulins have absorption maxima (λ max) and minima (λ min) near 278 and 251 mμ respectively. The λ max/λ min absorbance ratio is generally between 2.5 and 2.8 unless the sample is appreciably contaminated with antigen or nonimmunoglobulin serum protein. The OD_{280}/OD_{260} ratio generally approaches 2.0 and very little absorbance occurs between 320 and 340 mμ. Purified immunoglobulins have extinction coefficients ($E^{1\%}_{1cm}$) from about 13.5 to 16.8.

Table 6 shows the absorbances of two coho antibody solutions and human IgG at certain critical wavelengths.. The data from the salmon antibodies were obtained immediately after isolation and all proteins were in 0.15 M NaCl.

It can be seen that all solutions satisfied the above criteria. The minor variations between the two coho antibody solutions were of questionable significance.[38/] Antibody solutions 6 and 7 had λ max values of 278 and 277 mμ respectively; this difference might be real but could also be the result of experimental error. The detailed absorption spectrum of coho antibody solution 7 (Figure 16) further

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38/ Antibody solutions 6 and 7 were isolated under slightly different conditions (Table 5).

Table 6. Ultraviolet absorption properties of purified coho salmon antibodies and human IgG.

Immunoglobulin	OD at different wavelengths (mμ)						$\frac{OD_{280}}{OD_{260}}$	$\frac{OD_{278}}{OD_{251}}$	$E^{1\%}_{1cm}$ [b/] at 280 mμ
	251	260	276	278	280	320			
Coho ab. sol. [a/] 6	0.228	0.321	0.564	0.570	0.561	0.016	1.75	2.50	15.5 ± 0.3
Coho ab. sol. 7	0.227	0.329	0.602	0.600	0.590	0.011	1.79	2.64	17.0 ± 0.3
Human IgG	0.215	0.305	0.556	0.564	0.561	0.013	1.84	2.62	15.7 ± 0.3

a/ Coho antibody solution.

b/ These values are presented here for convenience. They were calculated from the protein concentrations given in Table 7 and OD_{280} values obtained in standard sized cuvettes: coho ab. sol. 6, 0.300; coho ab. sol. 7, 0.272; human IgG, 0.411.

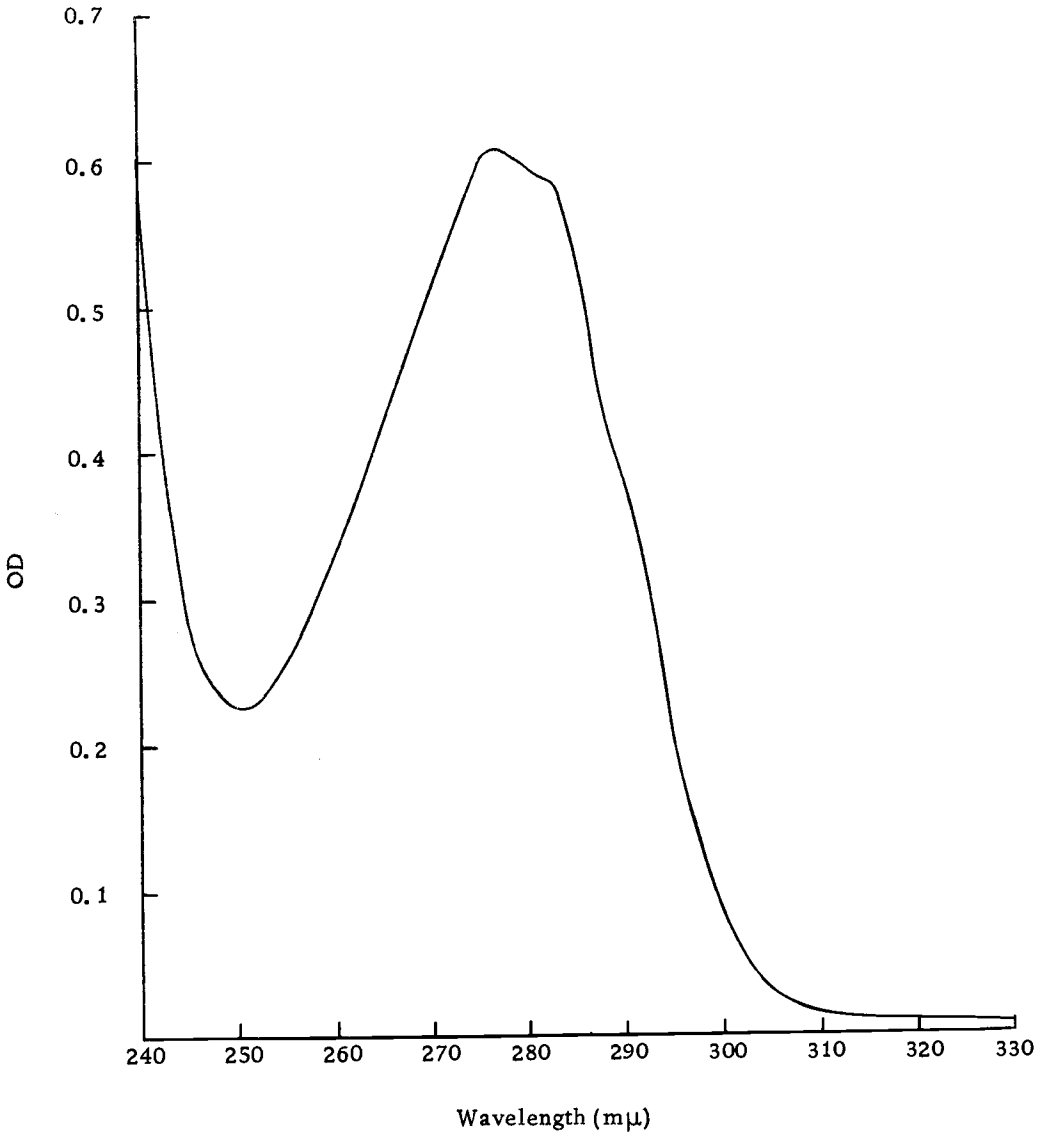


Figure 16. Ultraviolet absorption spectrum of coho antibody solution 7 in 0.15 M NaCl.

emphasized the absence of nonprotein, UV absorbing material in antibody solutions prepared by dissociation of cell wall immune complexes with 15 percent NaCl. The calculated $E^{1\%}_{1cm}$ values (Table 6) at 280 mμ seemed reasonable and their average was 16.2.

Polypeptide Chain Heterogeneity

One of the unique features of immunoglobulins is extreme molecular heterogeneity. An indication of this has come from the separation of both L and H chains into multiple electrophoretic subpopulations by electrophoresis in starch and acrylamide gels containing high concentrations of urea (Reisfeld and Small, 1966). Recent work with rabbit and human secretory IgA (Halpern and Koshland, 1970) and human IgM (Mestecky, Zikan and Butler, 1971; Frangione *et al.* 1971) indicates that these molecules contain a polypeptide chain which may have some function in the joining of 7S subunits. Halpern and Koshland (1970) suggested that this new component be designated as the "joining chain" (J chain). The presence of the J chain has apparently been overlooked in structural studies because its molecular weight is similar to that of L chains (~23,000). Acrylamide gel electrophoresis in the presence of urea is a valuable technic for the separation and presumptive identification of J chains. A thorough analysis of the purified coho antibodies for heterogeneity and the presence of J chains was beyond the scope of this study. However,

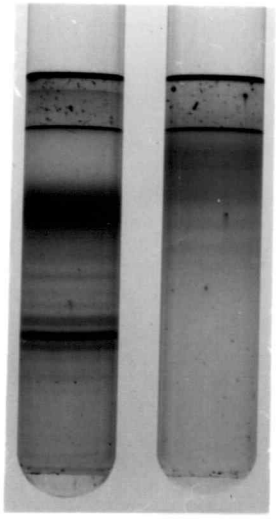
certain information concerning these subjects was obtained.

