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Humoral and Secretory Immunoglobulins of the Sheepshead, Archosargus probatocephalus, A Marine Teleost

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Craig Jethro Lobb

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ABSTRACT

Humoral and Secretory Immunoglobulins

of the Sheepshead,

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by

Craig J. Lobb, Doctor of Philosophy

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The sheepshead has two readily isolatable humoral immunoglobulins, a 16S tetrameric form and a 6S monomeric form. The 16S tetrameric form is composed of two subpopulations, one being a disulfide linked form (~ 700,000 daltons) and the other a noncovalently linked population of predominantly disulfide linked dimers (~ 350,000 daltons). The 6S immunoglobulin (~ 140,000 daltons) is composed of two noncovalently linked units (~ 70,000 daltons) each having one heavy and one light chain. The 6S immunoglobulin is antigenically deficient to the 16S immunoglobulin. This deficiency may be due to the heavy chain of the 6S protein lacking a ~ 25,000 dalton segment present in the heavy chain of the 16S molecule.

Cutaneous mucus and bile also contain immunoglobulins. The mucus contains three proteins that can be considered

immunoglobulins: a 6S form which is antigenically indistinguishable from the serum 6S immunoglobulin; a ~700,000 dalton form which does not have a "dimeric" subpopulation as observed with the serum 16S protein; and a dimeric form of ~350,000 daltons. The dimeric form may have a secretory piece since the reduced mucus dimeric protein shows an additional polypeptide chain at ~95,000 daltons. All of the cutaneous mucus high molecular weight immunoglobulins have heavy and light chains identical to the serum high molecular weight immunoglobulins (~ 70,000 and ~25,000 daltons).

Bile immunoglobulin is dimeric and composed of two noncovalently linked monomers of ~160,000 daltons. The bile heavy chains are ~55,000 daltons; the light chains are ~25,000 daltons. The bile immunoglobulin does not appear to be a different class of protein from that of the serum or mucus immunoglobulins.

In vivo administration of radiolabeled 16S and 6S serum immunoglobulins indicates that the 6S protein is not a degradation product of the 16S form. The half lives of the 16S and 6S forms are both ~16 days.

Furthermore, the secretory immunoglobulins of the bile and mucus are not due to simple transudation or active transport of the predominant serum immunoglobulins. This result suggests that the secretory immunoglobulins of the sheepshead may be locally synthesized.

(153 pages)

INTRODUCTION AND LITERATURE REVIEW

Immunoglobulins (Igs) together with the proteins of the complement system form the basis for the vertebrate humoral immune system. Igs are produced by the plasma cells of vertebrates in response to the presence of an antigen, a substance foreign to that particular animal. Antibodies are recognized by their ability to bind and complex to that specific antigen. The Igs themselves are composed of a complex system of structurally related proteins found in the sera of all vertebrates.

In mammals there are five classes of Igs: IgM, IgA, IgD, IgG and IgE. Class distinctions are made because of structural (amino acid) differences. Studies with mammalian Igs in the middle fifties to late sixties described the different classes, subclasses and genetic types, and showed that Igs differed not only in their chemical structure but in their biological properties as well. IgM and IgG are the predominant Igs in serum and are classically involved in the primary and secondary responses of an animal when exposed to an antigen (Smith et al., 1959; Nossal et al., 1964; and Pernis et al., 1971). IgD and IgE are found in trace quantities in serum (less than 0.03 mg/ml), and both are intimately associated with particular lymphoid cells capable of triggering powerful cellular reactions (reviewed by

Nisonoff et al., 1975). IgA, commonly referred to as the secretory antibody, is the predominant class in avascular secretions such as saliva, tears, gut mucosa and colostrum (reviewed by Tomasi and Bienenstock, 1968; Tomasi, 1976).

Topographically all Igs are composed of four polypeptide chains: two identical "heavy" (H) chains and two identical "light" (L) chains. This distinction between H and L chains is based on their molecular weights (for IgG, H = 50,000 daltons and L = 25,000 daltons) as well as their amino acid compositions. The basic 2H:2L structure is typical of monomeric IgG. IgM and IgA, however, are usually found in multiples of class specific monomeric units. IgM in mammals is composed of five such monomeric units. Its five subunits are arranged in a closed circle with the Fc domains extending inward (Chesebro et al., 1968). The most common form of polymeric IgA is dimeric, with its two Fc domains abutted end to end to form a dumbbell-shaped molecule (Munn et al., 1971).

Polymeric IgM and IgA have also been shown to contain another protein chain, designated the J chain (Halpern and Koshland, 1970; Mestecky et al., 1974). The J chain in polymeric species may have an important function in polymerization (Koshland, 1975). The J chain represents less than five percent of the total polymer protein with a molecular weight of 15,000 daltons.

In addition to the J chain secretory IgA contains an additional protein chain designated the secretory component or SC. The molecular weight of the human SC is approximately 58,000 to 72,000 daltons when determined by sedimentation equilibrium, or 75,000 to 85,000 daltons as obtained by gel filtration or polyacrylamide gels. The SC is not an immunoglobulin because its amino acid composition (high glycine, no methionine) differs from H or L chains. Unlike immunoglobulin chains, SC is produced in epithelial cells rather than B cells. The SC is complexed to IgA in a manner not yet known. Complexing may occur either as IgA is transported through the epithelial cell to the mucosal layer or outside the cell in the mucosa. SC increases resistance of dimeric IgA to proteolysis, and its supposed function is related to this enhanced resistance (reviewed by Tomasi and Bienenstock, 1968; Tomasi, 1976).

In recent years, bile has been shown to be a rich source of IgA in higher vertebrates. Bile of chickens, rats and humans has been shown to be heavily enriched with IgA (Bienenstock et al., 1973; Lemaitre-Coelho et al., 1977; Brown et al., 1979). Intravenously injected monoclonal rat IgA is cleared from rat serum into the bile at a very fast rate (93 percent in four hours). Rat liver perfusions demonstrated this rapid clearance was due to active secretion of serum IgA into

bile. This active process occurred against a strong concentration gradient which was not observed for rat IgG_{IIa} and albumin (Jackson et al., 1978). Rapid disappearance of injected IgA (isolated from serum or ascitic fluid) into bile did not occur in rats with ligated bile ducts. In quantitive *in vivo* clearance assays, labeled IgA appeared rapidly in the bile so that 25 percent of the injected dose was recoverable in three hours. At this three hour peak, specific radioactivity of the bile (cpm/mg protein) was about 200 times greater than serum (Orlans et al., 1978).

Intraperitoneal and intragastric immunization of rats with foreign red blood cells elicited antibodies in both bile and serum. Selective predominance of IgA in bile was observed for both immunization routes, with the intragastric giving a greater local response and a lower systemic response (Lemaitre-Coelho et al., 1978). *Brucella abortus* IgA antibodies were also observed in rat bile within four days (reaching substantial levels in eight days) following direct injection of the killed bacteria into the intestine. Therefore, bile may be an important means of directing IgA to the gut for protective functions.

A study analyzing the proteins of rat bile distinguished 16 proteins (Mullock et al., 1978). Thirteen of these proteins are immunologically related to those found in serum. However, the relative proportions of

these bile proteins differ from their proportions in serum. It therefore appears that the majority of bile proteins, although probably derived from serum, cannot be accounted for by direct leakage of serum into bile during collection procedures.

The distinct possibility exists that the IgA found in intestinal washings may indeed be derived from the bile. However, it would be presumptuous to assume that in native intestinal secretions, most of the IgA comes from indirect biliary origin and not from the intestinal epithelium. The strong interactions of IgA with the intestinal mucosa may not allow for efficient recovery, thus making bile the major readily isolated secretion for analyzing secretory immune activity in the gastro-intestinal tract.

IgM is second to IgA as the major class of immunoglobulin in certain external secretions of man (Tomasi and Bienenstock, 1968). Numerous studies suggest that IgM and IgA share similar (if not identical) mechanisms of secretion and transport through mucus membranes; local synthesis in mucosal plasma cells and selective transport into external body fluids have been demonstrated (Allen et al., 1976; Brandtzaeg et al., 1968; Brandtzaeg, 1971). In addition, some human patients selectively deficient in IgA have been shown to have an increase in the local synthesis and secretion of IgM (Brandtzaeg, 1971; Coelho et al., 1974).

Immunocytological studies have localized IgA, IgM and SC in identical cellular and ultrastructural sites in human intestinal epithelium (Brandtzaeg, 1975; Brown et al., 1976). IgM, similar to IgA, has been found to be linked to SC in certain external secretions (Brandtzaeg, 1975; Coelho et al., 1974). 19S IgM containing J chain has been shown to be as efficient as dimeric IgA in the formation of spontaneous, non-covalently bonded complexes with free SC in vitro (Brandtzaeg, 1974; Eskeland and Brandtzaeg, 1974). However, human IgM-SC complexes formed and incubated in vitro with intestinal fluids do not appear to be as resistant to proteolysis as IgA-SC complexes.

However, IgM-SC complexes from secretions showed that the complexes were weakly stabilized by covalent interactions (Brandtzaeg, 1975). Proteolytic resistance assays for secretory IgM have not yet been carried out. Analysis of IgM in intestinal fluids of man found that in one half of the seven samples analyzed there was IgM reacting material that had the same sedimentation coefficient as dimeric IgA (10S). Unfortunately, studies were not performed to assay the binding of this 10S IgM to the SC (Richman and Brown, 1977).

Individuals selectively deficient in IgA have a compensatory local immune mechanism expressed by enhanced local synthesis and secretion of IgM (Brandtzaeg, 1975; Brown et al., 1975). One case of

hypoαglobulinaemia studied showed that IgM was the only secretory immunoglobulin in parotid fluid (Brandtzaeg, 1975). IgM in this case antigenically contained both the J chain and the secretory component. Paired immunoflorescent staining showed that IgM was present in the columnar cells of the colonic glands corresponding exactly to IgA distribution. This was in sharp contrast to IgG which was not detected in the columnar epithelial cells. Interestingly, neonatally thymectomized rats lost the ability to make salivary IgA, but were able to compensate by secreting IgM into these secretions (Ebersole et al., 1979).

In summary, there are five primary classes of Ig in mammals, each distinguished by H chain primary structural differences. Of these classes IgM and IgA are polymeric, functioning in multiples by the possible role of J chain in polymerization. IgA, the predominant secretory Ig, contains SC which is hypothesized to be required for glandular transmission through epithelial cells and/or to confer protease resistance. IgM has also been shown to bind SC <u>in vitro</u> and <u>in vivo</u> and functions as a secretory Ig especially in IgA deficient mammals.

In contrast to studies of mammals and other higher vertebrates, studies of the immunoglobulins of fishes have delineated only one class of Ig. Whereas shark as well as higher vertebrates have a pentameric (19S)

IgM, teleosts have a tetrameric (16S) immunoglobulin.

Absolute assignment of lower vertebrate Ig classes to mammalian Ig classes depends on amino acid sequence data. Since no fish Ig has been sequenced to date, class assignment of teleost Igs has relied on the properties of the component polypeptide chains. The most extensive immunochemical analysis of teleost immunoglobulins to date has been with the giant grouper (Clem, 1971). Clem's analysis suggested that the teleost's Igs most closely resembled mammalian IgM because of the molecular weight of the intact macroglobulin (~700,000 daltons) as well as the H chains (~70,000 daltons). In addition the hexose content, as determined by the orcinol method, suggested that the relatively high sugar content most closely resembled IgM.

Studies analyzing the humoral Igs of the teleosts, the most "advanced" fishes, can be grouped into three major subdivisions.

1) Those studies which have defined only one form of IgM, commonly referred to as "high molecular weight" Ig.
2) Those studies which have defined two humoral Ig forms, the "high molecular weight" and a "low molecular weight" form. These two forms are in a polymeric - monomeric relationship, with the H chains antigenically indistinguishable.

3) One study defined both high and low molecular weight forms in which the low molecular weight form H chains "lacked" a ~30,000 dalton segment present on the high molecular weight H chains. This resulted in an antigenic relationship of partial identity.

The immunoglobulins of trout were studied in detail by Hodgins et al. (1967). The trout antibody produced to keyhole limpet hemocyanin was observed to be approximately 14S. No low molecular weight antibody was found, based upon precipitation of agglutination assays. However, natural agglutinins to foreign red blood cells were observed in the 14S population as well as a 4.2S low molecular weight population. Although the investigators hypothesized that a polymer-monomer Ig relationship may exist in the trout, they felt that this 4.2S population probably represented a complement type protein. The question of electrophoretic mobilities of trout Igs was also examined. Previously, Hodgins et al. (1965) showed that the trout possessed two populations of Ig differing in their electrophoretic mobilities. Contrasting trout Ig mobilities to human sera, a "fast" gamma as well as a beta globulin were identified. These two populations were also observed in the later study (Hodgins et al., 1967). The 14S Ig was observed to have the "fast" gamma mobility whereas the 4.2S protein demonstrated beta mobility.

The immunoglobulins of trout were also studied by Cisar and Fryer in 1974. Their research showed that the purified anti-Aeromonas salmonicida Ig was a 17S macroglobulin. Electron microscopy demonstrated a "+" shaped molecule, suggesting that the 17S Ig was composed of four IgM-like subunits. Although the specifically purified 17S Ig electrophoretically formed a precipitin arc with a predominant B mobility, two electrophoretic populations (a gamma and a beta) showing partial identity were observed. The gamma Ig population was not characterized, and the possibility that another Ig population exists besides the 17S Ig(s) was not precluded. So the question of whether or not trout have a low molecular weight Ig is unresolved, but the data reported suggest that the 14-17S Ig population may be heterogeneous.

Uhr et al. (1962) immunized goldfish with bacteriophage ΦX 174 and assayed their immunoglobulins. This investigation resolved goldfish serum into two fractions under conditions which separated human IgM (19S) gamma globulin from the 7S (IgG) gamma globulin. Codeterminations of antibody level in these two fractions indicated that antibody activity was evenly distributed between these fractions at 2 months post injection, but at 5 months was almost entirely contained in the 7S fraction. This apparent switch to the 7S antibody fraction was expedited by elevating the water temperature from 30 C to 32 C. Thus Uhr and colleagues concluded

that a 7S form not only exists but is a maturation type antibody in the goldfish. Since antigenic or biochemical identification was not performed, no class relationship was postulated for the 19S and 7S fractions.

Goldfish antibodies to bovine serum albumin (BSA) were examined in subsequent studies (Trump, 1970 and Trump and Hildemann, 1970). Passive hemagglutination assays determined that the Igs in goldfish were composed of two distinct populations, based upon preparative starch block electrophoresis methods. Both Ig populations when analyzed by analytical ultracentrifugation gave ~16S boundaries. Yet the electrophoretic populations were apparently not antigenically identical as defined by rabbit anti-whole goldfish serum. One population had a determinant which the other one did not, resulting in partial identity in double diffusion assays. Analysis of these two populations during primary and secondary immune responses did not detect appreciable differences in serum concentration; therefore, no dramatic switch-over from one to the other could be suggested. Unfortunately, data was not generated to support Uhr et al.'s (1962) finding that a 19S switch to a 7S Ig existed. This question was not directly addressed, although by hemagglutination no low molecular weight Ig was found.

Although a low molecular weight Ig in trout and goldfish has not yet been characterized, other fishes have been found to have this Ig species. The margate

Haemulon album has been found to have a high molecular weight Ig, when analyzed by hemagglutination or precipitation techniques (Clem and Sigel, 1966). The margate was reinvestigated later (Clem and McLean, 1975) to determine if low molecular weight Ig could be discovered using more sensitive assays. By monitoring margates immunized with BSA using hemagglutination and antigen binding assays, the margate was found to have both a 16S and a 7S Ig. The 16S population accounted for all hemagglution reactions observed. Antigen binding levels, however, gave peak reactions of ~82 for 16S Ig and ~35 for 7S Ig. The 16S Ig (~700,000 daltons) and the 7S Ig (~175,000 daltons) were antigenically identical. Using antisera to the 16S or 7S molecule, the H chains isolated from the 16S and 7S Igs were identical to each other and nonidentical to L chains. Likewise the two L chains were identical to each other. Thus the margate appeared to have a tetramer - monomer relationship.

The immunoglobulins of the giant grouper, Epinephelus itaira, have also been investigated (Clem and Small, 1970 and Clem, 1971). A 16S Ig was identified, as is typical in most fishes. What makes the grouper unique is the observation and characterization of the relationship of the low molecular weight Ig to the 16S Ig. A 6.4S Ig was identified and found to contain a heavy chain that was ~30,000 daltons smaller than the H chain from the 16S Ig. This deficiency was suggested to account

for the double immunodiffusion reaction of partial identity between the 6.4S and 16S populations. The L chains were identical. Peptide mapping and amino acid composition analyses of the H chains from the 16S and 6.4S Igs indicated there were minimal differences. This finding suggested that the relationship of the 6.4S Ig to the 16S Ig was similar to an F $(ab'\mu)_2$ like fragment. Additional studies to determine any metabolic (anabolic vs. catabolic) interrelationship between the groups 16S and 6.4S Igs were not done.

Few studies have been done to determine if teleosts have an avascular or secretory immunoglobulin system. Immunoglobulins of the plaice, Pleuronectes platessa, were detected in serum and in mucus samples from the intestine and body surfaces (Fletcher and Grant, 1969; and Fletcher and White, 1973). The plaice's antibodies to the bacterium Vibrio anguillarum were detected by passive hemagglutination. The plaice antibodies were not physiochemically characterized except that they were larger than 200,000 daltons (appeared in void volume of Sephadex G-200). Interestingly, in orally vaccinated plaice, titers were greater in intestinal mucus extracts than in serum, which is suggestive of a secretory immune system. This observation was reversed in fish immunized by subcutaneous or intraperitoneal injections.

The Australian catfish, Tachysurus australis, was

shown to have a protein in cutaneous mucus and intestinal washings which by double diffusion reacted with a pattern of identity to serum antibody (DiConza and Halliday, 1971). Intraperitoneal and intramuscular BSA injections of these catfish were unsuccessful in eliciting antibody production in mucus samples, although high serum titers were observed.

Bradshaw et al. (1971) demonstrated hemagglutinating antibody in cutaneous mucus from the gar, Lepisosteus platyrhincus. This mucus antibody was 2-mercaptoethanol sensitive and was greater than 200,000 daltons (voided Sephadex G-200 column). The mucus antibody was shown by double diffusion to be identical to the gar serum Ig.

It was with this background that the research discussed herein was undertaken. The objectives of this research were twofold. The first was to purify and characterize the immunoglobulins present in blood, mucus and bile of a marine fish. The second objective was to determine if the immunoglobulins present in mucus and bile were derived from blood.

MATERIALS AND METHODS

Collection of sheepshead

Sheepshead, *Archosargus probatocephalus*, were collected in the inland waterway near the Whitney Laboratory at Marineland, Florida. The sheepshead (~600 grams) were kept individually in 30 gallon Nalgene tanks supplied with fresh running seawater. Water temperature during the experiments presented herein was 26 C. Sheepshead were fed to satiation two to three times weekly with frozen shrimp and/or live fiddler crabs. All animals were held at least a month before experimentation. There were no visible lesions in any fish used, and the fish appeared healthy.

Preparation and collection of sheepshead serum, bile and cutaneous mucus

Blood was drawn with a 19 gauge needle from the caudal artery/vein. Serum was allowed to clot at room temperature for 1 hour, ringed with a wooden applicator stick and left overnight (8 to 12 hours) at 4 C. The blood was centrifuged at 2500 RPM for 15 minutes at 4 C and the serum collected. Serum was stored at -20 C until analyzed.

Cutaneous mucus samples were collected from sheepshead anaesthesized with tricane methanesulfonate

(Crescent Research Chemicals, Inc., Paradise Valley, Az.) at the level of 150 milligrams/liter. Fish were rinsed with 0.15 M NaCl-Tris pH 7.4 and blotted. Then using the same buffer the mucus was gently wiped from the fish with filter paper and collected into a funnel that drained into a 50 ml centrifuge tube which was on ice. The 50 ml of mucus washate was centrifuged at 2500 RPM at 4 C for 30 minutes. The supernate was decanted and frozen at -20 C until concentrated.

Bile was collected from fish that had not received food for three days. Bile was collected by first removing the gall bladders from exsanguinated fish. The gall bladders were then stripped of adhering mesentaries and fat, rinsed thoroughly in several changes of 0.15 M NaCl and blotted. The free flowing bile was collected by puncturing the posterior end of the gall bladder. Bile was frozen at -20 C until analyzed.

Concentration of samples

All samples except L chains were concentrated by positive pressure dialysis at 13 psi. L chains were concentrated at 10 psi.

Gel filtration

Gel filtrations were made on columns of A-5M (BioRad Laboratories, Richmond, Cal.) at room temperature (specific size as indicated in figures). Fractions were collected at a flow rate of 10 ml/hour. The buffer

for all the column profiles presented was 0.15 M NaCl-Tris pH 7.4, except for the column separation of H and L chains which used 5 M guanidine HCl.

Ion exchange chromatography

Sheepshead serum was dialyzed against 0.015 M Tris HCl, pH 8.0. Serum was then loaded onto 200 ml of DEAE-cellulose (DE 32) which had been equilibrated in the above starting buffer. Linear gradient elution was with 700 ml starting buffer and 700 ml limit buffer (0.4 M NaCl in 0.015 M Tris HCl, pH 8).

Sheepshead mucus was equilibrated also in starting buffer. The equilibrated mucus was then loaded onto 10 ml of DEAE-Sephadex. Elution was stepwise with increasing molarities of salt (as indicated in figures) in the starting buffer.

Antigenic analysis of sheepshead immunoglobulins

Immunization of New Zealand white rabbits was done according to procedures described by Clem and Small (1967).

Ouchterlony analysis was done in 1 percent agarose with 0.15 M barbitol buffer pH 7.4 at room temperature. Immunoelectrophoresis was done on microscope slides coated with 1 percent agarose in barbitol buffer pH 8.6. Electrophoresis was at 1.5 mAmps/slide for 1 hour at room temperature. Precipitin bands were allowed to

develop at 4 C.

Polyacrylamide gel electrophoresis

Vertical slabs of 4 percent acrylamide crosslinked with 0.7 percent N, N′-diallytartardiamide were used for sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE). This gel (described by Ziegler and Hengartner, 1977) was electrophoresed for 1 hour at 15 mAmp; then the current was increased to 25 mAmp for ~5 hours. Electrophoresis was at 18 C.

Proteins to be used were analyzed at a concentration of 50 to 100 µg. Proteins to be reduced were mixed with 2-mercaptoethanol to effect a concentration of 1 percent (v/v) (~ 0.14 M). 10 µl of 20 percent SDS was added to the reduced or unreduced proteins in a volume of ~100 µl. The samples were stoppered and immediately heated to 100 C for 3 minutes in a boiling water bath. 2 µl of 0.005 percent bromphenol blue and two drops of 80 percent sucrose were added to the sample before loading onto the slab gel.

After electrophoresis, gels were fixed in 7 percent glacial acetic acid/40 percent ethanol. If staining were desired, the gel was stained with 0.125 percent Comassie Blue R-250 (in the fixing solution) for 2 hours. Destaining was in 7 percent glacial acetic acid/10 percent ethanol.

For gels to be used for autoradiography, the gel

was fixed as above and then soaked for 30 minutes in 7 percent glacial acetic acid/1 percent glycerol. The slab was dried for 2 hours on a slab dryer (BioRad model 224) according to the manufacturer's instructions. The dried slab was juxtaposed with Kodak X-Omat R film in the dark and left in an X-ray cassette for 1 to 7 days. The film was then developed and fixed according to manufacturer's directions.

Analytical ultracentrifugation

Sedimentation velocity studies were made in a Spinco Model E ultracentrifuge under conditions of analysis as described in the figures.

Preparation of polypeptide chains

Sheepshead Ig in 0.15 M NaCl-Tris pH 7.4 was mildly reduced with 0.05 M 2-mercaptoethanol for 30 minutes at room temperature. The protein was alkylated overnight with 0.06 M iodoacetamide at 4 C. The sample was then extensively dialyzed with 0.15 M NaCl-Tris pH 7.4 at 4 C and equilibrated in 7 M guanidine HCl. Next the sample was gel filtered on A-5M in 5 M guanidine HCl to resolve the H and L chains.

Analysis for the presence of J chains was by the use of alkaline-urea gels. The gel was used as described by Reisfeld and Small (1966). The L chain pool was mildly reduced with 0.05 M 2-mercaptoethanol for 30 minutes at room temperature then alkylated with

.06 M iodoacetamide overnight at 4 C. Next the sample was briefly dialyzed against the alkaline-urea gel top buffer for 2 hours. Gel was loaded, fixed, stained and destained as described for SDS-PAGE.

Elution of immunoglobulins from acrylamide gels

SDS-PAGE was run on the sheepshead high molecular weight immunoglobulin as described. The elution method used was a modification of the method described by Bray and Brownlee (1973). The gel was fixed, stained and destained. The tetrameric and dimeric subpopulations were separately cut from the gel with a clean scalpel. The gel bands were chopped into very small pieces and mixed with a solution of 0.05 M phosphate pH 7.5, 0.1 percent SDS, and 0.1 percent periodate. The protein was precipitated with potassium chloride to a final concentration of 0.2 M and left on ice for 15 minutes. The precipitates were collected by centrifugation at 10,000 x g for 20 minutes. The sample was suspended in 0.15 M Tris NaCl, pH 7.4, dialyzed for 2 hours against the SDS-PAGE running buffer and then reanalyzed by SDS-PAGE.

Bile purification

After much experimentation a new method was developed for initially purifying bile. The method was termed immobilized zone preparative electrophoresis.

Whole bile was centrifuged for 15 minutes at 2500 RPM at 4 C and the supernatant decanted. The supernate was then dialyzed against three changes of 0.1 M sodium phosphate pH 6.5.

The preparative electrophoresis apparatus used three plastic tubes 16 cm X 2 cm. Each tube was sealed at the bottom with plastic wrap held in place with a rubber gasket. This tube was inserted into the apparatus. 15 ml of 1 percent agarose, 9 percent acrylamide (acrylamide/bisacrylamide = 37.5:1; polymerized with 0.06 percent ammonium persulfate and 0.06 N, N, N', N'-tetramethyl ethylenediamine [TEMED]) was poured into each tube, filling about one third of the tube. After polymerization, the plastic wrap was removed from the tubes, and the top and bottom resevoirs were filled with the above phosphate buffer. An amount of 80 percent sucrose equal to 5 percent of the bile volume was added to the bile. The bile was then pipetted onto the top of the agarose/acrylamide plug. Electrophoresis was at a constant current of 10 mAmps per tube for 8 to 10 hours. At the end of electrophoresis, the top buffer was discarded, and the liquid on top of the plug, which contained the bile Ig, was collected. The electrophoresed sample was centrifuged if needed to remove aggregated lipids at 2500 RPM for 15 minutes at 4 C, and the supernatant was concentrated.

Staph A immunoprecipitation

Staphylococcus aureus (Cowans I strain) was prepared according to manufacturer's directions (Enzyme Center, Inc., Boston, Mass.). For immunoprecipitation 50 µl of sample were mixed with 50 µl of buffer + NP-40 (0.15 M NaCl, 0.005 M EDTA, 0.05 M Tris, 0.02 percent sodium azide and 0.5 percent NP-40, pH 7.4). 10 µl of specific antisera was added and the reaction continued for 1 hour on ice. Pellets were washed three times in the above buffer and the bound material solubilized with 1 percent SDS. The sample was then prepared for SDS-PAGE analysis as described above.

Radioiodination of immunoglobulins

Two methods for iodination were used. The chloramine-T technique was done as defined by McConahey and Dixon (1966). Iodogen (Pierce Chemicals, Rockford, Illinois) was used to label proteins as recommended by the manufacturer. A Beckman model Gamma 7000 spectrometer was used for counting ^{125}I.

Trichloroacetic acetic acid (TCA) precipitation of serum and bile was done by mixing the labeled sample with 0.5 ml bovine serum albumin, then bringing the sample to 10 percent TCA. After 15 minutes on ice, the sample was centrifuged, and the pellets were washed three times with 10 percent TCA. The washed pellets were then counted.

Ether/alcohol extraction of TCA precipitates were

performed using the method of Entenman (1957).

Radial immunodiffusion analysis

All radial immunoassays were done in 1.5 mm slabs. The buffer was 0.15 M phosphate, pH 7.4. Anti-HMW specific antiserum (absorbed empirically with LMW Ig) was used at a dilution of 1:100 for serum and 1:200 for mucus and bile in 1 percent agarose.

LMW quantitations were done using the unabsorbed anti-HMW antiserum in 9 percent polyacrylamide (acrylamide/bisacrylamide = 20:1) / 1 percent agarose gels. Antisera dilutions were the same as above. Ig levels were determined from a standard curve of LMW or HMW purified standards.

RESULTS

Characterization of the humoral immunoglobulins of the sheepshead

Purification of sheepshead humoral immunoglobulins was a two step chromatographic process. The first step was purification with DEAE (DE-32), represented in Figure 1. Shown here are the protein and NaCl gradient profiles. No protein was eluted until the conductivity reached ~3,000 mhos. Pool 1, the first peak to elute, represents the serum protein eluted at the conductance range of 3,000 to 6,000 mhos. This pool was then concentrated and fractionated on A-5M in the second chromatographic step for purification.

Figure 2 shows the protein profile of DEAE Pool 1 on A-5M. Five peaks based upon the molecular weight sieving action of this resin were observed. Of these five peaks only two peaks reacted with rabbit anti-sheepshead Ig. These two peaks, designated Pool A and Pool B, were separately pooled then each was repassed on A-5M. Since this column had been precalibrated with known molecular weight standards, it was possible to determine estimated molecular weights for Pools A and B. Pool A eluted in the 700,000 to 800,000 dalton area and was referred to as the high molecular weight (HMW) sheepshead Ig. Pool B eluted in the 130,000 to

Figure 1. DEAE-cellulose chromatography of sheepshead serum. Conditions of fractionation: 100 ml serum was applied to a 5 X 40 cm column equilibrated with 0.015 M Tris, pH 8. A linear gradient was formed with limit buffer of 0.4 M NaCl, 0.015 M Tris, pH 8. Fraction volumes 14 ml.

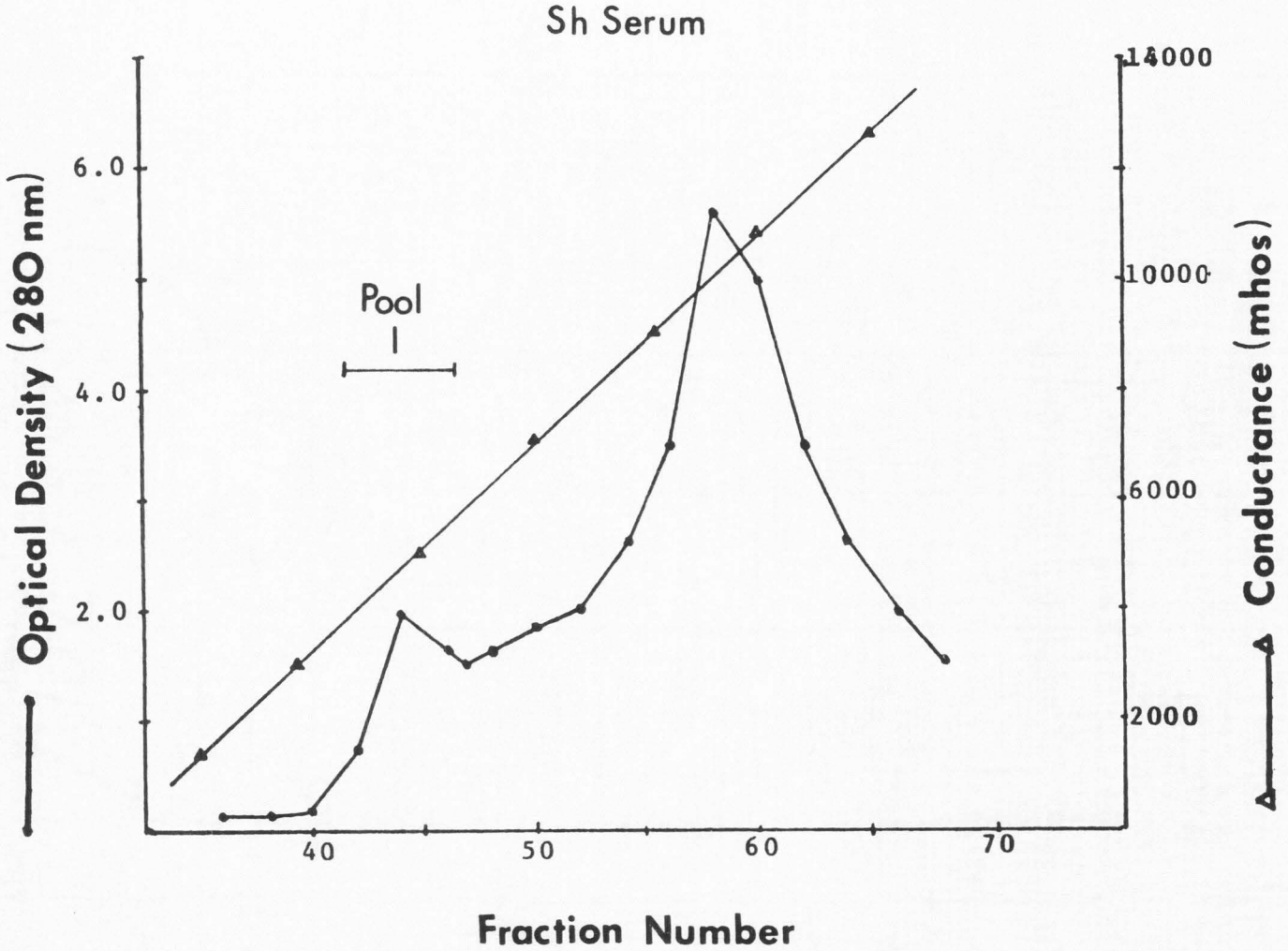
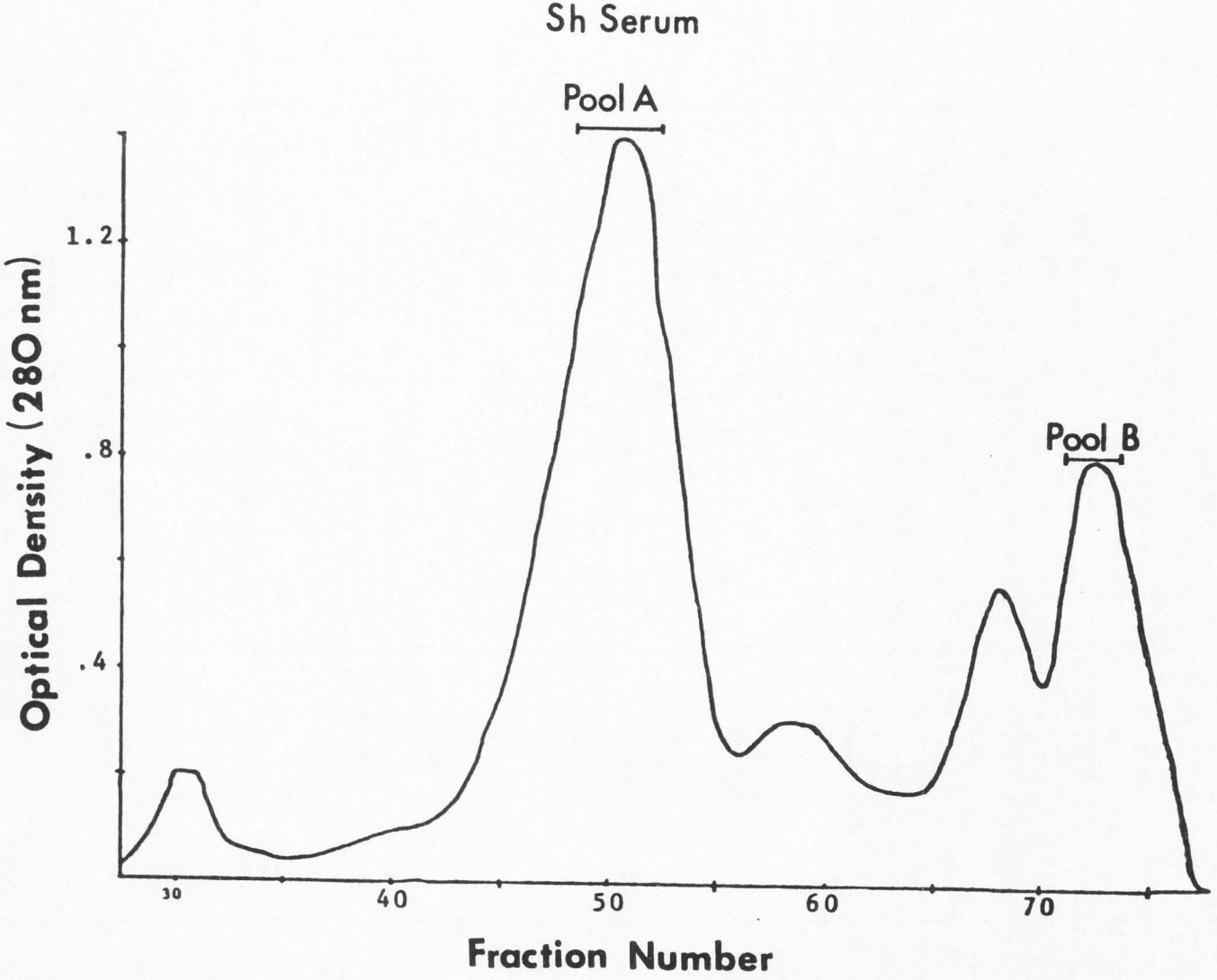

Sh Serum
Optical Density (280 nm)
6.0
4.0
2.0
Pool
I
14000
10000
6000
2000
Conductance (mhos)
40
50
60
70
Fraction Number

Figure 2. A-5M gel filtration of Pool 1 from the DEAE-cellulose column depicted in Figure 1. The column size was 2.5 X 100 cm and 5 ml fractions were collected. Pools A and B yielded purified high and low molecular weight sheepshead immunoglobulins respectively.