Extensively reduced and alkylated coho antibodies (Figure 17, A) and human IgG (Figure 17, B) were simultaneously separated. Based on the conditions of electrophoresis and the location of the pattern, (Halpern and Koshland, 1970), the diffuse bands in the IgG sample were tentatively identified as L chain subpopulations. From the results of Reisfeld and Small (1966), it was anticipated that the coho H chains would not enter the lower gel. However, this can not be ruled out and the broad slow migrating band in Figure 17 (A) may represent coho H chains. In the freshly stained gel 14 components were observed which migrated ahead of the broad band. Many of these probably represent electrophoretic subpopulations of coho L chains. In view of the mobility differences between rabbit and human L chains (Halpern and Koshland, 1970), it was not surprising to find that coho L chains apparently migrated faster than those from human IgG. The purified coho antibodies appeared to be quite a heterogeneous collection of molecules. This was expected, especially since the antibodies had been isolated from pooled serum.

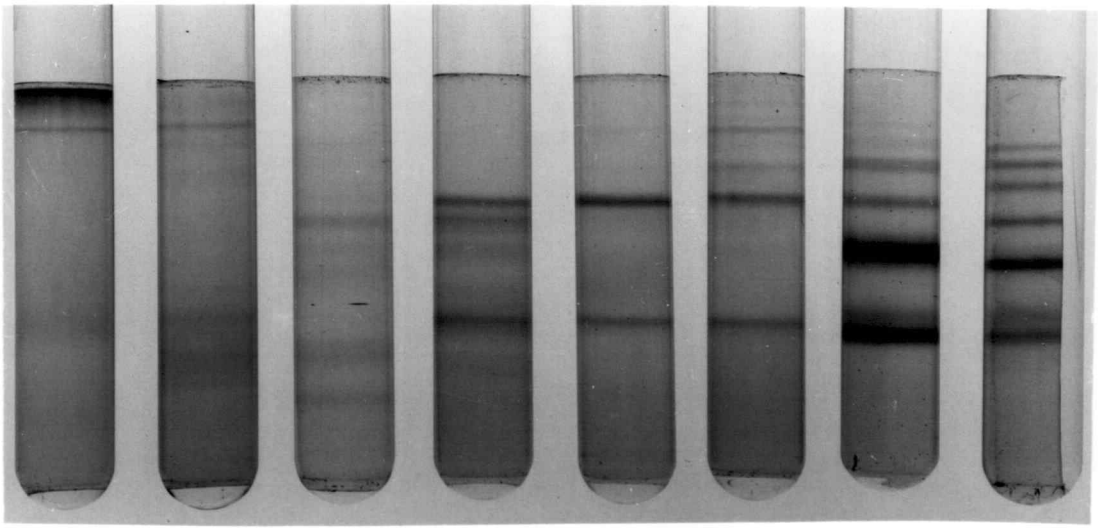
Of special interest were the three rapidly migrating dense bands. The location, number and prominence of these bands are similar to the appearance of J chains in published electrophoretic patterns (Halpern and Koshland, 1970; Mestecky _et al_. 1971).

Figure 17. Separation of extensively reduced and alkylated immunoglobulin chains with acrylamide gel electrophoresis in the presence of urea: A, coho anti- *A. salmonicida* antibodies; B, human IgG.

Figure 18. Molecular weight analysis of coho anti- *A. salmonicida* antibodies with SDS-acrylamide gel electrophoresis. The effects of different treatments on the salmon antibody molecule are illustrated: A, unreduced; B, reduced and alkylated in PBS by dialysis method; C, reduced and alkylated in saline (pH 8.0); D, reduced and alkylated in 8 M urea; E, reduced in 4 M urea; F, reduced and alkylated in 7 M guanidine HCl. The apparent molecular weight and identity of each band are given in Table 8. Preparations of human IgG are included for comparisons; G, reduced and alkylated in 8 M urea; H, reduced and alkylated in saline (pH 8.0).



A B



A B C D E F G H

The present results certainly do not establish the existence of J chains in the salmon macroglobulin, but they are suggestive.

Molecular Weight Analysis

Previous results indicated that the coho salmon anti- *A. salmonicida* antibodies were purified to the point where a molecular weight analysis was possible. From results with human IgG it also seemed that the SDS-acrylamide gel technic might yield direct information concerning the molecule's polypeptide chain structure.

Various procedures were employed to reductively cleave the coho macroglobulin prior to electrophoresis (Figure 18). Extensively and partially reduced samples of human IgG (Figure 18, G and H respectively) were included for comparative purposes. Average relative mobilities were determined for each band and molecular weight estimates were calculated from the two previously determined standard equations (Table 7). Values from Standard Equation A were considered as the best molecular weight estimates when intact intra-chain disulfide bonds were anticipated (A, B and C, Figure 18 and Table 7). Likewise, in extensively reduced and alkylated samples (D, E and F, Figure 18 and Table 7), the best estimates were generally considered to be those from Standard Equation B.

After alkylation of coho antibodies in 4 M urea at 45 C (A, Figure 18 and Table 7), the majority of the protein failed to enter the

Table 7. Molecular weight data obtained from purified coho salmon antibodies.

Preparation of antibody sample prior to electrophoresis	Identity of electrophoretic band[a]	Number of independent determinations	Average relative mobility	Apparent molecular weight from Standard Equation A	Apparent molecular weight from Standard Equation B
A. Unreduced, alkylated in 4 M urea	3H + 2L(?)[c]	3	0.062	252, 600[b]	211, 800
	L-H-H-L	3	0.198	179, 600[b]	152, 600
B. Reduced and alkylated in PBS by dialysis method	L-H-H-L	2	0.203	177, 400[b]	150, 800
	H-H[c]	1	0.267	151, 100[b]	129, 200
	L-H[c]	1	0.400	108, 300[b]	93, 800
C. Reduced and alkylated in saline (pH 8.0)	H(?)	4	0.525	79, 200[b]	69, 400
D. Reduced and alkylated in 8 M urea	H-H[c]	2	0.213	173, 000	147, 200[b]
	L-H[c]	1	0.365	118, 200	102, 000[b]
	H	7	0.488	86, 800	75, 900[b]
	H(?)	5	0.556	73, 200[b]	64, 400
	L	7	0.933	28, 500	26, 000[b]
E. Reduced in 4 M urea at 45 C	H-H	2	0.221	169, 600	144, 400[b]
	H	5	0.497	85, 100	74, 400[b]
	H(?)[c]	1	0.570	70, 700[b]	62, 300
	L	5	0.940	28, 000	25, 500[b]
F. Reduced and alkylated in 7 M guanidine HCl	L-H-H-L	3	0.110	224, 000	188, 700[b]
	L-H-H	3	0.171	192, 200	162, 900[b]
	H-H	3	0.219	209, 700	145, 100[b]
	L-H	3	0.356	120, 900	104, 300[b]
	H	3	0.470	90, 900	79, 200[b]
	L	3	0.922	29, 300	26, 700[b]

a/ From best estimate of molecular weight.

b/ Best estimate, based on method of sample preparation and final analysis of the data.

c/ Not visible in Figure 18.

gel. However, two bands appeared which probably resulted from a slight breakdown of the macroglobulin. The molecular weight of the fastest moving component (~180,000) suggested that it was the immunoglobulin subunit. In the final analysis the slower migrating band was identified as a subunit plus one H chain; however, the lack of markers in this region of the gel made this a questionable designation.