Sh Serum
Pool A
Pool B
Optical Density (280 nm)
1.2
.8
.4
30
40
50
60
70
Fraction Number

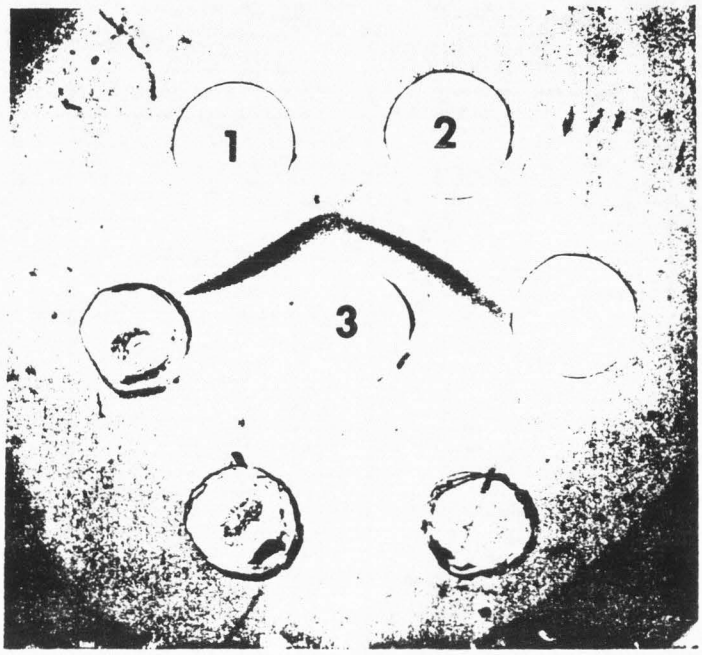
160,000 dalton area and was referred to as the low molecular weight (LMW) sheepshead Ig.

The antigenic relationship of the HMW Ig (Pool A) to the LMW Ig (Pool B) is shown in Figure 3. The Ouchterlony reaction observed was one of partial identity. The HMW Ig contained antigenic determinants not present in the LMW Ig.

To assess the purity of the HMW and LMW Ig pools, immunoelectrophoresis was performed. The electrophoretic patterns observed using HMW and LMW Igs, sheepshead serum, anti-sheepshead HMW Ig (anti-HMW Ig) and rabbit anti-sheepshead whole serum (anti-whole serum) showed three major points: 1) the anti-HMW Ig detected only one major protein band in whole serum; 2) the LMW and HMW Igs were pure by immunoelectrophoretic criteria; and 3) the electrophoretic mobilities of the LMW and HMW Igs differed. The LMW Ig had a slightly faster electrophoretic mobility than the HMW Ig (Figures 4 and 5).

The LMW and HMW Igs were reduced and analyzed by SDS-PAGE (Figure 6). Nurse shark H and L chains (which had been previously characterized by Clem and Small (1967), bovine serum albumin, and bovine IgG were used for comparison molecular weights. This analysis showed that the sheepshead Igs were composed of two polypeptide chains. The HMW heavy chain was ~70,000 daltons, and the L chain was ~25,000 daltons. The LMW Ig had a

Figure 3. Ouchterlony analysis comparing sheepshead high and low molecular weight immunoglobulins. The antiserum was prepared against the high molecular weight immunoglobulin and was used undiluted. The immunoglobulins were used at 2 mg/ml.



1 Sh HMW Ig
2 Sh LMW Ig
3 Anti-HMW Ig

Figure 4. Immunoelectrophoretic analysis of sheepshead serum and purified high molecular weight sheepshead immunoglobulin. The rabbit antiserum and the sheepshead serum were used undiluted. The sheepshead immunoglobulin was used at 2 mg/ml. The anode is to the left.

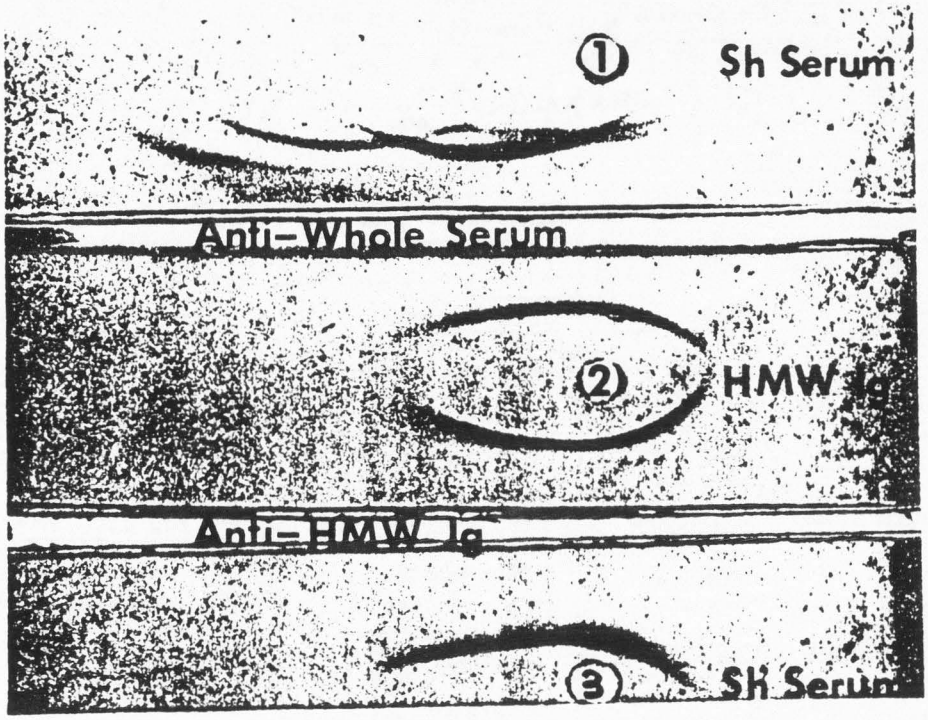
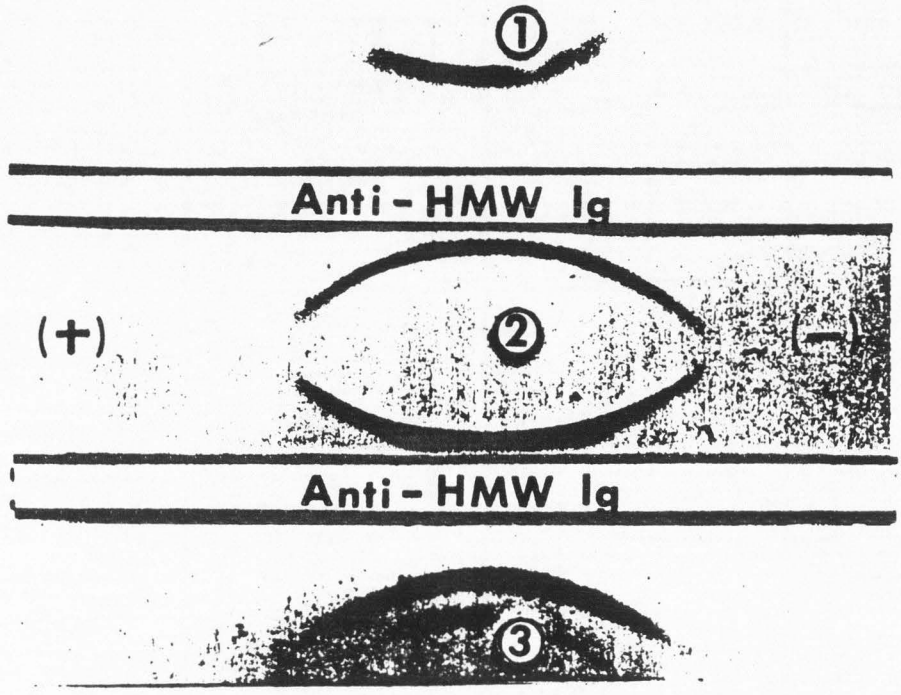
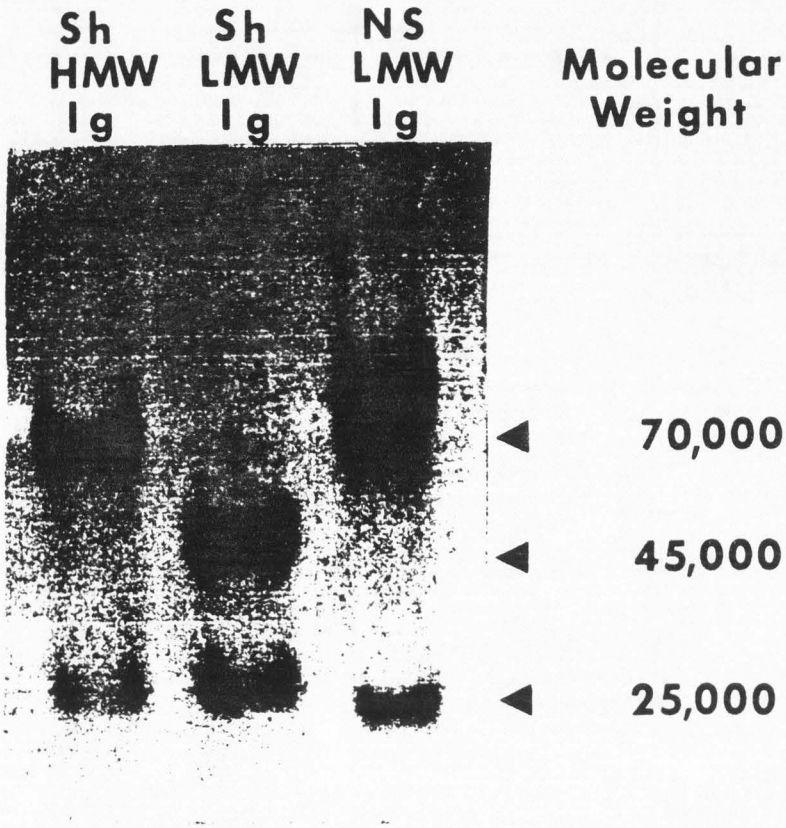

① Sh Serum
Anti-Whole Serum
② HMW Ig
Anti-HMW Ig
③ Sh Serum

Figure 5. Immunoelectrophoretic analysis of sheepshead high molecular weight and low molecular weight immunoglobulins. Antisera to the high molecular weight immunoglobulin was used undiluted. Immunoglobulins were used at 2 mg/ml.



① HMW Ig
② LMW Ig
③ HMW + LMW Ig

Figure 6. SDS-PAGE of purified reduced sheepshead immunoglobulins. Protein concentrations were 1 mg/ml. Nurse shark low molecular weight immunoglobulin is shown for comparison.


Sh HMW Ig
Sh LMW Ig
NS LMW Ig
Molecular Weight
70,000
45,000
25,000

L chain of ~25,000 daltons, but the H chain was ~45,000 daltons. This difference of ~25,000 daltons in the H chains probably accounted for the Ouchterlony reaction of partial identity shown in Figure 3.

Analysis of intact HMW and LMW Igs by SDS-PAGE revealed a heretofore undisclosed property of teleosts' immunoglobulins: the dissociation of sheepshead Igs (Figure 7). The HMW Ig was observed to stain with bands corresponding to the molecular weight of the tetramer (~700,000 daltons) as well as a band corresponding to the molecular weight of a dimer (~350,000 daltons). Only very small amounts of trimer and monomer were evident. The LMW Ig was also observed to dissociate on SDS-PAGE into a single component of ~ 70,000 daltons. This band represented halfmers or heavy-light pairs. These same immunoglobulin samples, however, chromatographed on A-5M as homogeneous peaks at 700,000 to 800,000 daltons for the HMW Ig and 130,000 to 160,000 daltons for the LMW Ig. Thus the SDS data was not due to breakdown of the protein during storage.

This unexpected dissociation of the sheepshead Igs was further characterized by ultracentrifugation analyses. Analytical ultracentrifugation of HMW Ig in physiological buffer indicated a single boundary with a sedimentation coefficient ($S_{20,w}$) of 14S (at 7 mg/ml) (Figure 8). The LMW Ig likewise indicated a single boundary with a sedimentation coefficient

Figure 7. SDS-PAGE of purified sheepshead immunoglobulins. Concentrations were 1 mg/ml. Nurse shark low molecular weight immunoglobulin is shown for comparison.

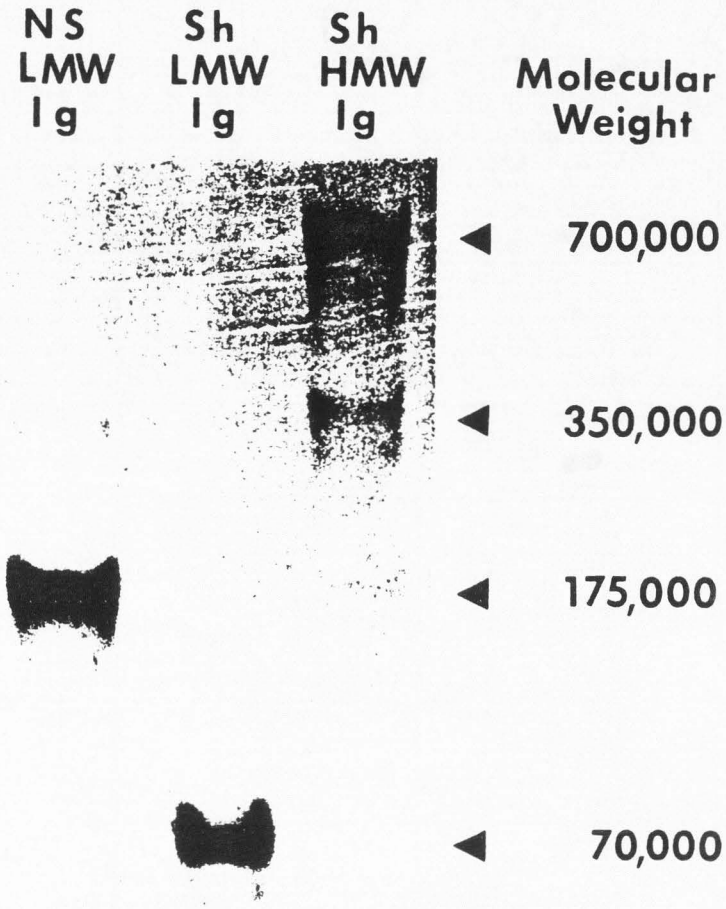
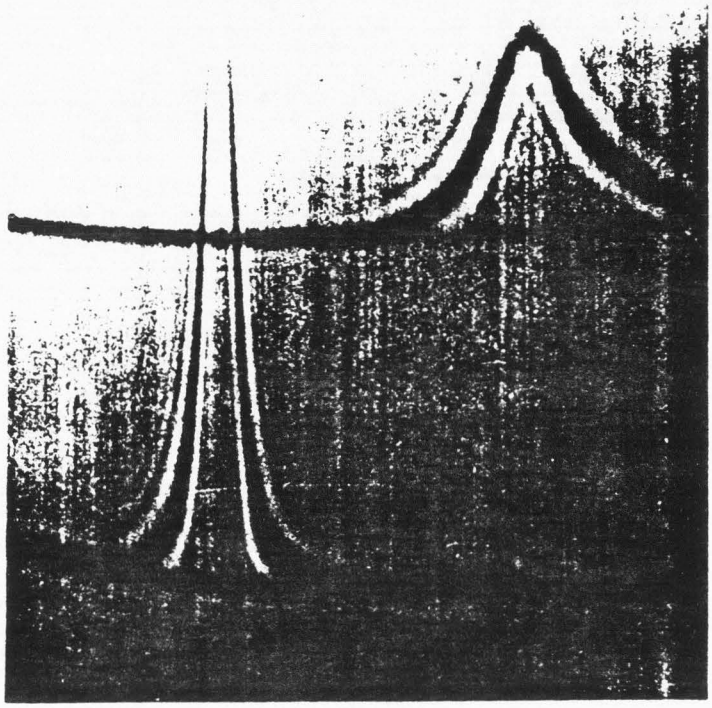

NS LMW Ig
Sh LMW Ig
Sh HMW Ig
Molecular Weight
700,000
350,000
175,000
70,000

Figure 8. Schlieren patterns of purified sheepshead immunoglobulins. Top, sheepshead 16S immunoglobulin (7 mg per ml), $S_{20,w}$ = 14S. Bottom, 6S sheepshead immunoglobulin (5.6 mg per ml), $S_{20,w}$ = 6.1S. The solvent was 0.15 M NaCl, Tris-HCl, pH 7.4. Sedimentation is shown from right to left.

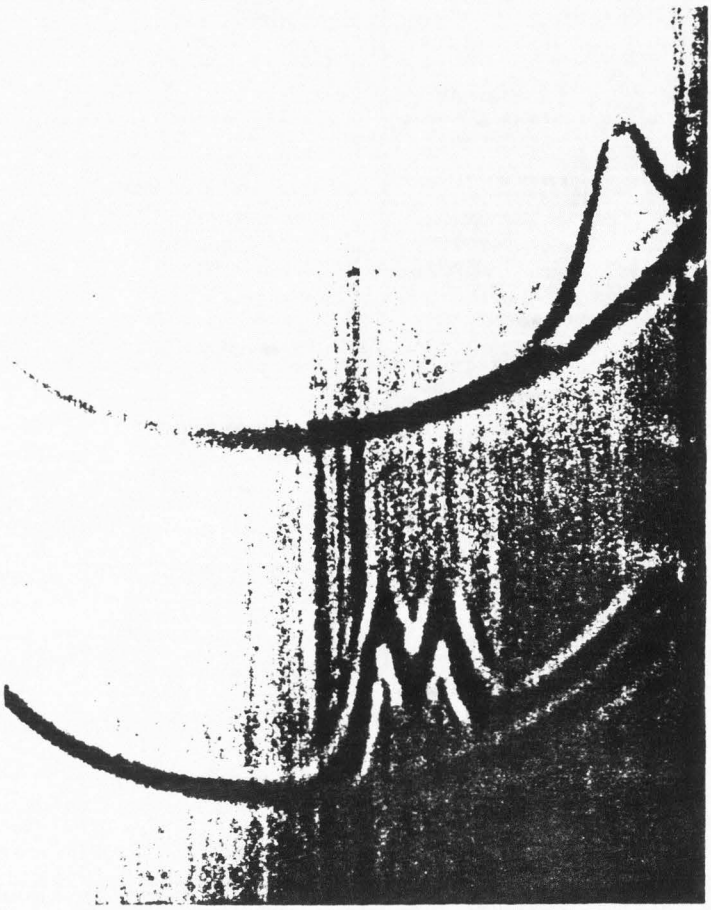


Top: Sh LMW Ig
Bottom: Sh HMW Ig

($S_{20,w}$) of 6.1S (at 5.6 mg/ml). Thus both the HMW and LMW Igs showed no evidence of intermolecular heterogeneity or unstability in physiological buffers. However, when the HMW and LMW Igs were equilibrated in 4 M guanidine HCl and reanalyzed, dissociation was observed (Figure 9). The denatured HMW Ig was observed to dissociate into a 14S boundary (corresponding to the tetramer) and a 11S boundary (corresponding to the dimer). The LMW Ig dissociated, giving a single boundary of 4S (corresponding to heavy-light pairs).

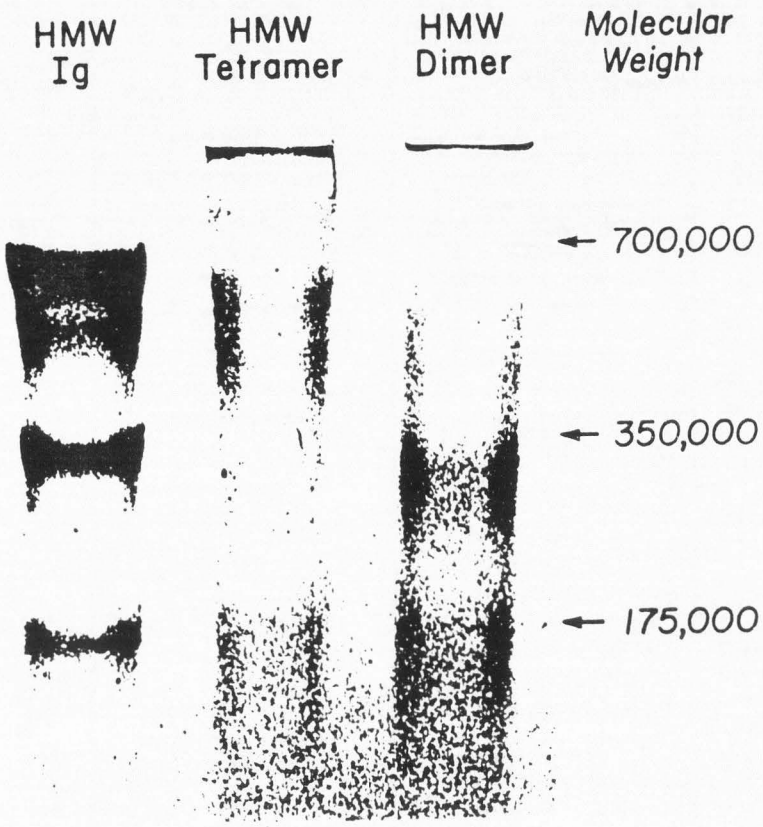
The dissociation of the HMW Ig into two subpopulations and the dissociation of the LMW Ig into halfmers did not appear to be an <u>in vitro</u> equilibrium situation. Two major observations supported this viewpoint. First, no detectable differences could be detected in the HMW or LMW Igs which had been through several freeze/thaw cycles over up to 1 year when compared to freshly purified Ig samples either by denaturing SDS-PAGE or by chromatography in physiological buffers. Secondly, to address this question more directly, the HMW tetrameric and dimeric populations were separated and analyzed individually. SDS-PAGE was performed and the resulting tetrameric and dimeric bands were cut out, solubilized and the subpopulations reanalyzed by SDS-PAGE. The tetrameric Ig population did not further dissociate to give the dimeric band (however, some monomer was seen) (Figure 10). The dimeric population did not reassociate

Figure 9. Schlieren patterns of purified sheepshead immunoglobulin in 4 M guanidine HCl. Top, sheepshead 6S immunoglobulin (5.6 mg per ml), $S_{20,w}$ = 4.1S. Bottom, sheepshead 16S immunoglobulin (7 mg per ml); 1st peak $S_{20,w}$ = 10.8S. The solvent was 4 M guanidine hydrochloride. Sedimentation is shown from right to left.



Top: Sh LMW Ig
Bottom : Sh HMW Ig

Figure 10. SDS-PAGE of tetrameric and dimeric high molecular weight immunoglobulin subpopulations. Subpopulations were eluted from previous SDS gels. The proteins were solubilized and reanalyzed. Unfractionated high molecular weight immunoglobulin is shown for comparison.


HMW
Ig
HMW
Tetramer
HMW
Dimer
Molecular
Weight
700,000
350,000
175,000

into a tetrameric band but essentially remained dimeric with monomers evident. Thus the tetrameric and dimeric subpopulations were not in equilibrium.

Further analysis of the component H and L chains was performed on the HMW sheepshead Ig. Mildly reduced and alkylated HMW Ig was chromatographed on A-5M equilibrated in 5 M guanidine HCl (Figure 11). Heavy and light chain peaks were resolved and pooled separately. The H chain peak comprised ~62 percent of the eluted protein whereas the light chain peak comprised ~38 percent.

Immunoelectrophoresis was performed on the HMW H and L chain pools. The light chains were shown to remain at the origin whereas the heavy chains were relatively fast moving. As such, H chains can be readily distinguished from the intact immunoglobulin, which has a slight anodic mobility (Figure 12).

SDS-PAGE of the pooled heavy chains revealed that most of the protein was in heavy chain dimers of ~140,000 daltons. The H chain pool when reduced migrated in entirety at 70,000 daltons. The light chain pool migrated at 25,000 daltons (Figure 13).

Since J chains usually migrate with L chains in SDS-PAGE or chromatography, experiments were conducted to determine J chain presence in the L chain pool of the HMW Ig. Alkaline-urea gel analysis showed the J chain was present in the L chain pool (Figure 14).

Figure 11. Gel filtration of mildly reduced high molecular weight immunoglobulin chromatographed in 5 M guanidine HCl. High molecular weight immunoglobulin was reduced with 0.05 M 2-mercaptoethanol and alkylated with 0.06 M iodoacetamide.

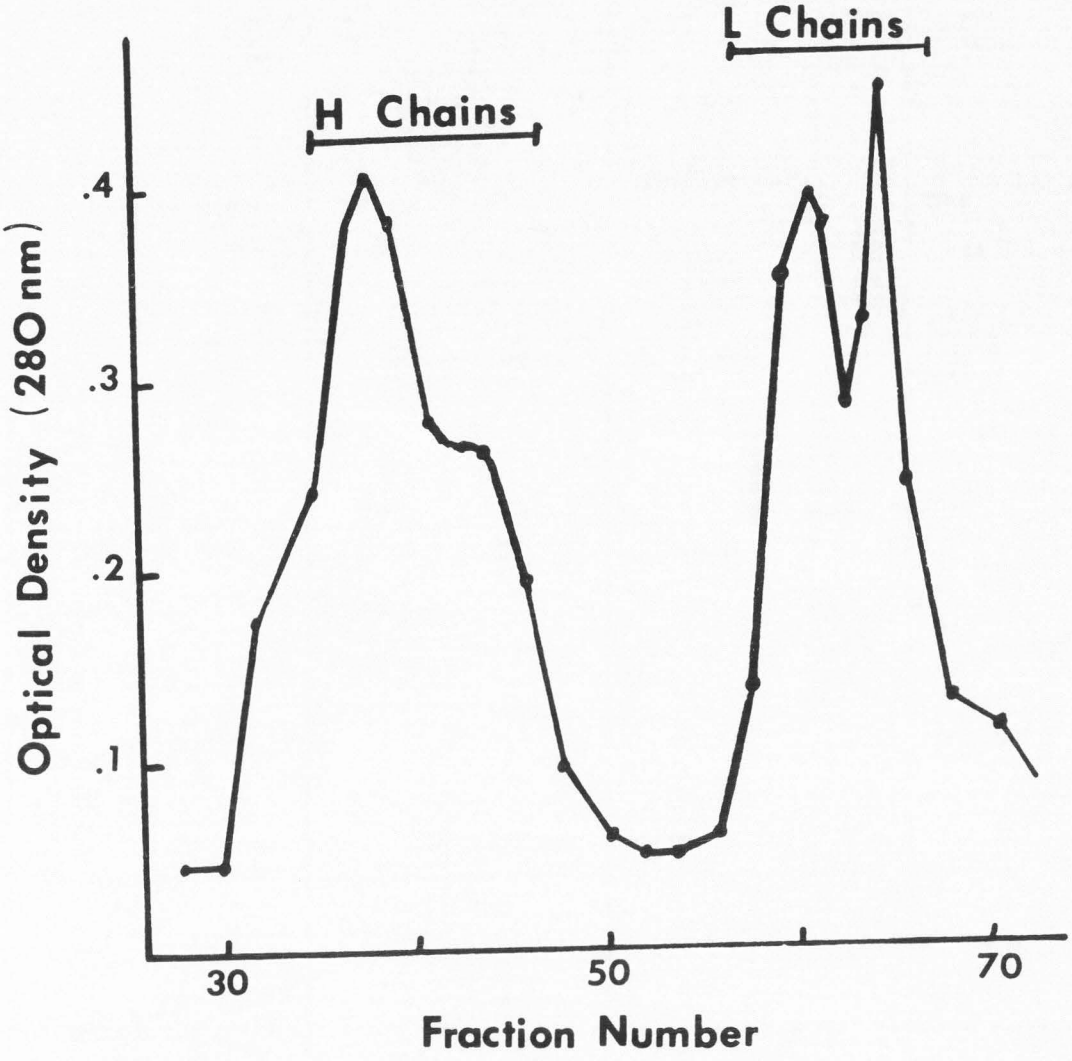
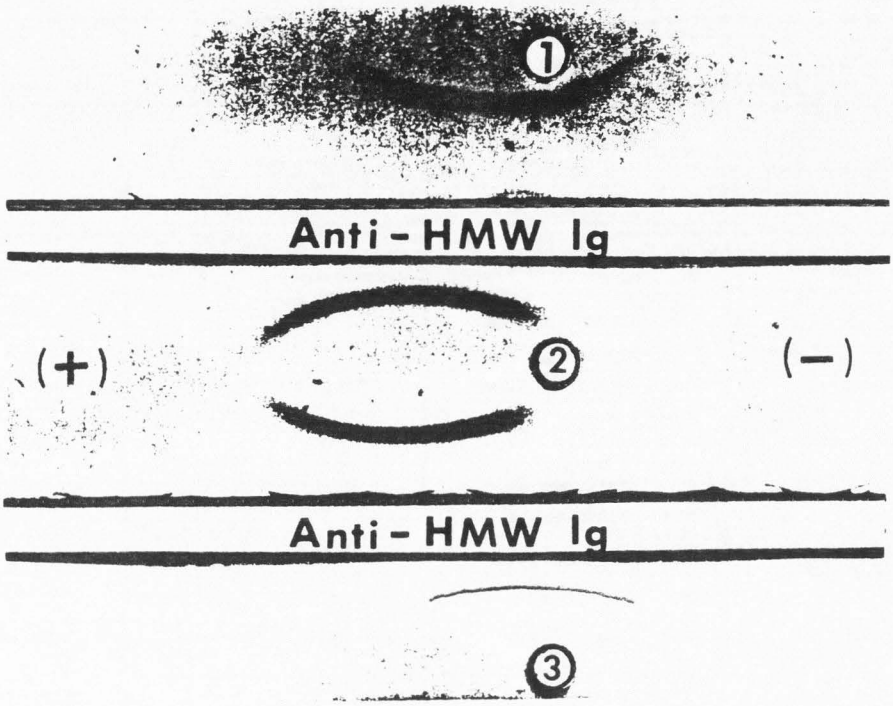

L Chains
H Chains
.4
.3
.2
.1
Optical Density (280 nm)
30
50
70
Fraction Number

Figure 12. Immunoelectrophoresis of reduced and alkylated sheepshead heavy and light chains. Protein concentrations were approximately 2 mg per ml. The intact high molecular weight immunoglobulin is shown for comparison.



1 HMW Ig
2 H Chains
3 L Chains

Figure 13. SDS-PAGE analysis of extensively reduced sheepshead high molecular weight immunoglobulin heavy and light chains. Protein concentrations were 1 mg/ml. Nurse shark low molecular weight immunoglobulin is shown for comparison.

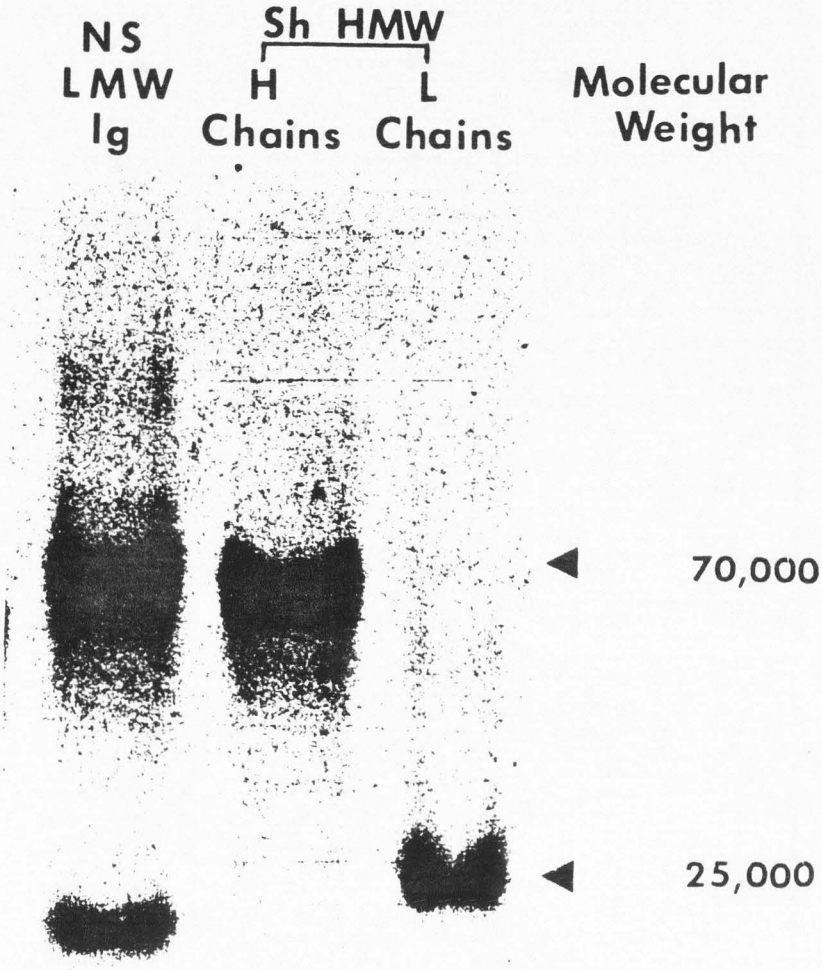
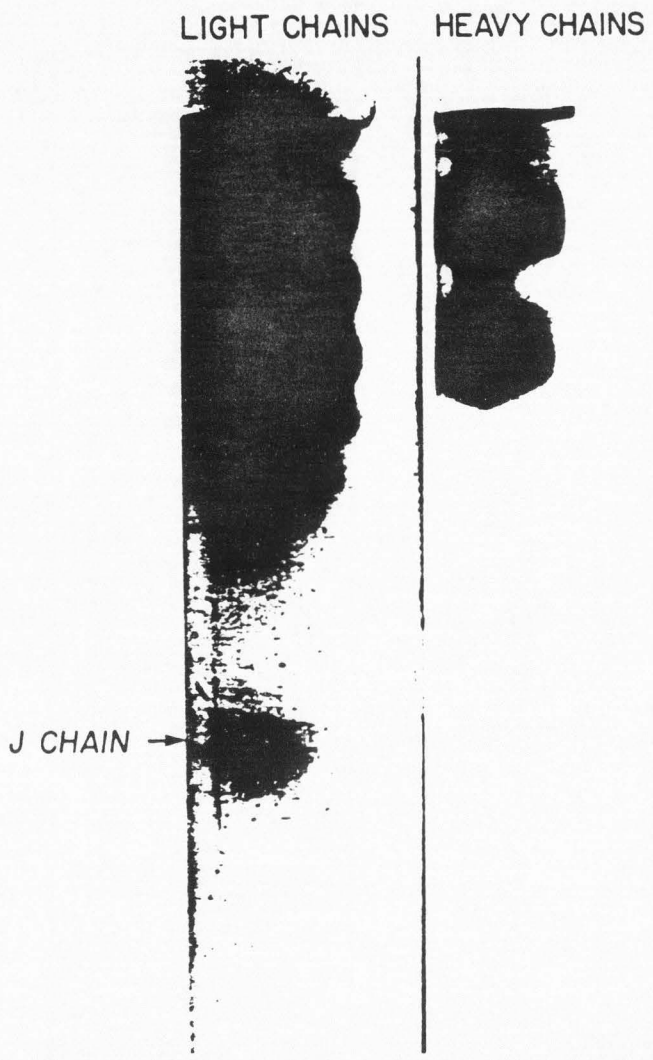

NS
LMW
Ig
Sh HMW
H
Chains
L
Chains
Molecular
Weight
70,000
25,000

Figure 14. Alkaline-urea gel electrophoretic analysis of sheepshead high molecular weight immunoglobulin heavy and light chains. Protein concentrations were 1 mg/ml. The anode is to the bottom.

LIGHT AND HEAVY CHAINS
FROM HMW Ig
ALKALINE UREA GEL ANALYSIS



This figure also showed the great heterogeneity present in the light chains of the sheepshead HMW Ig.

Characterization of the secretory immunoglobulins of the sheepshead

Characterization of the immunoglobulins in cutaneous mucus. Cutaneous mucus samples from seven sheepshead were pooled, concentrated and gel filtered on A-5M. The resulting column profile is shown in Figure 15. Six major peaks were identified and pooled individually. Of the six concentrated pools, only Pool 2 gave a strong Ouchterlony (double immunodiffusion) reaction. Pool 2 was rechromatographed on a smaller A-5M column to "clean up" contaminants seen by preliminary SDS-PAGE (Figure 16). Three major peaks were resolved by rechromatography. Of these three, only peak 2 was positive by Ouchterlony analysis. The antigenic relationship of mucus Ig to serum HMW and LMW is shown in Figure 17. Mucus Ig is antigenically identical to HMW serum Ig.

Immunoelectrophoresis was also performed to further characterize the mucus Ig. Mucus immunoglobulin was observed to migrate with the same mobility as the HMW serum Ig. The mucus Ig mobility observed was not similar to the serum LMW Ig (Figure 18).

Due to the small amount of partially purified mucus Ig (the entire pool contained 0.3 mg protein), the A-5M Pool 2 (rechromatographed) was radiolabeled with ^{125}I by the chloromine-T method. Chromatography

Figure 15. A-5M gel filtration of sheepshead cutaneous mucus. The column size was 2.5 X 100 cm and 5 ml fractions were collected. Pools were made as indicated at the top.