Inconsistent results were obtained from partially reduced and alkylated coho antibodies. With one sample, reduction and alkylation at pH 7.2 (B, Figure 18 and Table 7) appeared to cleave the coho macroglobulin into subunits (molecular weight ~177,000). Electrophoresis of a second sample prepared by this method suggested the presence of half subunits (H-L) along with H and L chains. This finding seemed to be more consistent with data obtained from other IgM molecules (Frank and Humphrey, 1969; Metzger, 1970). From a structural standpoint, the appearance of subunits in the first sample was of interest even though they may have resulted from reoxidation.

A multiple band pattern was anticipated after partial reduction and alkylation of the coho immunoglobulin in 0.15 M NaCl at pH 8.0 (C, Figure 18 and Table 7). One sample prepared by this method did not form any bands, and a second attempt yielded a faint but definite component which had a molecular weight of approximately

79,000 (Standard Equation A). This band was tentatively designated as the coho H chain.

Certain points could not be explained regarding partially reduced preparations of coho immunoglobulin (B and C in Figure 18 and Table 7). When patterns were observed, the bands were faint and it seemed that a major portion of each sample was unaccounted for. Although bands were observed which seemed to represent L and H chains, faint, diffuse bands were also noted which had estimated molecular weights (Standard Equation A) of approximately 16,000, 34,000 and 62,000. These were considered as preparation artifacts since they were never observed when chains were separated immediately after extensive reduction.

In contrast to partial reduction and alkylation, the effects of extensive reduction and alkylation were quite consistent. Prominent H and L chain bands (~76,000 and ~26,000 respectively) appeared following reduction and alkylation in 8 M urea (D, Figure 18 and Table 7). Occasionally faint slower moving bands were noted and identified as half subunits (~102,000) and H chain dimers (~147,000). A faint band was often observed which migrated slightly faster than the extensively reduced H chain. Standard Equation B yielded a molecular weight estimate of 64,000 for this component while Standard Equation A gave an H chain-like value of about 73,000. It seemed possible that the band represented a small fraction of H

chains which had not undergone extensive reduction. This interpretation drew a certain amount of support from the report that reduced rabbit γ-chains, in the presence of SDS, existed in two different sized fractions depending on the degree of intrachain disulfide bond reduction (Koshland, Englberger and Shapanka, 1966). The nature of a protein-SDS complex also indicates that its size is restricted by intrachain disulfides (Reynolds and Tanford, 1970b; Fish, Reynolds and Tanford, 1970).

Reduction of the coho immunoglobulin in 4 M urea at 45 C (E, Figure 18 and Table 7) resulted in H and L chains (~74,000 and ~26,000) respectively. Trace amounts of H chain dimers were occasionally observed (~144,000). The sharp separation of H and L chains from this type of preparation made it best suited for densitometry. The H chain band appeared to bind approximately three times[39/] more amido black than the L chain band. Since the H to L molecular weight ratio was also approximately three, it appeared that the coho antibody contained equal numbers of H and L chains. This finding, although expected, lended support to the overall molecular weight analysis. The dye binding results were only approximate and did not offer any information concerning the possible presence of J chains.

39/ In two separate determinations, the actual H to L chain dye binding ratios were 2.6 and 3.1.

After extensive reduction and alkylation in 7 M guanidine HCl, an antibody sample displayed six bands (F, Figure 18 and Table 7). By analogy with previous findings from human IgG, it was possible to test the assumption that the six components resulted from partial cleavage of a L-H-H-L subunit. Since the actual molecular weights were unknown, the L and H chains were assigned relative masses of 1.0 and 3.0 respectively. The relationship of the six bands was tested with a semilogarithmic plot of predicted relative mass against measured mobility (Figure 19). The linear relationship between the six components indicated that the assumptions of the analysis were probably correct (i.e. L-H-H-L subunit with a H to L mass ratio of about three).

Examination of the apparent molecular weights from this sample suggested that the values for the L and H chains (~27,000 and ~79,000 respectively) were too high. The subunits estimated molecular weight (~189,000) seemed to be in fairly good agreement with the previous values (~180,000) obtained from partial reduction.

Results from the molecular weight analysis indicated that the coho antibody was composed of IgM-like subunits. It appeared that the subunit's molecular weight was between 180,000 and 190,000. The average molecular weight estimate for the H chain (from H) was about 76,000. However, 73,000 was the average estimate from half the molecular weight of the H chain dimer (H-H). The coho L

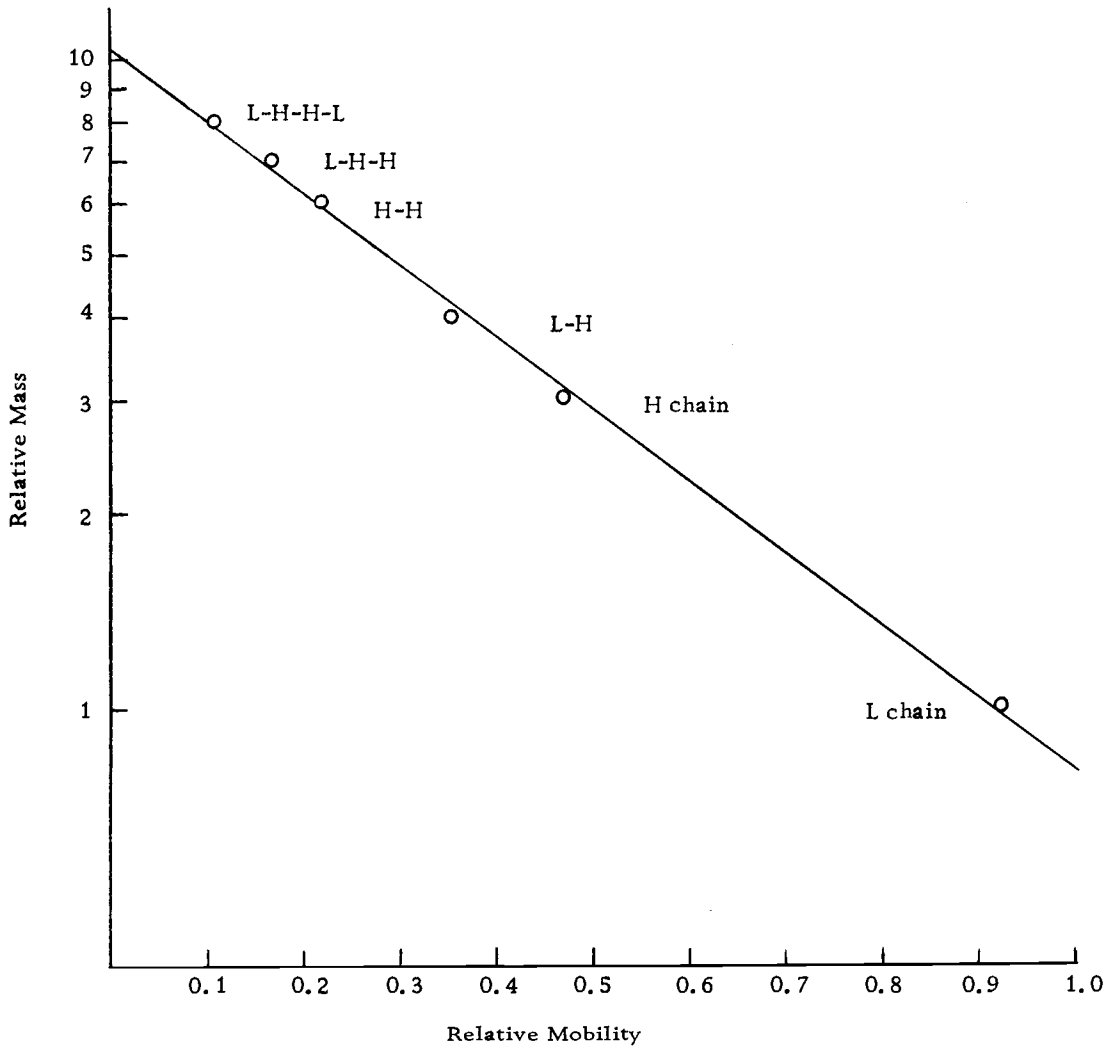


Figure 19. Semilogarithmic plot of predicted relative mass vs. relative mobility showing the relationship of the six components in a sample of extensively reduced and alkylated coho antibodies (F, Figure 18 and Table 7). Predictions were based on a H chain and L chain mass ratio of 3.0.