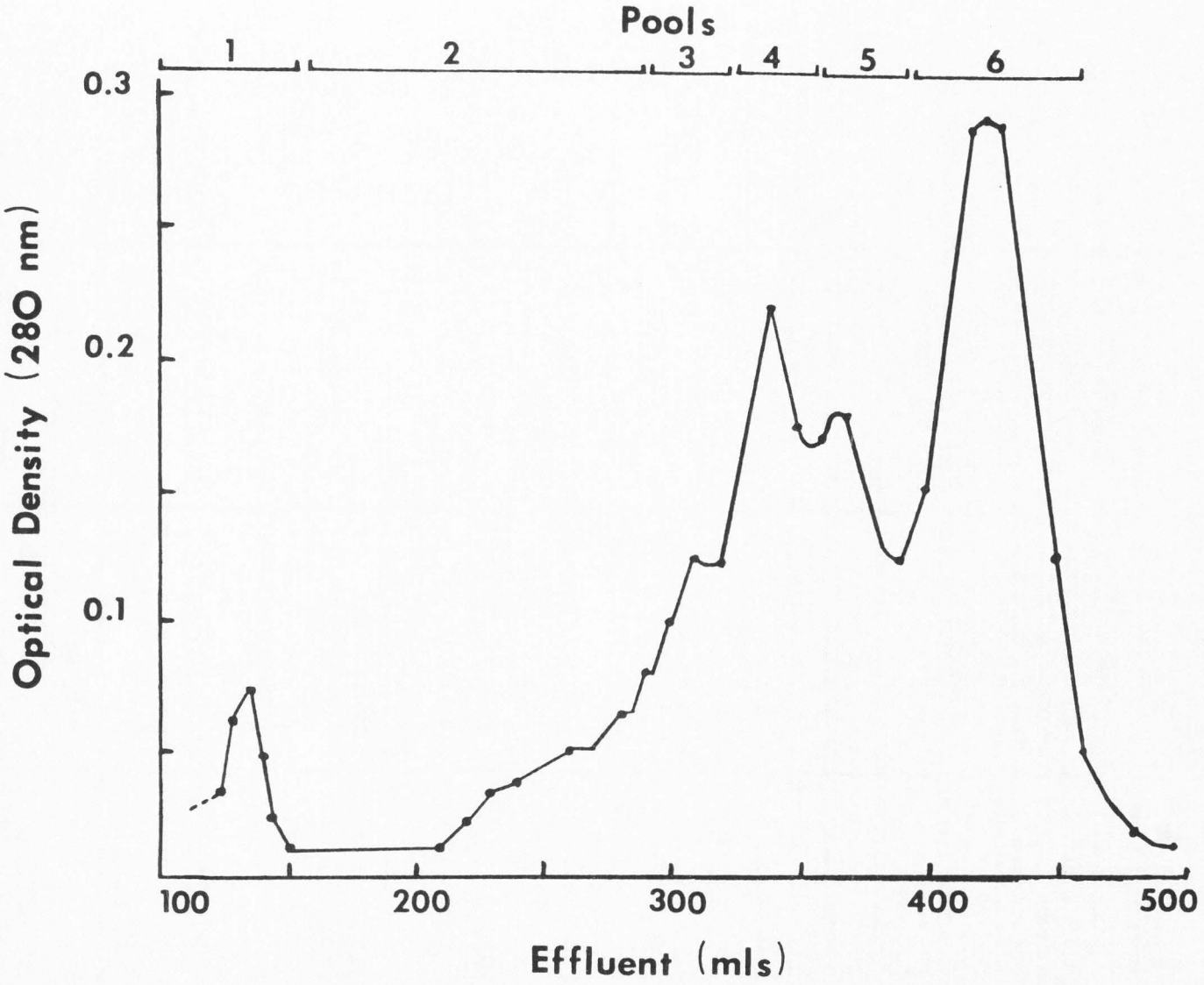

Pools
1
2
3
4
5
6
0.3
0.2
0.1
Optical Density (280 nm)
100
200
300
400
500
Effluent (mls)

Figure 16. A-5M gel filtration of cutaneous mucus Pool 2 obtained from the elution profile depicted in Figure 15. The column size was 1.5 X 58 cm and 1 ml fractions were collected. Pools were made as indicated at the top.

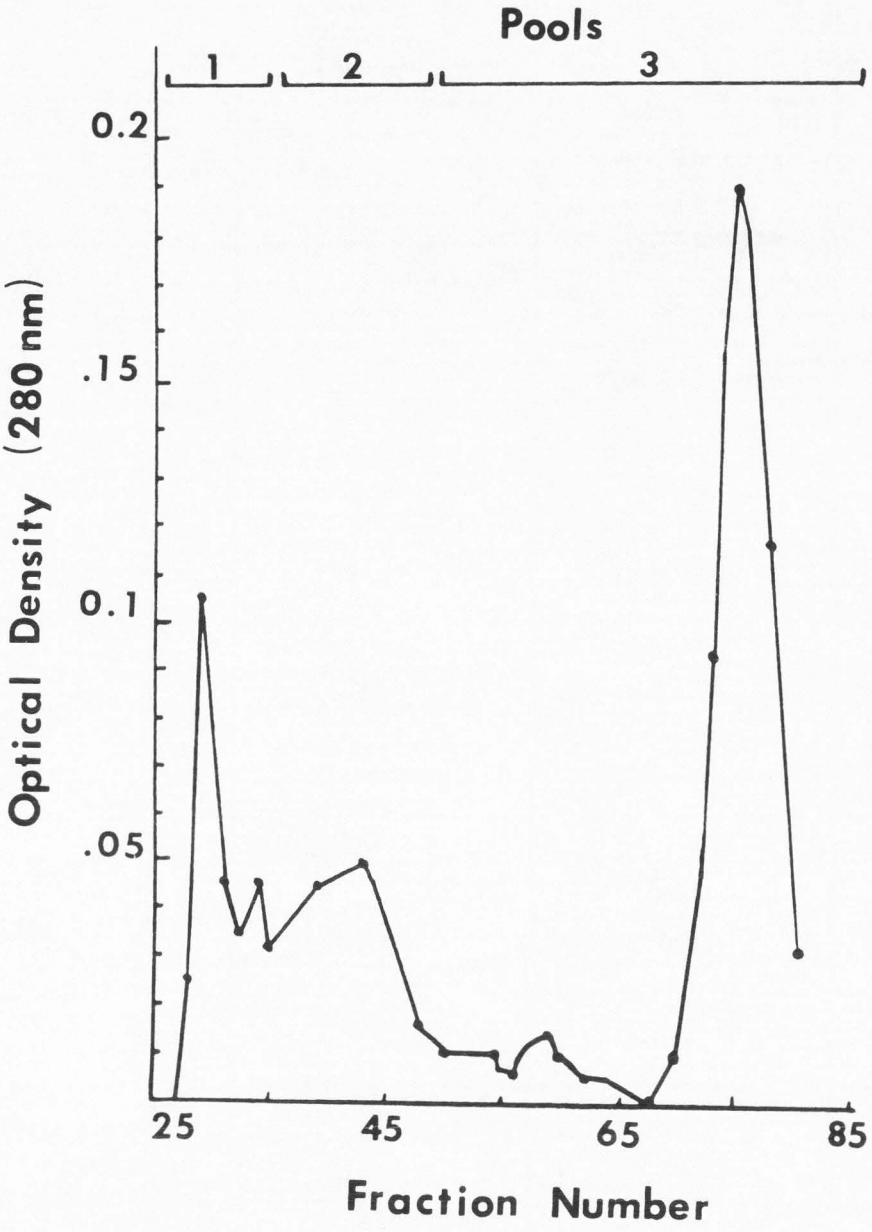
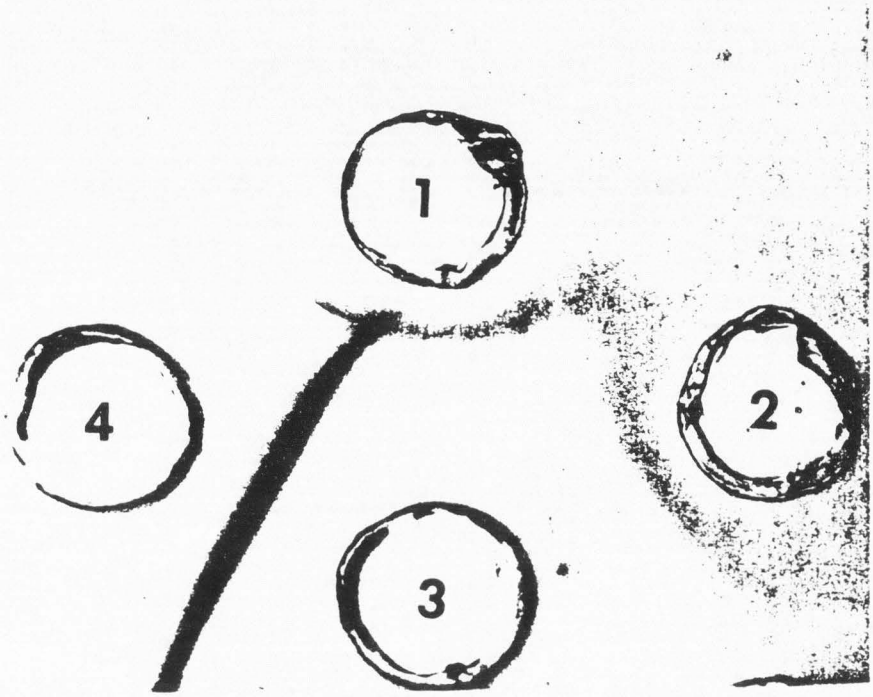
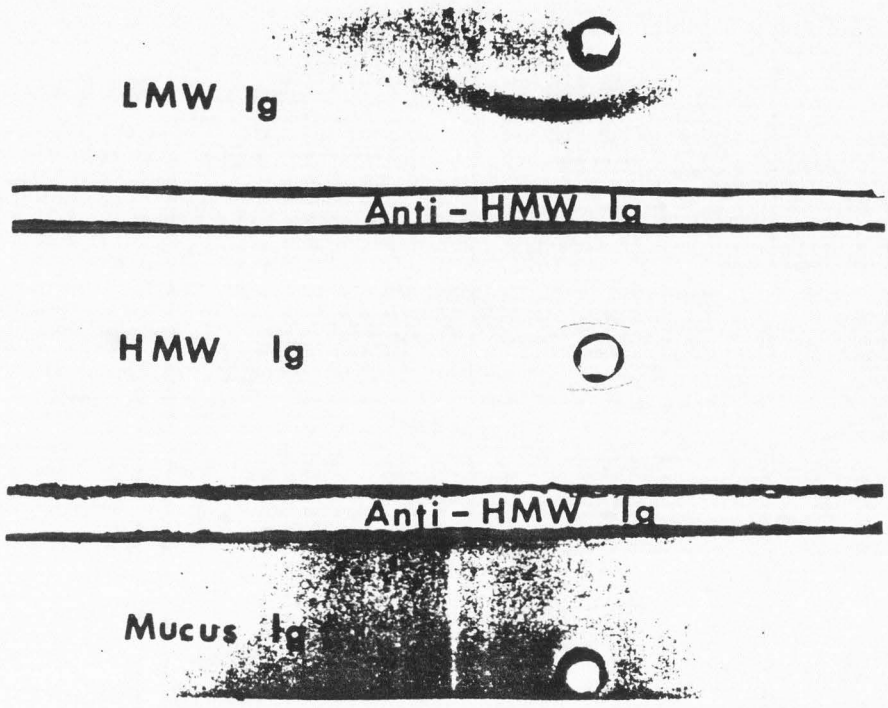

Pools
1
2
3
0.2
.15
0.1
.05
Optical Density (280 nm)
25
45
65
85
Fraction Number

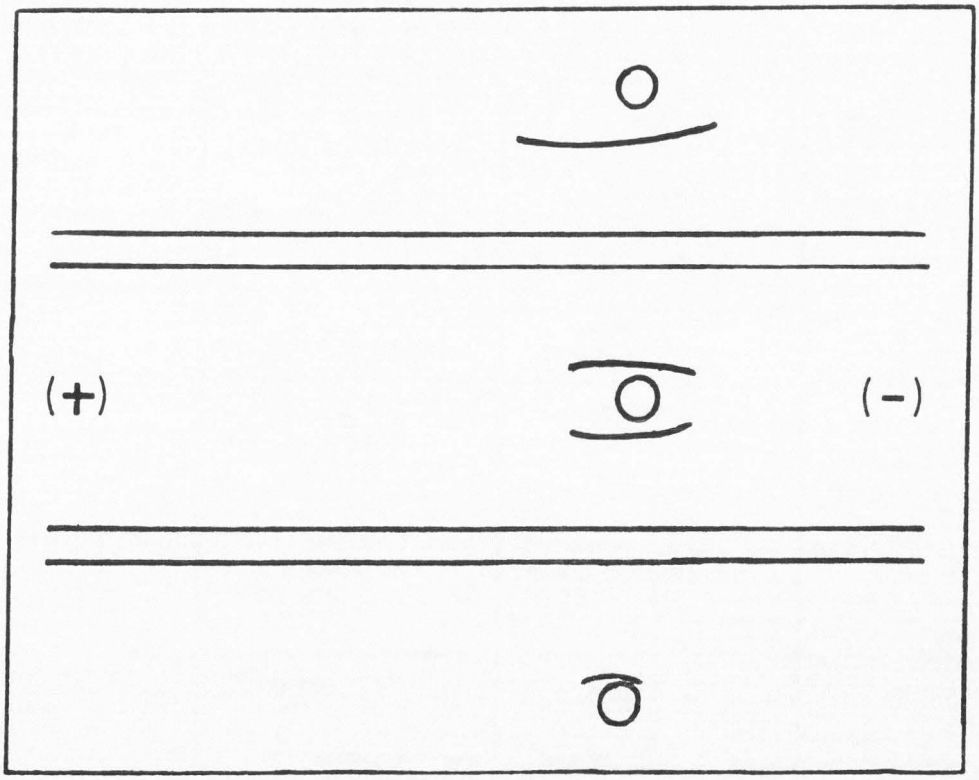
Figure 17. Ouchterlony comparison of mucus immunoglobulin (Pool 2, Figure 16) with serum high and low molecular weight immunoglobulins. Protein concentrations used were .25 mg/ml.



1 Sh HMW Ig
2 Mucus Ig
3 Anti - HMW Ig
4 LMW Ig

Figure 18. Immunoelectrophoretic analysis of sheepshead mucus immunoglobulin (Pool 2, Figure 16). Proteins were used at .25 mg/ml. Due to the faintness of some of the precipitin arcs, a sketch is also shown.


LMW Ig
Anti-HMW Ig
HMW Ig
Anti-HMW Ig
Mucus Ig


(+)
(-)

on A-5M revealed that the labeled mucus was very heterogeneous (Figure 19). Due to this variability, DEAE ion exchange chromatography was utilized to further purify the mucus Ig. DEAE-Sephadex was eluted stepwise with increasing molarities of NaCl (.10 M, .15 M, .25 M, .50 M). The resulting stepwise fractions were pooled and concentrated. These four pools showed considerable variability when analyzed by SDS-PAGE autoradiography (Figure 20). The 0.10 M mucus pool was seen to be of high molecular weight, $>$ 700,000 daltons. The fraction when reduced (not shown) did not reveal heavy or light chains. Based upon this evidence, it was assumed that the 0.10 M pool represented mucoid glycoproteins. The 0.15 M, 0.25 M and 0.5 M pools all contained proteins which migrated in the same position as the serum HMW Ig subpopulations. The 0.5 M pool was observed to have many proteins not in common with either the serum HMW and LMW Ig or the 0.15 M and 0.25 M pools. These additional contaminating proteins made this pool unacceptable for further analysis. Further characterization of the mucus Igs was performed with the 0.15 M and 0.25 M pools.

An aliquot of the 0.15 M mucus pool was chromatographed on A-5M (Figure 21). This figure shows the elution peaks for serum HMW (16S) and LMW (6S) Igs for reference. The majority of this pool was eluted in an area of the column corresponding to $\sim$350,000 daltons. This elution area corresponded to the area one would

Figure 19. A-5M gel filtration of radiolabeled mucus immunoglobulin (Pool 2, Figure 16). The column size was 1.5 X 58 cm, and 1 ml fractions were collected. The reference elution positions for serum 16S and 6S immunoglobulins are indicated by the arrows.

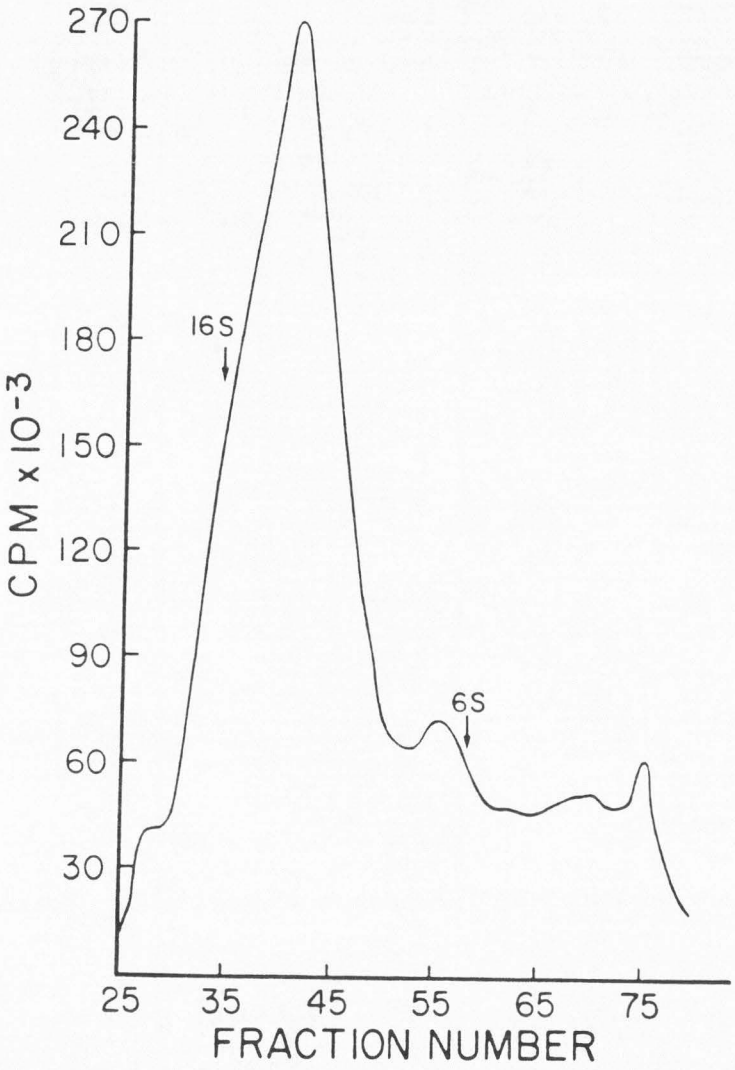

270
240
210
180
150
120
90
60
30
CPM x 10-3
16S
6S
25
35
45
55
65
75
FRACTION NUMBER

Figure 20. SDS-PAGE autoradiograph of radiolabeled mucus immunoglobulin from DEAE-Sephadex. DEAE "cuts" were made by stepwise elution with increasing concentrations of NaCl as indicated. High molecular weight and low molecular weight serum immunoglobulins are shown for comparison.

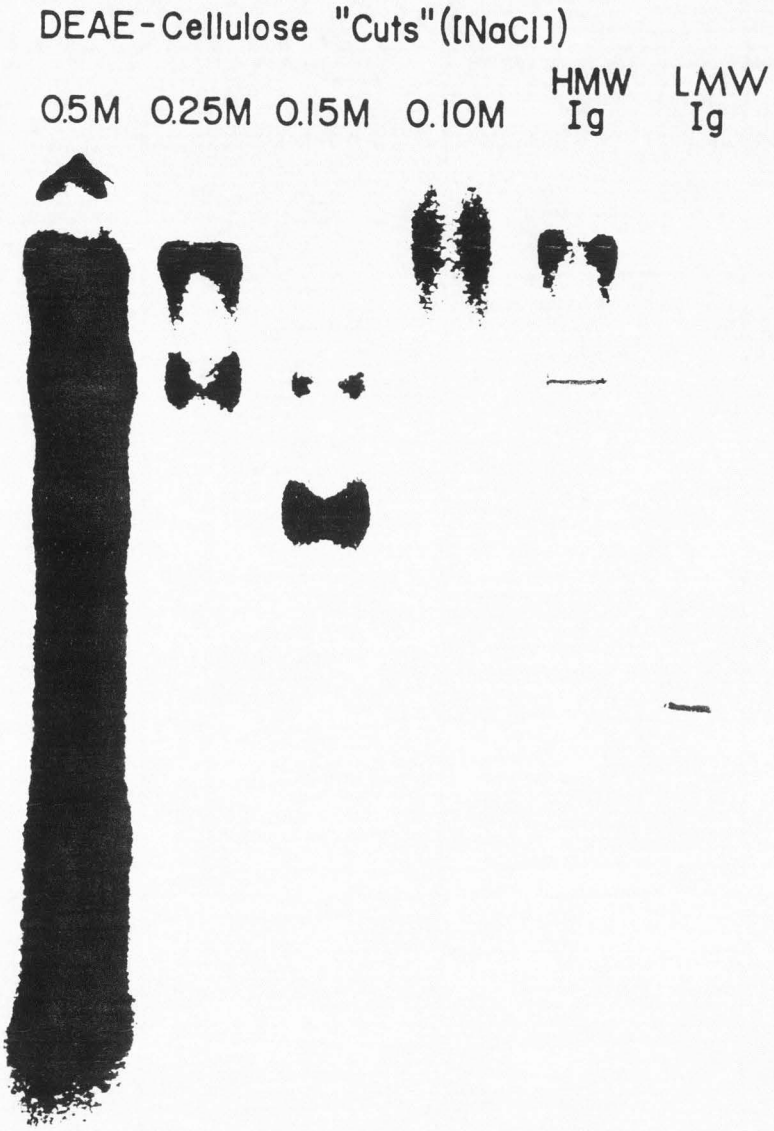
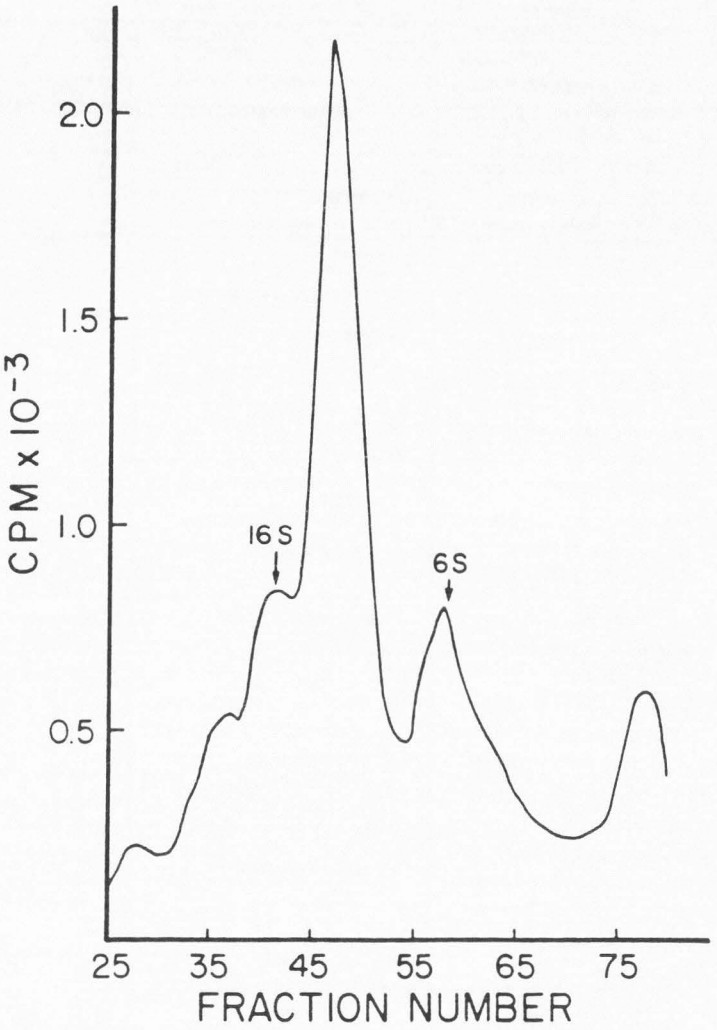

DEAE-Cellulose "Cuts"([NaCl])
0.5M
0.25M
0.15M
0.10M
HMW Ig
LMW Ig

Figure 21. A-5M gel filtration of radioiodinated mucus proteins obtained by elution from DEAE-Sephadex with 0.15 M NaCl. The column size was 1.5 X 58 cm, and 1 ml fractions were collected. The reference elution positions for serum 16S and 6S immunoglobulins are indicated by the arrows.

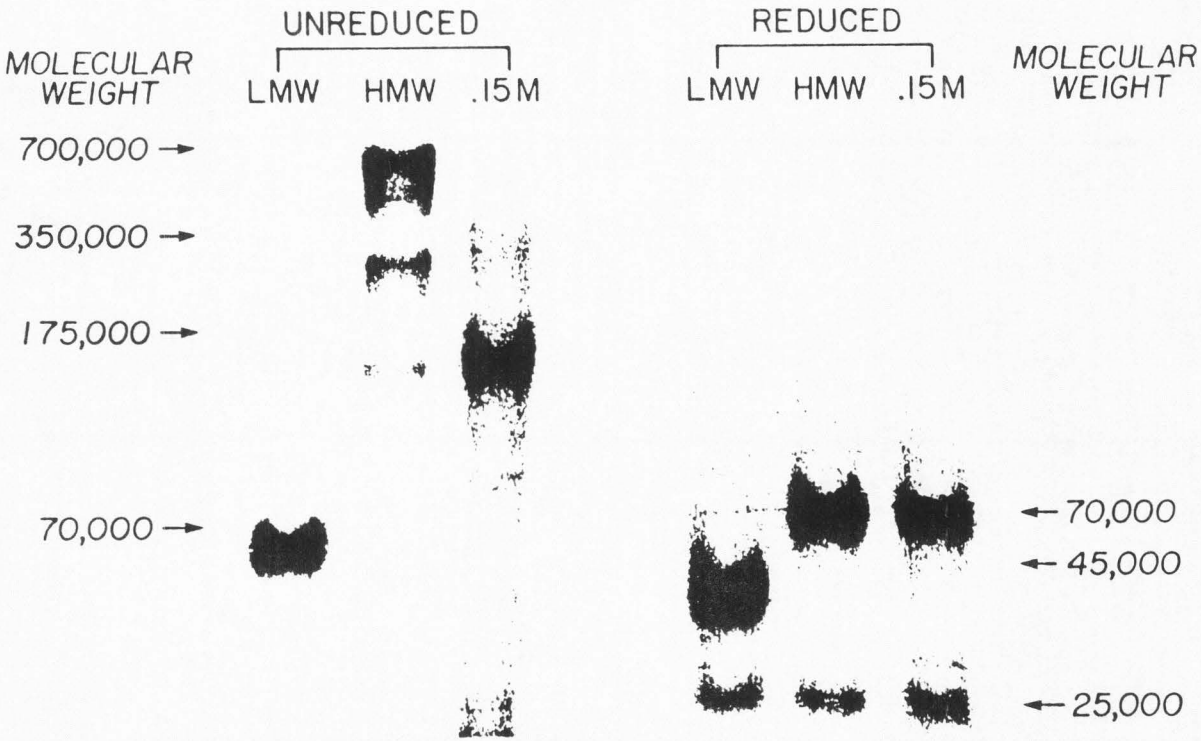

2.0
1.5
1.0
0.5
CPM x 10-3
16S
6S
25
35
45
55
65
75
FRACTION NUMBER

expect a dimeric immunoglobulin to occur. In addition, significantly smaller peaks are observed in areas corresponding to the elution area of the HMW serum Ig and in a peak just slightly ahead of the elution area for the LMW serum Ig.

SDS-PAGE autoradiography of both reduced and unreduced 0.15 M mucus Ig revealed several important facts (Figure 22). The unreduced protein was observed to have the same mobility as the monomer of the HMW serum Ig. No evidence was seen to indicate any relationship to the LMW serum Ig. When the 0.15 M mucus Ig was extensively reduced, two bands were observed. The first was the H chain which migrated at 70,000 daltons; and the second was the L chain which migrated at 25,000 daltons. Some density was observed at the buffer front which represents protein $<5,000$ daltons. (This same small polypeptide density was observed in unreduced proteins.) The 0.15 M mucus Ig H and L chains migrated identically to the H and L chains of the serum HMW Ig. No evidence for LMW H chains was observed. Thus, in physiological buffers the 0.15 M mucus Ig was predominantly dimeric; however, under denaturing conditions such as SDS, the dimeric mucus Ig was observed to dissociate into monomeric units. This finding suggests that the 0.15 M mucus Ig is bonded under physiological conditions by noncovalent interactions.

The 0.25 M mucus pool was analyzed next. Preliminary

Figure 22. SDS-PAGE autoradiograph of radioiodinated reduced and unreduced mucus proteins obtained by elution from DEAE-Sephadex with 0.15 M NaCl. The serum high and low molecular weight immunoglobulins are shown for comparison.


UNREDUCED
REDUCED
MOLECULAR WEIGHT
LMW
HMW
.15M
LMW
HMW
.15M
MOLECULAR WEIGHT
700,000
350,000
175,000
70,000
70,000
45,000
25,000

chromatography indicated that the pool could be separated into two peak fractions. Based upon this fact, the entire 0.25 M pool was chromatographed on A-5M (Figure 23). Two major peaks were resolved. The predominant peak eluted in the same place as the HMW serum Ig. A secondary peak, just behind the 16S marker, was also observed. These two peaks were concentrated separately. The major peak, fractions 31-41, contained ~350,000 cpm. The secondary peak, fractions 42-50, contained ~100,000 cpm.

SDS-PAGE autoradiography was used to analyze the 0.25 M mucus pools (Figure 24). Unreduced pool 31-41 was observed to be indistinguishable from HMW serum Ig. The pool dissociated into subpopulations in a manner that was apparently identical to the HMW serum Ig. When pool 31-41 was reduced, H and L chains were seen. The H chains migrated at 70,000 daltons, whereas the L chains migrated at 25,000 daltons. This was identical to the HMW serum H and L chains. However, upon reduction the "dimeric" band did not reduce to H and L chains. This dimeric band was either a contaminant or represented a 2-mercaptoethanol resistant form of Ig. No evidence for aggregated LMW serum Ig was observed in either the unreduced or reduced protein.

Pool 42-50, unreduced, predominantly migrated at an area slightly greater than the dimeric form of serum HMW Ig. There was also some density in the monomeric area. When the pool protein was reduced, there were

Figure 23. A-5M gel filtration of radioiodinated mucus proteins obtained by elution from DEAE-Sephadex with 0.25 M NaCl. The specifics of this fractionation are given in the legend to Figure 21.

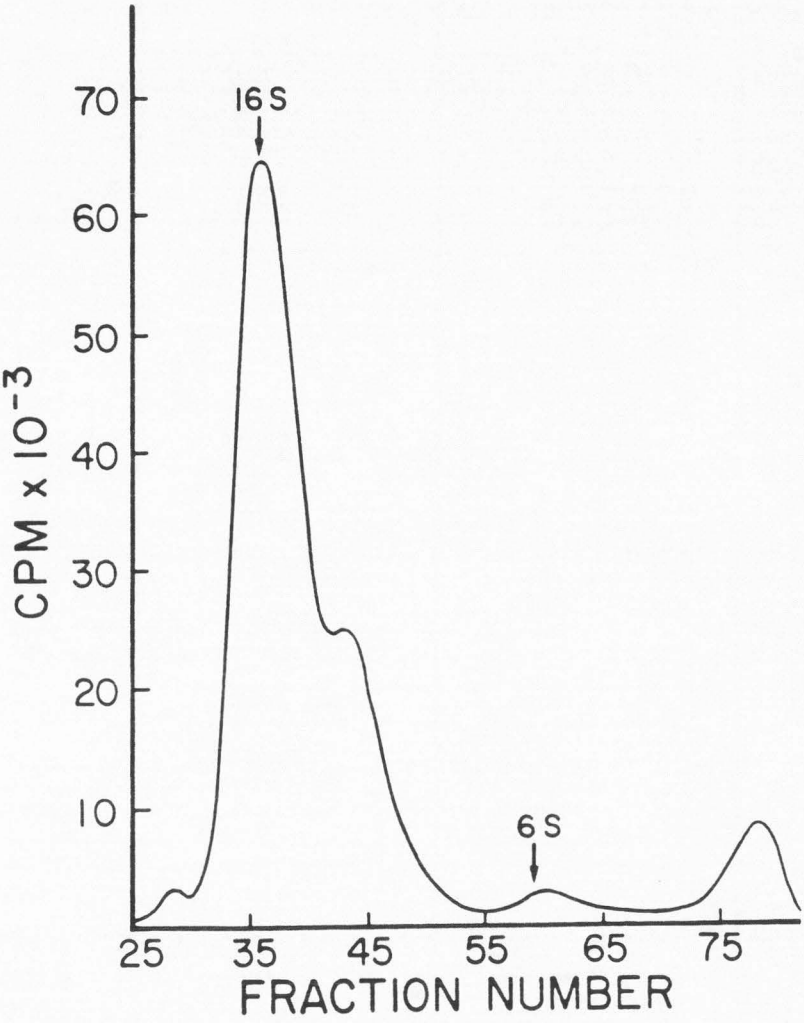
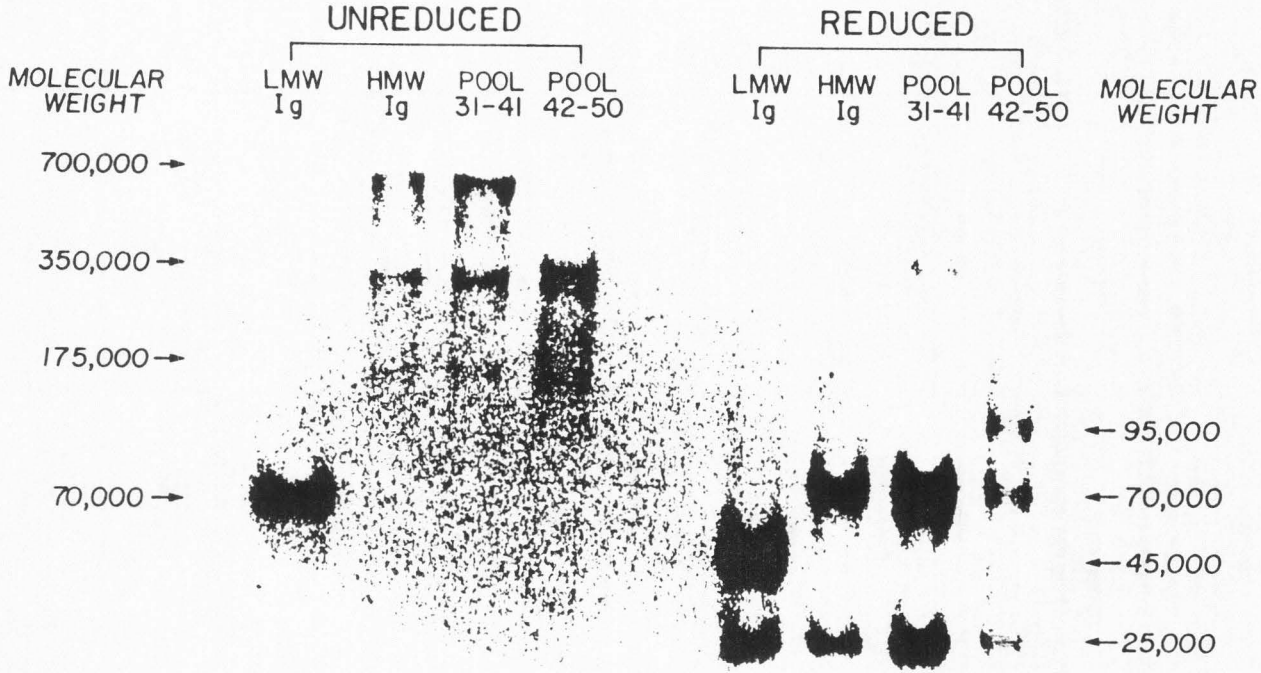

70
60
50
40
30
20
10
CPM x 10⁻³
16S
6S
25
35
45
55
65
75
FRACTION NUMBER

Figure 24. SDS-PAGE autoradiograph of reduced and unreduced radiolabeled mucus immunoglobulin which had been gel filtered and pooled as indicated from the A-5M elution profile depicted in Figure 23.


UNREDUCED
REDUCED
MOLECULAR WEIGHT
LMW Ig
HMW Ig
POOL 31-41
POOL 42-50
LMW Ig
HMW Ig
POOL 31-41
POOL 42-50
MOLECULAR WEIGHT
700,000
350,000
175,000
70,000
95,000
70,000
45,000
25,000

three predominant bands observed. H and L chains, molecular weights of 70,000 and 25,000 daltons, were observed which were indistinguishable from the H and L chains from HMW serum Ig. In addition, another protein chain migrated at 95,000 daltons. This chain may well represent the teleost equivalent to the mammalian secretory component. There was no evidence to indicate any relationship of this mucus Ig pool to the LMW serum Ig.

Characterization of the immunoglobulins in bile. Preliminary investigations demonstrated immobilized zone preparative electrophoresis to be a quick and efficient method for the initial purification of bile Ig. Experiments were performed to insure that if bile Igs similar to serum HMW or LMW Igs were present, they would not be "lost" during electrophoresis. A 9 percent polyacrylamide/1 percent agarose concentration was established empirically and was shown to prevent penetration of the serum HMW Ig into the gel. However, since this gel porosity allowed simple diffusion of the LMW Ig, it was important to establish if the serum LMW Ig penetrated the gel during electrophoresis. ^{125}I-LMW serum Ig was electrophoresed in the identical method used for the bile Ig purification. By quantitating the cpm applied and recovered, 80 percent of the counts were recoverable. After concentration, the ^{125}I-LMW Ig chromatographed on A-5M in an identical manner to

non-electrophoresed LMW Ig. Thus, it was concluded that if bile Igs resembled the HMW or LMW serum Igs, these bile immunoglobulins would be recoverable after electrophoresis.