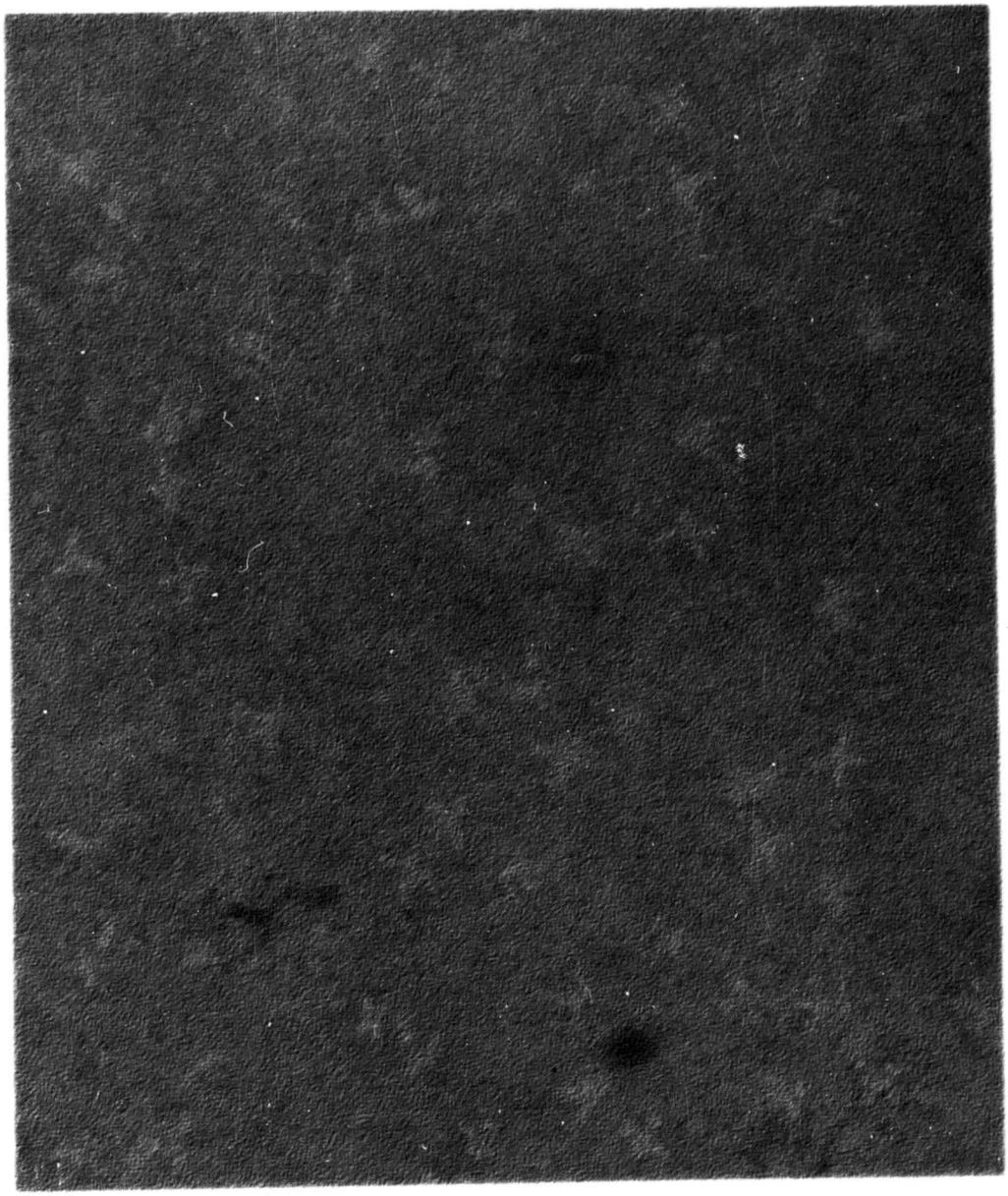
chain appeared to have a molecular weight of about 26,000 although this may be too high. As shown earlier, the human L chain consistently appeared to be heavier than it actually was (by approximately 1,000 molecular weight units in extensively reduced and alkylated preparations). Results from coelectrophoresis of identically prepared human IgG and coho antibodies (extensively reduced and alkylated in either 8 or 10 M urea) suggested that coho L chains were slightly larger than human L chains. Additional points concerning the molecular weight analysis are discussed later.

Electron Microscopy

An unanswered question from the molecular weight analysis concerned the number of subunits in the intact macroglobulin. Electron microscopy has proven to be a valuable technic for visualizing macromolecules and has recently provided evidence for tetrameric immune macroglobulins in carp (Shelton and Smith, 1970) and catfish (Acton et al. 1971a). In this study the method was also chosen for determining the number of subunits in the intact coho antibody.

Figure 20 shows negatively stained coho antibody molecules. The macroglobulin appeared to consist of four arms (subunits) which were attached to give an "+" shaped particle. The identification of each arm as a subunit is valid in view of work with other

Figure 20. Electron photomicrograph of negatively stained coho anti- *A. salmonicida* antibodies showing the tetrameric structure of these molecules (274, 800 X).



macroglobulin antibodies (Green, 1969). From the limited number of molecules which could be accurately measured, the average distance across the span of two arms appeared to be about 330 Å. Other investigators reported that the span for carp (Shelton and Smith, 1970) and catfish (Acton et al. 1971a) tetramers was between 370 and 400 Å. No significance was attached to the slightly shorter measurement obtained from the coho immunoglobulin.

Agglutination Activity

Previous results indicated that the isolated coho salmon antibodies were in a rather high state of physical purity. An experiment was performed to estimate the amount of biologically active protein in purified antibody solutions. This was done by measuring the loss of protein (OD at 280 mμ) and activity (agglutination titer) after adsorption of antibody solutions with *A. salmonicida* cell walls (Table 8).

A single adsorption of both coho antibody solutions removed about 93 percent of the available protein and greater than 99 percent of the agglutination activity for *A. salmonicida* cells. Results from a saline control indicated that no UV absorbing material was released from the cell walls during the adsorption procedure. A human IgG solution was included in the experiment as a test for non-specific adsorption of immunoglobulin. The removal of approximately 37 percent of the IgG was unexpected. In a similar kind of experiment with rabbit anti- *Salmonella typhimurium* antibodies, it

Table 8. The agglutination activity of purified coho salmon anti- *A. salmonicida* antibodies.

Immunoglobulin	OD_{280}	% OD_{280} removed	$Titer^{-1}$	% activity removed	µgm protein per ml[a]	µgm protein per agglutinating unit[b]
Coho ab. sol. 6 unadsorbed	0.298		5, 120	>99	193 ± 4	0.035
		92.7				
Coho ab. sol. 6 adsorbed	0.022		10			
Coho ab. sol. 7 unadsorbed	0.276		2, 560-5, 120[c]	>99	160 ± 3	0.058-0.029[c]
		93.2				
Coho ab. sol. 7 adsorbed	0.019		5			
Human IgG unadsorbed	0.404		<5	--	262 ± 5	--
		36.6				
Human IgG adsorbed	0.256		<5			

a/ From duplicate nitrogen analyses.

b/ Calculated from the assumption that 93 percent of the protein was responsible for 100 percent of the agglutination activity.

c/ Uncertainty due to ± reaction at end point of macroscopic tube agglutination test.

appeared that nonspecific adsorption accounted for the loss of about 6 percent of the total protein (Robbins, Kenny and Suter, 1965). Adsorption of rabbit antibody solutions with homologous antigen resulted in a 90 to 95 percent loss of protein.