A pool of 26 mls of sheepshead bile (representing the bile from 12-15 sheepshead) was prepared by immobilized zone preparative electrophoresis. The electrophoresed bile was concentrated and chromatographed on A-5M (Figure 25). As indicated, four pools were made based upon this elution profile. Ouchterlony analysis of these four pools showed that only Pool 2 was positive. The relationship of the bile Ig to HMW serum Ig was a reaction of identity (Figure 26). Because of its asymetrical elution profile, Pool 2 was rechromatographed on A-5M to more definitively establish the elution molecular weight range of the bile Ig. The resulting chromatograph was pooled according to molecular weight ranges, since no major peak was obvious. Four pools were made: Pool 1 (greater than 1,000,000 daltons), Pool 2 (1,000,000 to 600,000 daltons), Pool 3 (600,000 to 200,000 daltons) and Pool 4 (200,000 to 20,000 daltons). Of these rechromatographed pools, only Pool 3 was positive for Ig by Ouchterlony analysis.

Due to the small amount of partially purified protein in Pool 3 ($\sim$.24 mg), the pool was radiolabeled with ^{125}I by the chloramine-T method to allow for further analysis. Staph A immunoprecipitation was used to finally

Figure 25. A-5M gel filtration of sheepshead bile prepared by immobilized zone preparative electrophoresis. The column size was 1.5 X 58 cm, and 1 ml fractions were collected. Pools were made as indicated at the top.

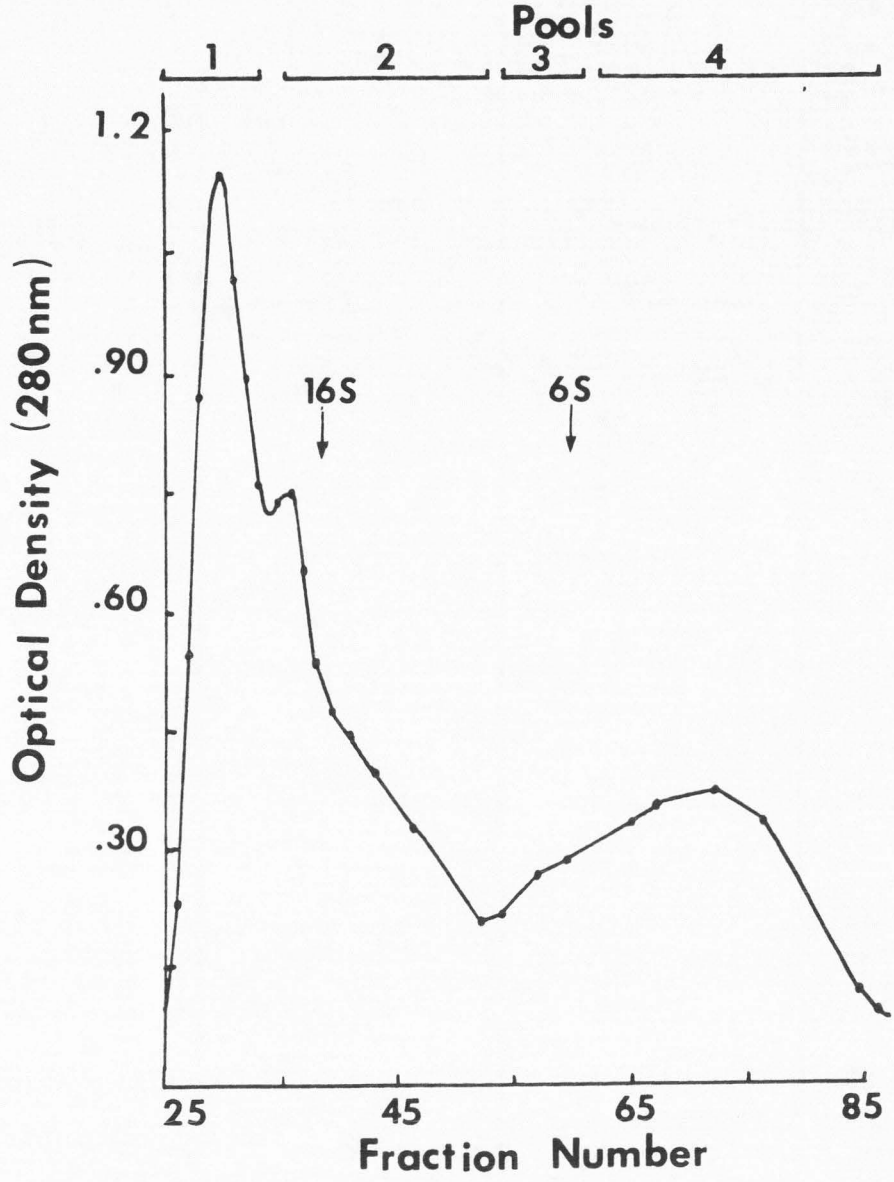
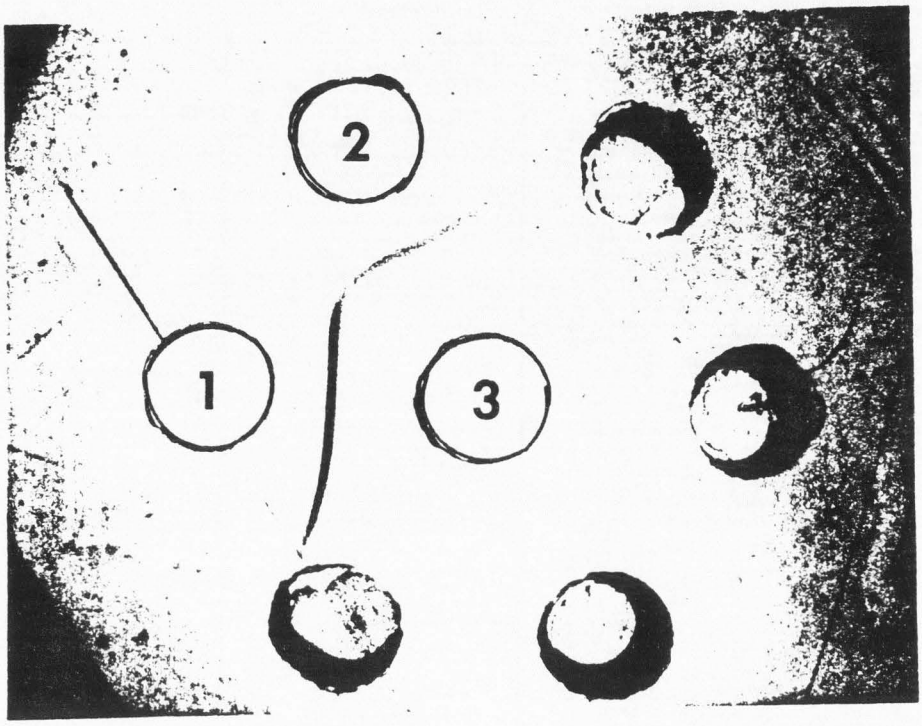

Pools
1
2
3
4
1.2
.90
.60
.30
Optical Density (280nm)
16S
6S
25
45
65
85
Fraction Number

Figure 26. Ouchterlony comparison of sheepshead bile immunoglobulin (Pool 2, Figure 25) and serum high molecular weight immunoglobulin. The protein concentrations were ~1 mg/ml.



1 Sh HMW Ig
2 Bile Ig
3 Anti–HMW Ig

characterize the bile Ig. Increasing amounts of the labeled Pool 3 were immunoprecipitated with anti-HMW Ig. The solubilized precipitates were analyzed by SDS-PAGE autoradiography. The bile Ig migrated in two major bands (Figure 27). These bands, when compared to the HMW and LMW serum Igs, migrated in areas corresponding to ~350,000 and ~170,000 daltons. Increasing density of the bands resulted from increasing the volume of the labeled bile Ig immunoprecipitated. No bands were observed in control (preimmune serum) precipitation using the highest sample volume of bile (50 λ). There was no evidence to suggest that bile contained Ig similar to the LMW serum Ig. Thus it appeared that the bile Ig most closely resembled the HMW serum Ig. The weights of the bile Ig components appeared similar, although the intensity of the bands was not the same as that of the serum HMW Ig.

Was this different density pattern of the immunoprecipitated bile Ig due to some inherent property of bile? To answer this question samples of whole bile were "spiked" with labeled HMW and LMW serum Ig and stored at -20 C and 4 C for three days. At the end of this period the "spiked" bile was analyzed by SDS-PAGE autoradiography. There was no apparent effect of bile on the reduced or unreduced serum Igs. The intensity of the subpopulations of the HMW serum Ig in bile was not affected at either temperature (Figure 28).

Figure 27. SDS-PAGE autoradiograph of increasing amounts of bile immunoglobulin obtained by immunoprecipitation with rabbit anti-high molecular weight immunoglobulin using Staph A. Control immunoprecipitation was with preimmune serum. Low molecular weight and high molecular weight serum immunoglobulins are shown for comparison.

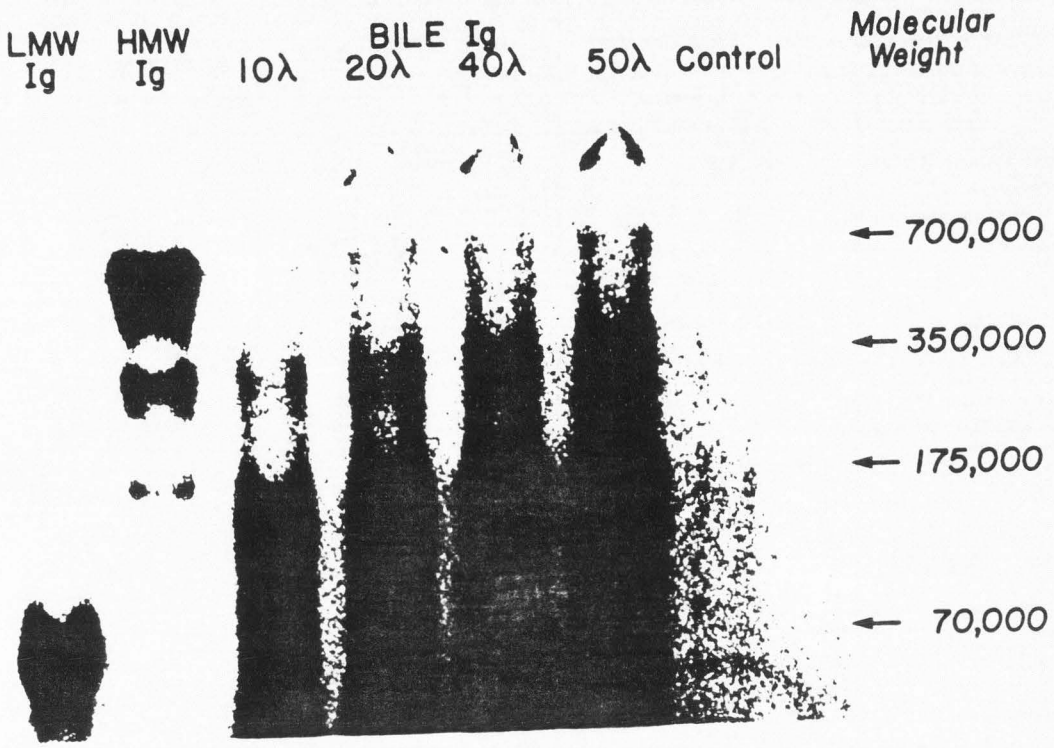
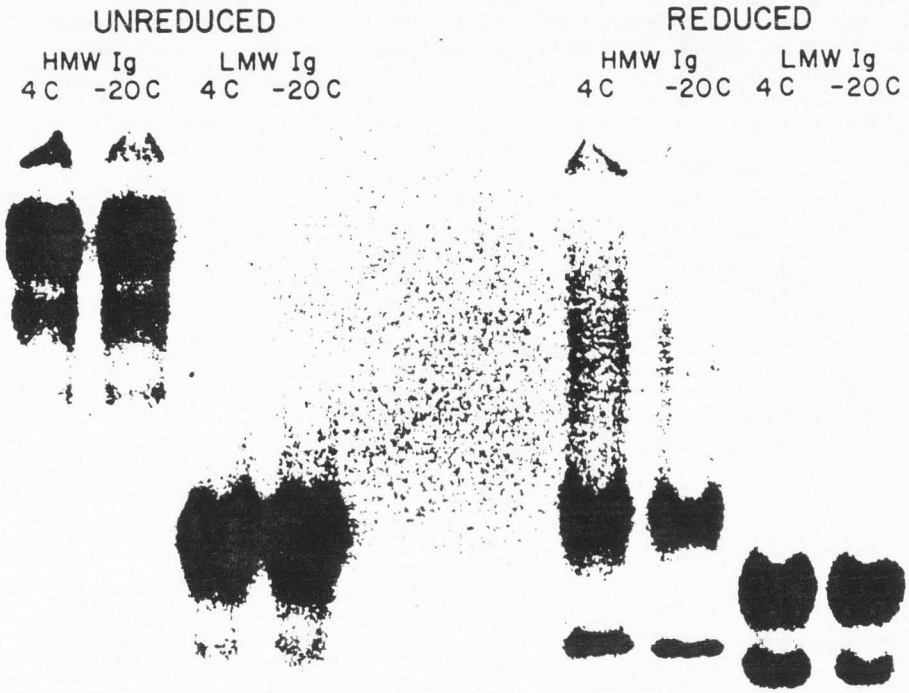

LMW Ig
HMW Ig
BILE Ig
10λ
20λ
40λ
50λ
Control
Molecular Weight
700,000
350,000
175,000
70,000

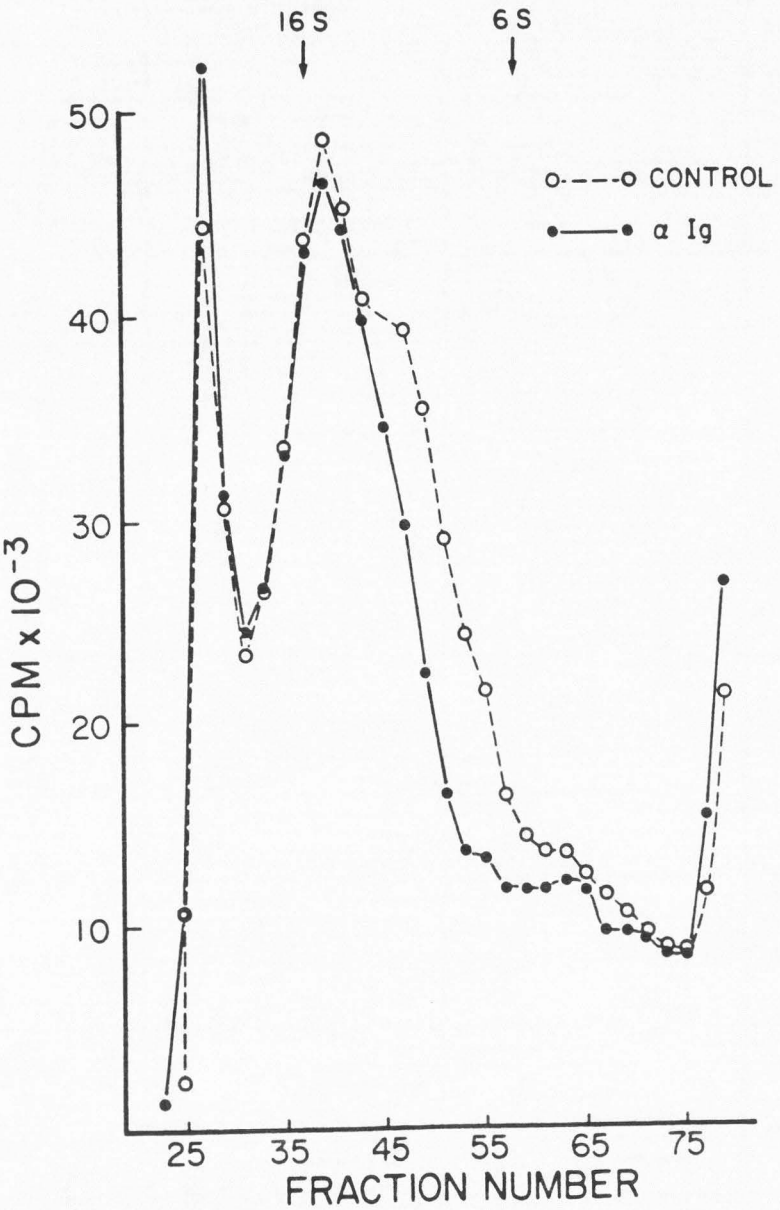
Figure 28. SDS-PAGE autoradiograph of radiolabeled serum high molecular weight and low molecular weight immunoglobulins mixed with bile under different storage conditions. Radiolabeled serum immunoglobulins were mixed with bile and stored for 3 days at the temperatures indicated.


UNREDUCED
HMW Ig
4 C -20 C
LMW Ig
4 C -20 C
REDUCED
HMW Ig
4 C -20 C
LMW Ig
4 C -20 C

Since the immunoprecipitated bile Ig was represented by two major bands (Figure 27), it was important to determine if these bands were due to the denaturing properties of SDS or if two physiologically distinct bile Igs were being precipitated simultaneously. In addition, the initial Staph A immunoprecipitation had shown a considerable amount of nonspecific density (Figure 27). Thus, it was felt there might be bile lipids still associated with the bile Ig. To increase the resolution of the bile Ig precipitations, and to get a better understanding of the physiological molecular weight of bile Ig, Staph A analysis was performed in the presence of the non-ionic detergent NP-40.

The supernatants from control and anti-HMW Ig Staph A immunoprecipitations were chromatographed on A-5M to determine the physiological molecular weight of the bile Ig. The supernatant from the control immunoprecipitation would still have contained the bile Ig whereas the supernatant from the anti-HMW Ig immunoprecipitations would not have contained bile Ig. This experiment showed that the supernatants from the two immunoprecipitations were indistinguishable by chromatography except for one major peak (Figure 29). This peak (at ≈350,000 daltons) was evident in the control precipitation supernate, yet was absorbed by the Staph A when immunoprecipitated with anti-HMW Ig (anti-Ig). This finding supported the initial

Figure 29. A-5M gel filtration of the supernatants of radiolabeled bile after precipitation with antiserum to high molecular weight immunoglobulin (αIg) or with normal rabbit serum (control). Staph A was used to remove immune complexes. The reference elution volumes of the serum 16S and 6S immunoglobulins are indicated by the arrows.