In the present study the possibility existed that a sizable fraction of coho antibody was nonspecifically adsorbed to the cell walls. The extremely high agglutination activity of the purified coho antibodies made this seem unlikely. If 93 percent of the protein in each antibody solution was active (no nonspecific adsorption), then the calculated minimum antibody concentration required for bacterial agglutination was around 0.04 μgm/ml. This was the same estimate obtained from coho anti- A. salmonicida serum with the quantitative and macroscopic tube agglutination tests (0.8 mg antibody/ml, 1:20,480 agglutination titer). The agreement of both estimates could not be used as proof that the purified coho antibodies were 93 percent active. It did indicate, however, that the coho anti- A. salmonicida antibody was an efficient agglutinin. From the work with purified coho antibodies, it appeared that a minimum antibody concentration of 0.04 μgm/ml for A. salmonicida agglutination was either correct or too high. Any nonspecific adsorption of inactive antibody to the cell walls would make the actual minimum antibody concentration for agglutination less than 0.04 μgm/ml. As discussed later, 0.04 μgm antibody/ml is less than the generally reported sensitivity

of the bacterial agglutination test.

The Effect of Reduction and Alkylation on Agglutination Activity

Previous work with high titer immune serums revealed that reduction and alkylation in PBS (pH 7.2) resulted in a substantial loss of antibody activity and also the appearance of the prozone phenomenon. Studies with purified antibodies indicated that the reduction procedure probably cleaved most of the interchain disulfide bonds. Prior to any explanation of the data obtained from coho antiserums, it was necessary to examine the effects of the reduction and alkylation procedures on the activity of purified coho antibodies.

Purified coho antibodies and immune serum (1:10 dilutions) were subjected to the same treatments employed in previous studies with coho antiserums. Care was taken to insure that activity (agglutinating units/ml) in the purified antibody solution was at least as high as that in immune serum. The results of the experiment are shown in Table 9. All control samples (treatments A and B) behaved as expected. The slight loss of activity which resulted from exposure of the purified antibodies to 2 M urea was of questionable significance.

Reduction and alkylation (treatment C) decreased activity in both immune serum and the purified antibody solution. The sample of reduced and alkylated immune serum displayed prozones in the

Table 9. Effects of partial reduction and alkylation on the activity of purified coho salmon anti- *A. salmonicida* antibodies.

Sample	Treatment[a/]	Agglutinating units/ml		Prozone Phenomenon
		Before Treatment	After Treatment	
Purified antibody	A	2, 560-5, 120[b/]	2, 560-5, 120	--
Purified antibody	B	2, 560-5, 120	2, 560	--
Purified antibody	C	2, 560-5, 120	320	--
Purified antibody	D	2, 560-5, 120	40	--
Immune serum[c/]	A	2, 048	2, 048	--
Immune serum	B	2, 048	2, 048	--
Immune serum	C	2, 048	256-512[b/]	+
Immune serum	D	2, 048	<2	--

a/ Treatments: A, dialysis against PBS; B, exposure to 2 M urea; C, reduction and alkylation in PBS; D, reduction and alkylation in PBS followed by exposure to 2 M urea.

b/ Uncertainty due to ± reaction at end point of macroscopic tube agglutination test.

c/ 1:10 dilution of pooled coho anti- *A. salmonicida* serum (agglutination titer, 1:20, 480).

first four dilutions of the macroscopic tube agglutination test. In contrast, no indication of a prozone was observed in the reduced and alkylated antibody solution. The absence of a prozone in the antibody solution suggested that anti- A. salmonicida antibody fragments were not solely responsible for prozones in reduced and alkylated antiserum. There was a possibility that in antiserum samples, interactions occurred between anti- A. salmonicida antibody fragments and reduced normal immunoglobulin. In the absence of further data it does not seem worth speculating about the possible types of interactions which might result in prozones.

The activity which remained in both the immune serum and the purified antibody sample following reduction and alkylation was decreased by exposure to 2 M urea (treatment D). However, the loss of activity from exposure to 2 M urea appeared to be significantly less in the purified antibody sample. It seemed possible that this reflected the greater chance for noncovalent reassociation of anti- A. salmonicida fragments in the absence of other proteins. Non-antibody immunoglobulin fragments and other serum proteins have been shown to decrease the activity of reassociated human IgM antibodies (Schrohenloher, Kunkle and Tomasi, 1964; Frank and Humphrey, 1969).

In summary, the effect of the partial reduction and alkylation treatments on antibody activity seemed to be dependent on whether

they were performed with immune serum or purified antibody solutions. Although it was not proven, it appeared that in immune serum nonantibody protein was interacting with anti- A. salmonicida antibody fragments. Residual agglutination activity in reduced and alkylated antiserum and antibody solutions seemed to be maintained by noncovalent forces.

Electrophoretic Comparisons of Immune and Normal Coho Salmon Serum

Work with purified coho antibodies inevitably led to the identification of the immune macroglobulin in alkaline polyacrylamide gel and immunoelectrophoretic patterns of coho serum. Certain investigators (Summerfelt, 1966; Anderson and Klontz, 1970) have correlated increased levels of specific antibody with differences in electrophoretic patterns of fish serums. Other workers have not detected differences between immune and nonimmune serums (Clem and Sigel, 1963; Post, 1963). It was of interest to determine if pooled coho anti- A. salmonicida serum[40/] could be distinguished from pooled normal serum[41/] on the basis of electrophoretic patterns.

Alkaline polyacrylamide gel and immunoelectrophoretic

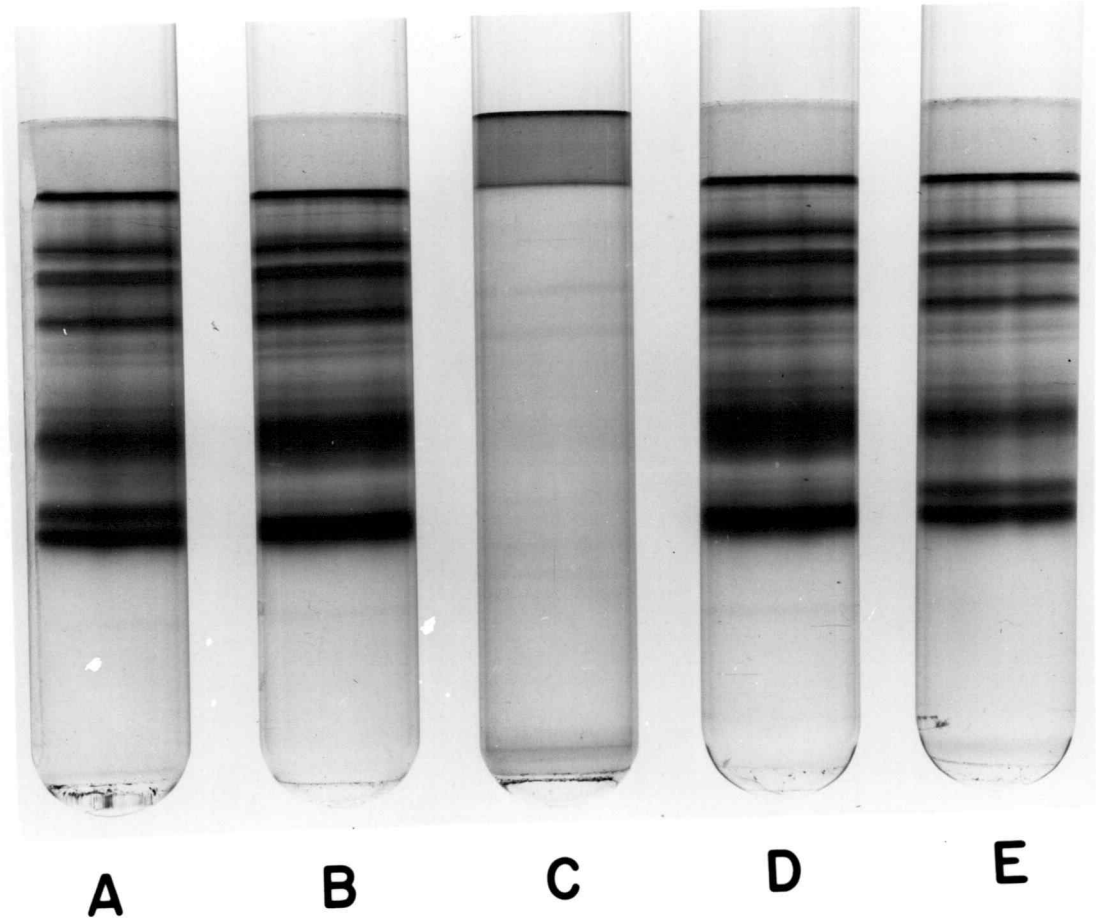
40/ The antiserum had an agglutination titer of 1:20, 480 and contained approximately 0.8 mg/ml of specific antibody.

41/ Pooled normal serum had an anti- A. salmonicida agglutination titer of 1:40.

patterns of adsorbed and unadsorbed immune and normal serums were analyzed. Serums (immune and normal) and PBS were identically adsorbed three times with A. salmonicida cells; the final agglutination titer of immune serum was 1:20. Adsorbed PBS served as a control for detecting bacterial components which were released into serums during adsorption.

Alkaline polyacrylamide gel electrophoretic patterns of the samples are shown in Figure 21. There were no obvious differences between unadsorbed samples of immune and normal serums (A and E respectively). Likewise, adsorbed immune and normal serums (B and D respectively) also appeared to be identical. A few bacterial components were visible in the adsorbed PBS sample (C), but these did not seem to complicate the interpretation of patterns from adsorbed serums. Differences existed between adsorbed and unadsorbed samples of immune or control serums. The most obvious change was the absence of a rapidly migrating component in adsorbed samples (B and D). In addition, both adsorbed samples displayed an apparent broadening of the pattern above and possibly below the missing band. It seemed probable that the missing component was either removed or that its mobility was altered by adsorption. The later possibility could explain the change(s) which occurred adjacent to the missing component. Of importance in this study was the absence of detectable differences in the slower migrating proteins

Figure 21. Alkaline polyacrylamide gel electrophoresis of adsorbed and unadsorbed coho anti- *A. salmonicida* serum (immune serum) and normal serum: A, immune serum; B, adsorbed immune serum; C, adsorbed PBS; D, adsorbed normal serum; E, normal serum.


A
B
C
D
E

including the immunoglobulin band (the slowest migrating serum protein).

Immunoelectrophoretic comparisons of the various serum samples are shown in Figure 22. Prior to considering these results, it should be pointed out that the adsorbed PBS sample contained six A. salmonicida antigens when tested against rabbit anti- A. salmonicida serum. None of these antigens were detected with the rabbit antiserum against coho serum which was employed to develop the patterns in Figure 22.

There appeared to be no significant differences between immune serum (A, upper well) and normal serum (A, lower well) patterns.[42/] Obvious changes occurred in adsorbed normal serum (B, upper well). The most cathodicly migrating component was absent from the pattern and the electrophoretic mobility of the prealbumins appeared to increase. There was a general loss of pattern resolution although a distinct immunoglobulin arc persisted. Adsorption had similar effects on immune serum (C, lower well); however, the entire immunoglobulin arc was altered. There seemed to be a general lack of pattern resolution in the area surrounding the sample well. Comparison of adsorbed normal serum (D, upper well) and

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42/ The coho immunoglobulin arc is clearly visible in both patterns as a long, flat, skewed arc which passes closest to the sample walls.

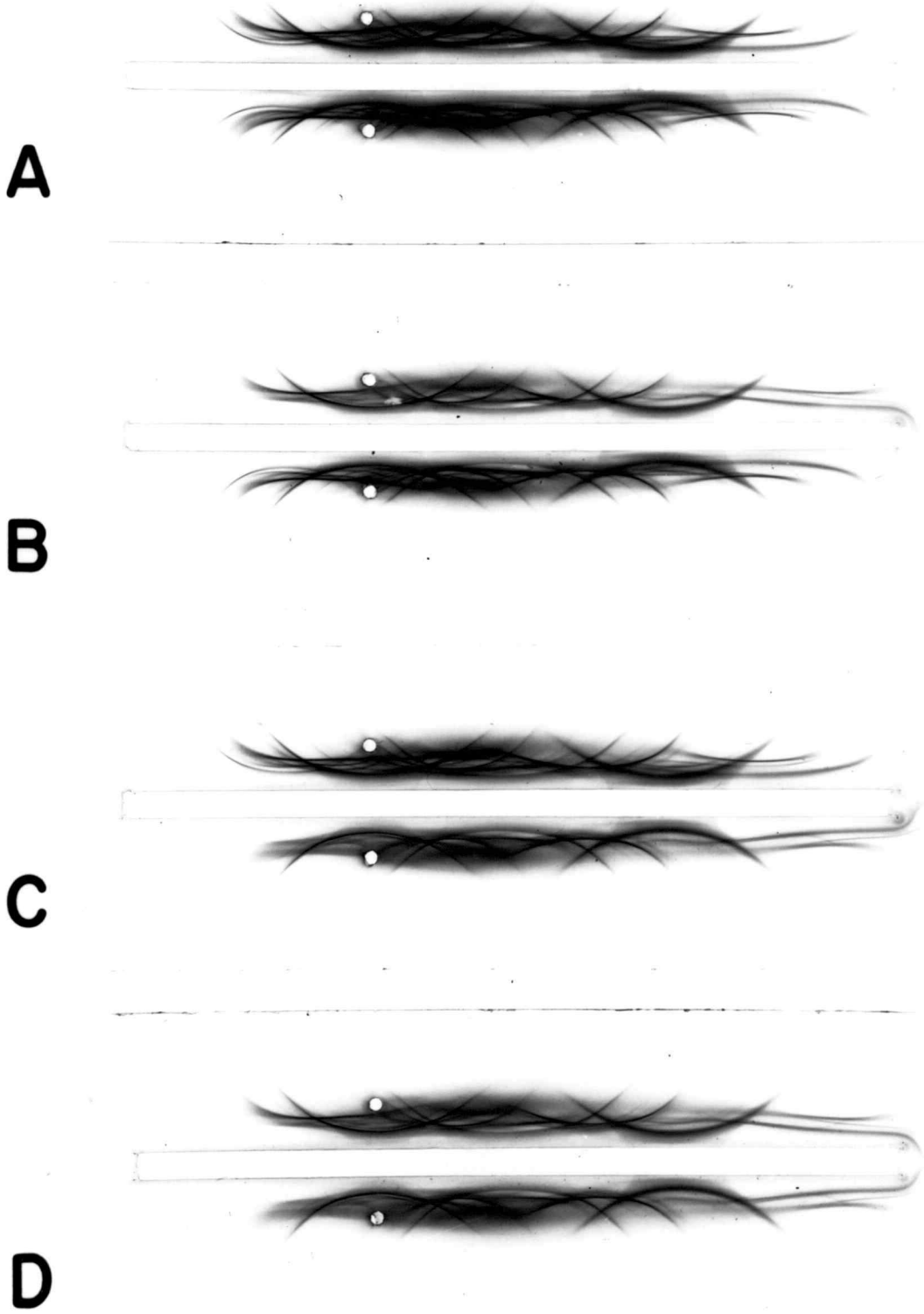
Figure 22. Immunoelectrophoretic comparisons of adsorbed and unadsorbed coho anti- *A. salmonicida* serum (immune serum) and normal serum (anode to the right).

A. Upper well: coho immune serum
Center trough: rabbit antiserum to coho serum
Lower well: coho normal serum

B. Upper well: adsorbed coho normal serum
Center trough: rabbit antiserum to coho serum
Lower well: coho normal serum

C. Upper well: coho immune serum
Center trough: rabbit antiserum to coho serum
Lower well: adsorbed coho immune serum

D. Upper well: adsorbed coho normal serum
Center trough: rabbit antiserum to coho serum
Lower well: adsorbed coho immune serum


A
B
C
D

adsorbed immune serum (D, lower well) revealed an obvious difference in the immunoglobulin arc. The possibility existed that the presence of A. salmonicida antigens in adsorbed samples altered immunoelectrophoretic patterns. Proof for this would require further experimentation.

It appeared that unadsorbed immune and normal serums were indistinguishable from their electrophoretic patterns. Adsorption of both immune and normal serums resulted in certain common changes. Immunoelectrophoresis but not alkaline polyacrylamide gel electrophoresis was capable of differentiating between adsorbed samples of immune and normal serum.

DISCUSSION

The work with coho salmon antibodies can be considered in three areas. The first concerned the number of readily distinguishable coho immunoglobulins. The second dealt with the overall structural features of the coho immune macroglobulin. The final consideration involved the macroglobulin's biological activity.

As pointed out earlier, work with other ray-finned fishes (subclass Actinopterygii) has not revealed the universal presence of high and low molecular weight immunoglobulins. In this study there was no evidence for the existence of a low molecular weight coho anti- *A. salmonicida* antibody. All the agglutination activity in coho antiserum was associated with the macroglobulin serum fraction (proteins with molecular weights > 200,000). This finding does not rule out the possibility that a low molecular weight monovalent antibody was present. The best evidence against a low molecular weight form came from work with specifically purified antibodies. Results from analytical ultracentrifugation, acrylamide gel electrophoresis, and immunoelectrophoresis all indicated that the purified antibodies were macroglobulins. The possibility existed that the isolation procedure selected against a low molecular weight form; however, this seemed unlikely.

Since the nature of an antigen can influence the kinds of

antibodies synthesized (Pike, 1967), the absence of low molecular weight antibodies is not conclusive evidence for the absence of low molecular weight serum immunoglobulin. The critical experiment would involve fractionation of coho serum proteins on the basis of size, followed by the determination of each fraction's ability to react with a potent rabbit antiserum against coho L chains. This kind of analysis was employed to show that gar serum contained only high molecular weight immunoglobulins (Bradshaw, Clem and Sigel, 1971).

Trump (1970) reported that goldfish antiserum contained two populations of immune macroglobulins which were distinguishable on the basis of net charge and antigenic composition. In the present study, the shape of the immunoglobulin arc in certain immunoelectrophoretic separations of serum suggested that a similar situation might exist for coho macroglobulins. The presence of a diffuse spur in the immunoglobulin arc after development with the anti-immune complex reagent was especially suggestive of this. Ion exchange chromatography of coho serum macroglobulins might offer definite proof for the existence of more than one immunoglobulin population.

One observation of particular interest was the apparent restriction of the purified coho antibodies to the anodic portion of the entire immunoglobulin arc. This restriction could have occurred during antibody synthesis or antibody purification. In either case, the purified anti- A. salmonicida antibodies were still a very heterogeneous

group of molecules. If the coho salmon can be shown to synthesize distinguishable populations of immunoglobulins, it would be worthwhile to examine the distribution of anti- A. salmonicida antibodies within each population.

Structural features of the coho anti- A. salmonicida antibody were quite similar to those reported for immune macroglobulins from the carp (Shelton and Smith, 1970), catfish (Acton et al. 1971a), giant grouper (Clem, 1971), gar (Acton et al. 1971b; Bradshaw, Clem and Sigel, 1971) and paddlefish (Acton et al., 1971a). Based on the structural properties of the coho antibody it should also be designated as a tetrameric IgM molecule.

Molecular weight estimates obtained from SDS-acrylamide gel electrophoresis suggested that coho L chains were slightly larger than human L chains (molecular weight, 23,000). The coho H chain appeared to have a molecular weight slightly greater than 70,000 (commonly accepted value for the human μ-chain). Coelectrophoresis of coho H chains and human μ-chains would be required prior to reaching any conclusion about the relative sizes of the two H chains. It should be pointed out that minor differences in apparent molecular weights of coho and human chains might result from factors other than actual size differences.

Clem (1971) was unable to obtain reductive macroglobulin subunits in his work with giant grouper immunoglobulins. In this study

the sensitivity of the SDS-acrylamide gel technic offered a molecular weight estimate of the coho macroglobulin subunit (~180,000 to 190,000). Of equal importance were the observations of L and H chain combinations. These strongly suggested that the subunit was typical (L-H-H-L). The use of SDS gels for the analysis of polypeptide chain structure is certainly not unique to this work. Other investigators (Sutherland, Zimmerman and Kern, 1970) have employed a similar approach to examine intracellular IgG assembly.

Certain additional points regarding the SDS gel electrophoresis technic seem worthy of comment. The relationship between a protein's mobility and its molecular weight is apparently dependent on two phenomena (Reynolds and Tanford, 1970a, b). The hydrophobic binding of SDS to the polypeptide chain imparts a change which is proportional to the size of a protein. The second phenomenon is that a protein-SDS complex assumes a rod shaped form which is also directly related to the protein's size. From even this simplified view, it seems obvious that intact disulfide bonds (especially intrachain disulfides) would influence the size of a protein-SDS complex. In the present study an attempt was made to compensate for the presence of disulfide bonding in the coho salmon antibody. This was done by employing standard curves which effectively estimated the molecular weights of partially and extensively reduced human IgG chains and chain combinations.

The possible existence of J chains in the coho antibody is of special interest. This component has recently been reported in human IgM (Mestecky et al. 1971; Frangione et al. 1971) and is thought to play a role in the joining of immunoglobulin subunits (presumably by disulfide attachment to H chains). Mestecky et al. (1971) have indicated that the J chain has a molecular weight of approximately 26,000 and is present in a ratio of one chain per IgM molecule.

The presence of coho J chains was suggested by the characteristic appearance of three rapidly migrating bands in electrophoretic separations of extensively reduced and alkylated coho antibody. However, analysis of the various immunoglobulin chain combinations in SDS-polyacrylamide gels failed to offer any evidence for coho J chains.[43/] In spite of this, the technic could prove to be of value in the detection of J chains. It would be worth-while to carefully analyze the various chain combinations produced by very mild reducing conditions. In this respect it should be noted that good data were not obtained from the partially reduced and alkylated coho immunoglobulin. Further studies concerning the presence of J chains (or J-like chains) are required with the coho antibody and other lower vertebrate immune macroglobulins.

43/ For instance, a band with L_2H-like mobility (actually L-H-J) was not observed.

The finding that the activity of the coho antibody was partially susceptible to reduction and alkylation raised the question as to what this meant in terms of antibody structure. Since the residual activity was lost after exposure to 2 M urea, it appeared that it was the result of noncovalent bonding rather than unreduced disulfides. Noncovalent reassociation followed by some restoration of antibody activity may prove to be a general property of IgM antibody subunits (Frank and Humphrey, 1969). From this standpoint, there seemed to be little value in differentiating the coho anti- A. salmonicida antibody from certain other lower vertebrate macroglobulins whose activities have been reported to be completely sensitive to reduction and alkylation.

The data concerning the activities of partially reduced coho antibodies also emphasized the need for working with purified antibody solutions. This is especially critical in studies designed to relate antibody structure to antibody function. The influence of extraneous serum proteins (including nonantibody immunoglobulin) on the activity of reduced preparations of human and rabbit IgM has been shown (Schrohenloher et al. 1964; Frank and Humphrey, 1969). This possibility deserves attention in studies with lower vertebrate immunoglobulins.

For various reasons the agglutinating activity of the coho antibody for A. salmonicida cells was of interest. Numerous

observations have shown that salmonid fishes develop very high agglutination titers to parenterally administered A. salmonicida cells. Results from this laboratory have indicated that titers of 1:40,960 are not uncommon in immunized juvenile coho (W. D. Paterson, personal communication). In addition, serum titers of 1:160 are often observed in juvenile coho which have had no apparent exposure to A. salmonicida.

The finding that an antibody concentration of approximately 0.04 μgm/ml can agglutinate A. salmonicida cells is essential for the proper interpretation of the above titers. Somatic agglutination of S. typhosa appears to have the greatest reported sensitivity of any previously studied bacterial agglutination reaction and requires approximately 0.12 μgm IgM/ml (Marrack, 1963; Pike, Schulze and Chandler, 1966). The A. salmonicida agglutination system thus seems to be about three times more sensitive than the S. typhosa system at detecting antibody. The reported minimum antibody concentration of 0.03 μgm/ml for the passive hemagglutination technic (Gill, 1970) further emphasizes the extreme sensitivity of the A. salmonicida system.

Certain points emerge from the above discussion. It appears that the high agglutination titers are not an indication of the salmon's ability to synthesize large amounts of anti- A. salmonicida antibody. Likewise, the high A. salmonicida titers can not be used as evidence

that this bacterium possesses exceptional antigenic powers in coho salmon. Quantitative information is required before any conclusions are possible regarding the relative antigenicity of different fish pathogens and antigen preparations. With respect to A. salmonicida, it would be of interest to know what percent of the serum immunoglobulin represents specific antibody in immunized coho.

A better understanding of orally induced immunity in juvenile coho salmon will require a knowledge of two problems. The first concerns the location of the protective barrier(s) in immunized fish. The second involves the series of events which results in the successful disposal of an invading pathogen. Knowledge of both phenomena might suggest a rapid means of quantitating orally induced, protective immunity. These are certainly not simple problems, and any meaningful answers will have to rest on information obtained from a variety of different experimental approaches.

An ability to identify the coho immunoglobulin and other serum proteins in electrophoretic separations should be of value in the examination of external secretions. In addition, a quantitative understanding of the A. salmonicida agglutination reaction will be helpful in the interpretation of mucus antibody titers. If antigenic and size comparisons indicate that antibodies in mucus and serum are similar, it would be reasonable to employ purified serum antibodies in studies of antibody function. It seems possible that the actual

protective mechanism involves one or both of the following: (1) a complement mediated, bactericidal effect, (2) an increased rate of phagocytosis (opsinization). Work with other systems has shown that IgM antibodies are very efficient in both the bactericidal reaction and the opsonic effect (Robbins *et al.* 1965; Pike, 1967). With rabbit IgM antibodies against *S. typhimurium*, both of these protective reactions can be demonstrated when the antibody concentration is less than that required for visible agglutination.

SUMMARY AND CONCLUSIONS

1. From work with pooled antiserums, it appeared that anti- *A. salmonicida* agglutination titers reached their maximum values between 10 and 16 weeks after intra-abdominal inoculation of juvenile coho salmon with *A. salmonicida* cells in Freund's Complete Adjuvant (water temperature, 54 C). The synthesis of a 2-ME resistant population of agglutinins was not observed during the primary immune response (observation period, 20 weeks).

2. Partial reduction and alkylation of coho salmon anti- *A. salmonicida* antiserum markedly lowered but did not completely destroy agglutination activity. Work with immune serum and purified antibodies suggested that the residual activity was maintained by noncovalent bonding. The effects of partial reduction and alkylation on antibody activity seemed to be influenced by the presence of nonantibody serum protein.

3. A detectable loss of anti- *A. salmonicida* agglutination activity occurred after heating immune serum for 30 minutes at 50 C. No loss of activity was observed after 30 minutes exposure to 45 C.

4. All the agglutination activity in coho salmon anti- *A. salmonicida* serum was associated with the large molecule

(molecular weight > 200,000) fraction of serum.

5. Juvenile coho salmon serum was resolved into 24 components by alkaline polyacrylamide gel electrophoresis and approximately 23 components with immunoelectrophoresis. Immune and normal serums were indistinguishable by either technic. Immunoelectrophoresis showed differences between samples of immune and normal serums which had been adsorbed with A. salmonicida cells.

6. Immunoelectrophoresis revealed a minimum of three proteins in immune serum which could bind to A. salmonicida cells. Only one of these appeared to be immunoglobulin. The asymmetrical shape of the immunoglobulin arc in immunoelectrophoretic separations of coho salmon serum suggested some type of heterogeneity.

7. Coho salmon anti- A. salmonicida antibodies were isolated by dissociation of bacterial cell wall immune complexes with 15 percent NaCl. The procedure resulted in the recovery of about 30 percent of the anti- A. salmonicida antibody.

8. Analytical ultracentrifugation, alkaline polyacrylamide gel electrophoresis, immunoelectrophoresis, and UV absorption spectroscopy all indicated that the isolated antibodies were in a high state of purity.

9. The $S^0_{20,W}$ of the coho antibody was about 17.1S

10. In alkaline polyacrylamide gels, the immune macroglobulin was identified as the slowest migrating serum protein.

11. Immunoelectrophoretic analysis indicated that the purified antibodies were restricted to the anodic portion of the serum immunoglobulin arc. The reason for this restriction was not determined.

12. The coho salmon immune macroglobulin was composed of four covalently joined subunits. The estimated molecular weight of the subunit was between 180,000 and 190,000. Each subunit was composed of 2 H and 2 L chains which were covalently attached in the usual manner (L-H-H-L). The molecular weight of the H chain appeared to be in the neighborhood of 74,000 ± 2,000. The estimated molecular weight of the L chain was 26,000, although this value may be slightly high. It did appear that coho L chains were slightly larger than human L chains. From the above considerations, the coho salmon antibody was designated as a tetrameric IgM molecule.

13. The purified anti- A. salmonicida antibodies were a very heterogeneous collection of molecules. This was shown by the finding that L chains from an antibody preparation were separable into a minimum of 11 electrophoretic subpopulations.

14. There was a suggestion that the coho salmon macroglobulin contained a polypeptide chain which was similar to the recently described J chain in secretory IgA and human IgM. Further work is required before any firm conclusions are possible concerning the presence of J-like chains in the coho salmon antibody.

15. Results obtained from immune serum and work with purified antibody solutions indicated that approximately 0.04 μgm/ml was the minimum coho salmon antibody concentration required for the agglutination of A. salmonicida cells. The extreme sensitivity of this agglutination reaction is an important consideration in any interpretation of coho salmon anti- A. salmonicida agglutination titers.

16. Information obtained in this study will help in the elucidation of problems which are critical for the understanding of orally induced immunity.

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