16 S
6 S
50
40
30
20
10
CPM x 10⁻³
o----o CONTROL
•——• α Ig
25
35
45
55
65
75
FRACTION NUMBER

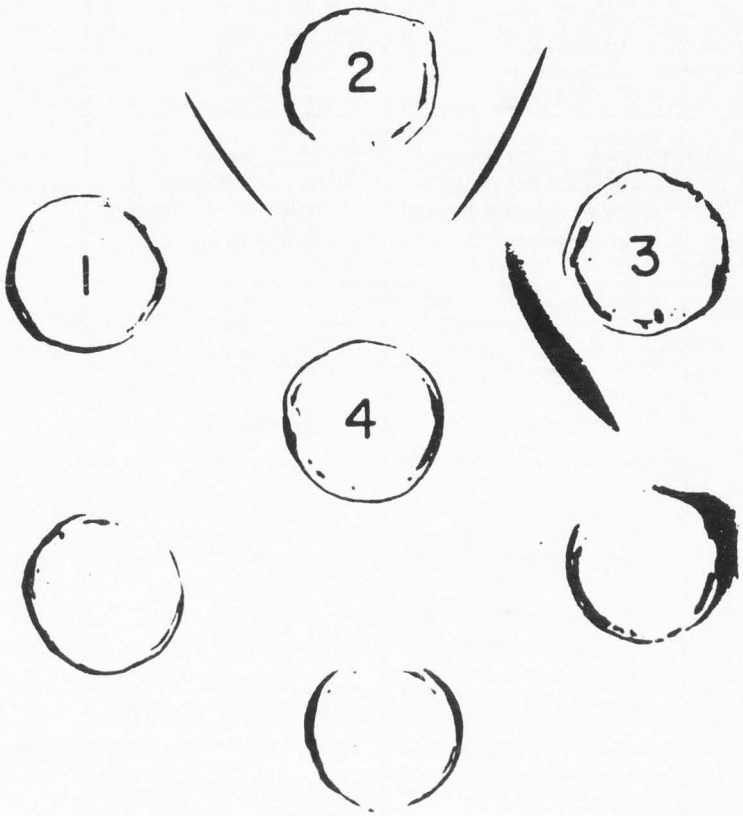
chromatography experiments which identified bile Ig in the same elution area of the column profile. This experiment strongly suggested that bile Ig is physiologically dimeric.

Experiments to conclusively show the relationship of the bile Ig to the serum HMW and LMW Igs required antisera which was specific for the serum HMW Ig. Anti-HMW (anti-Ig) antisera was absorbed empirically with LMW Ig. The resulting antisera (anti-HMW specific) did not react with LMW Ig (Figure 30).

Using anti-Ig and anti-HMW specific antisera, immunoprecipitations in NP-40 were done on identical 50 λ aliquots of the labeled bile pool. The immunoprecipitates were solubilized and analyzed by SDS-PAGE autoradiography (Figure 31). Several important facts were learned from this experiment:

1) The NP-40 significantly lowered the nonspecific density observed in Figure 27 and increased the resolution of the bile Ig bands.
2) There were no apparent differences between immunoprecipitations using the anti-Ig or the anti-HMW specific antisera.
3) The unreduced bile Ig migrated in a predominant band at ~160,000 daltons with a much less dense band at ~325,000 daltons. Both of these bands migrated faster than the HMW serum dimeric and monomeric subpopulation (350,000 and 175,000

Figure 30. Ouchterlony analysis comparing the relationship of absorbed anti-high molecular weight serum immunoglobulin to the unabsorbed anti-high molecular weight immunoglobulin (anti-Ig). Protein concentrations were ~2 mg per ml.



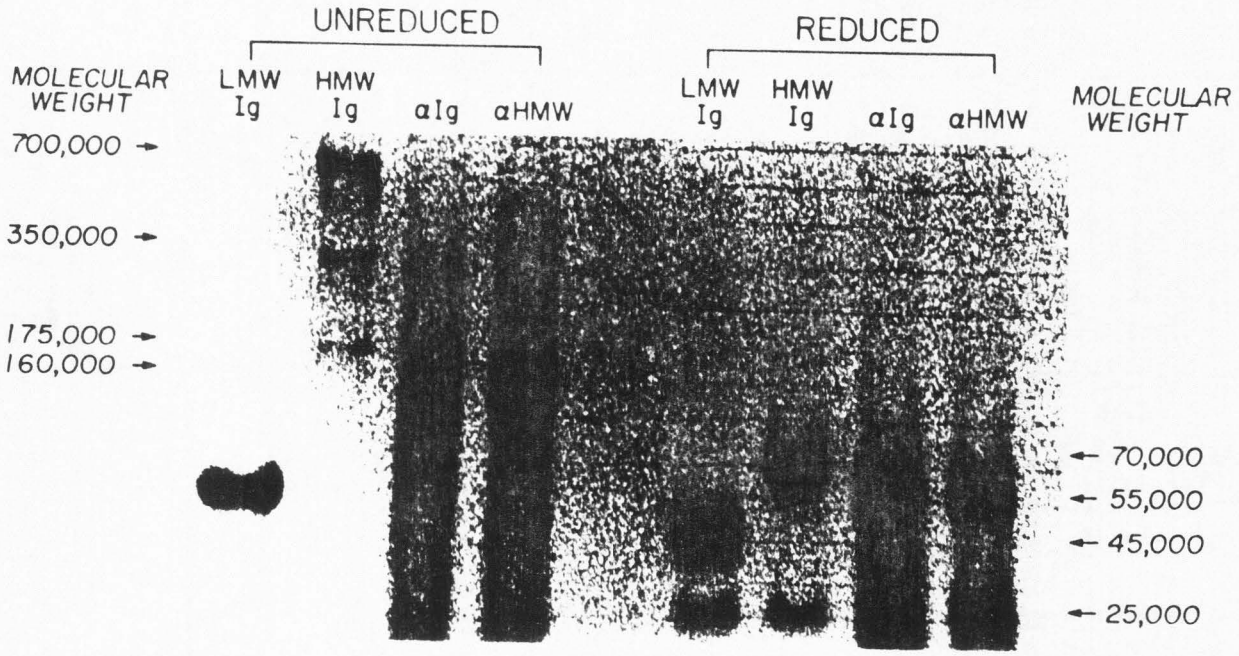
1 - α HMW Ig

2 - HMW Ig

3 - α Ig

4 - LMW Ig

Figure 31. SDS-PAGE autoradiograph of Staph A immunoprecipitated bile immunoglobulin using absorbed anti-high molecular weight serum immunoglobulin specific (αHMW) and unabsorbed anti-high molecular weight serum immunoglobulin (αIg). Low molecular weight and high molecular weight serum immunoglobulins are shown for comparison.


UNREDUCED
REDUCED
MOLECULAR WEIGHT
LMW Ig
HMW Ig
αIg
αHMW
LMW Ig
HMW Ig
αIg
αHMW
MOLECULAR WEIGHT
700,000
350,000
175,000
160,000
70,000
55,000
45,000
25,000

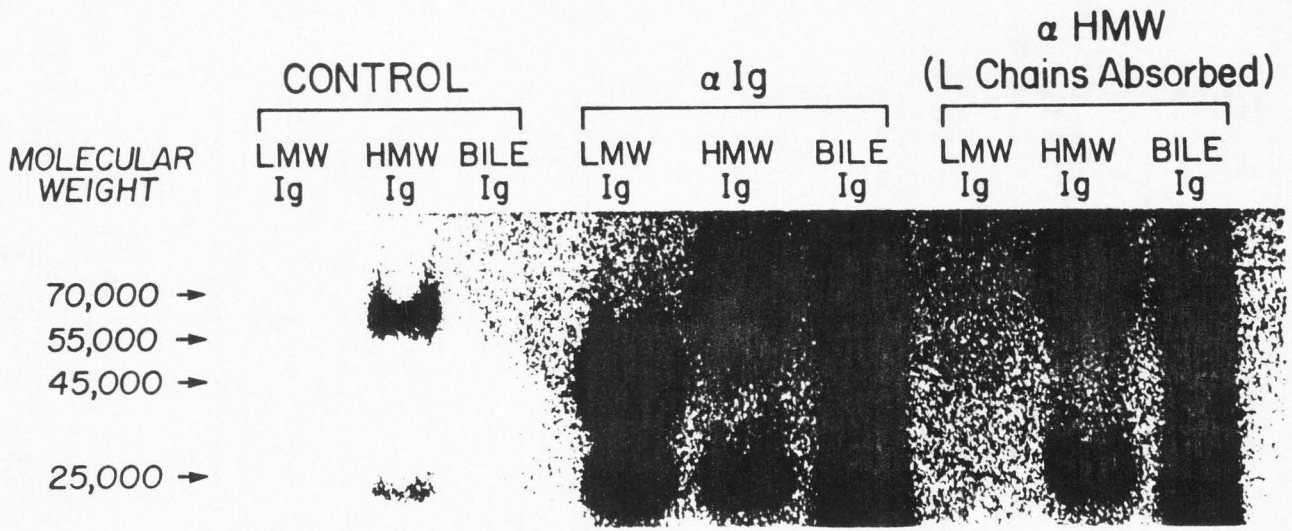
daltons).

4) The reduced bile Ig was composed of two component polypeptide chains. The H chain migrated at 55,000 daltons. This size was intermediate to the serum HMW and LMW H chains. The bile light chain migrated at 25,000 daltons. This was identical to the migration of the serum HMW and LMW L chains.

This surprising, unexpected difference in the bile H chains compared to the HMW and LMW serum H chains was reconfirmed in a more carefully controlled experiment. To insure that the anti-HMW specific antiserum had no L chain activity, the antiserum was heavily absorbed with purified L chains from the HMW serum Ig. In addition, to be sure that the Staph A immunoprecipitation did not peculiarly affect the migration of reduced serum H and L chains, the LMW and HMW Igs were also Staph A immunoprecipitated in the NP-40 buffer. The results of this experiment are shown in Figure 32. This experiment showed several important facts:

1) The HMW serum Ig bound to Staph A without specific antisera. The LMW and bile Igs did not.
2) The anti-Ig immunoprecipitated the serum HMW Ig, the serum LMW Ig and the bile Ig.
3) The anti-HMW absorbed with L chains did not immunoprecipitate serum LMW Ig, but immunoprecipitated the serum HMW and bile Ig.

Figure 32. SDS-PAGE autoradiograph of Staph A immunoprecipitated serum and bile immunoglobulins. Three antisera were used: preimmune (control); anti-high molecular weight serum immunoglobulin (anti-Ig); anti-high molecular weight serum absorbed with low molecular weight immunoglobulin and light chains (anti-high molecular weight, light chains absorbed).


CONTROL
α Ig
α HMW
(L Chains Absorbed)
MOLECULAR WEIGHT
LMW Ig
HMW Ig
BILE Ig
LMW Ig
HMW Ig
BILE Ig
LMW Ig
HMW Ig
BILE Ig
70,000
55,000
45,000
25,000

4) The experiment reconfirmed that the bile Ig definitely had a lower molecular weight H chain than the HMW serum Ig. This difference was ~15,000 daltons.

5) The L chains of the HMW, LMW and bile Igs had identical molecular weights of ~25,000 daltons.

The in vivo relationships of the humoral and secretory immunoglobulins of the sheepshead

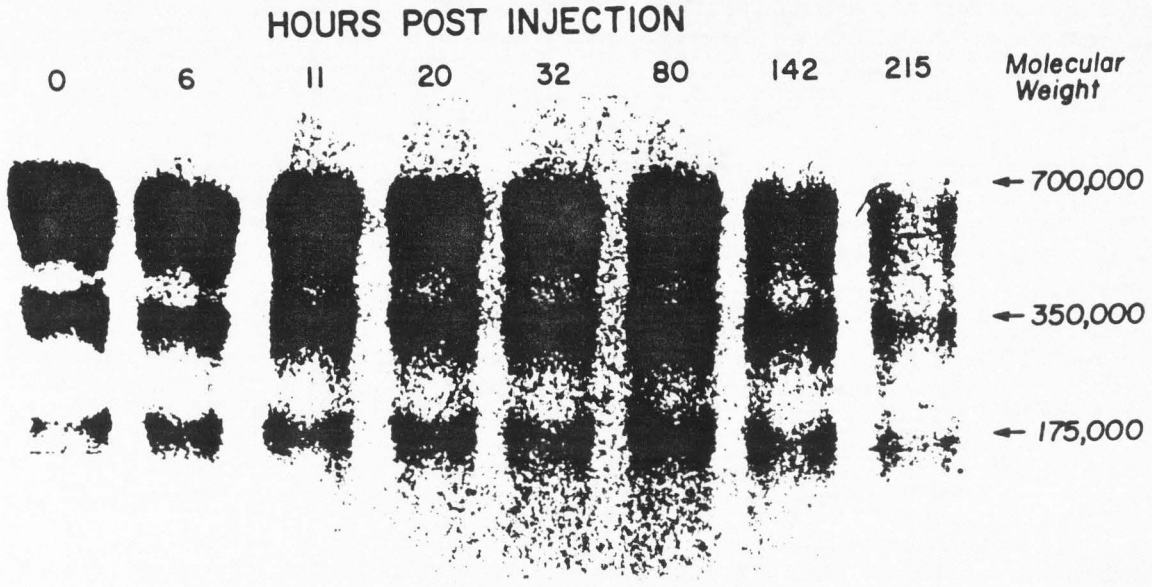
With the immunoglobulins of the serum and secretions defined, it was now possible to address several important questions. The main questions that were posed were:

1) Do the HMW and LMW Igs in serum represent aggregation and/or a degradation product of the same immunoglobulin?

2) Are the Igs in secretions derived by transudation of serum Ig into both mucus and bile?

In vivo clearance of sheepshead HMW serum Ig. Two fish were injected with ^{125}I-HMW serum Ig which was prepared by the chloramine-T method. At various time points, the fish were bled, and cutaneous mucus samples were taken. (Secretions will be discussed in a later section.) Analysis of the serum by SDS-PAGE autoradiography at various bleeds is shown in Figure 33. After 215 hours there was no *in vivo* degradation to a 70,000 dalton band which might be the LMW Ig (or possibly free H chains).

Figure 33. SDS-PAGE autoradiograph of sheepshead serum at various times post-injection of radiolabeled high molecular weight serum immunoglobulin.


HOURS POST INJECTION
0
6
11
20
32
80
142
215
Molecular Weight
700,000
350,000
175,000

A-5M chromatography of serum after 796 hours *in vivo* showed that the ^{125}I-HMW Ig remained in the same elution volume (Figure 34). There was no apparent degradation to yield a LMW Ig peak in either fish injected.

Although there was no apparent shift of the HMW to the LMW Ig, densimetric scans of the autoradiograph of Figure 33 suggested that there may be *in vivo* changes that occurred in the HMW subpopulations (Figure 35). There was a definite change in the ratios of the 700,000 dalton subpopulation to the 350,000 dalton subpopulation when compared over time. Unfortunately, this data does not tell whether the 700,000 dalton tetramer was removed more quickly or alternatively was converted into the 350,000 dalton dimer (or monomer).

In vivo clearance curves for the ^{125}I-HMW Ig were constructed (Figure 36). Linear regression analysis on each fish, comparing the time post-injection to the cpm/0.4 ml serum, was made. (Since fish were bled at different times statistical analysis of pooled data would be invalid.) The regression lines nearly overlapped and the slopes were almost identical. The half-life was ~360 hours or about 15 days.

The possibility that ^{125}I labeling of the HMW Ig with chloramine-T may have masked a protease binding site required for degradation and therefore may not truly have reflected metabolism of the HMW Ig was considered to be a valid critism of this clearance

Figure 34. A-5M gel filtration of sheepshead serum 796 hours post-injection of radiolabeled serum high molecular weight immunoglobulin. Column size 1.5 X 58 cm; 1 ml of sheepshead serum chromatographed; 1 ml fractions were collected. Both optical density and radioactivity levels of the serum are indicated.

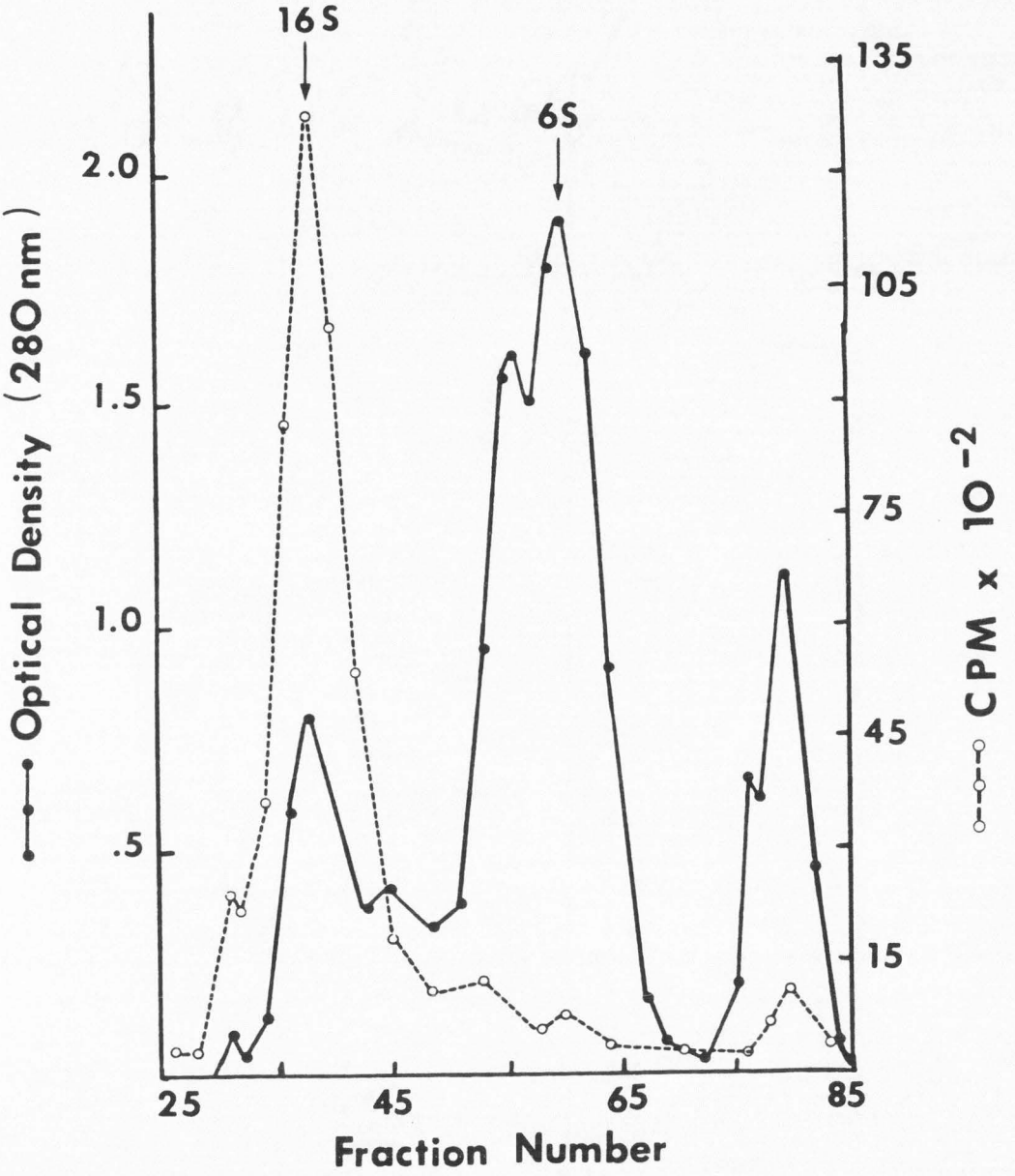

16S
6S
2.0
1.5
1.0
.5
Optical Density (280 nm)
135
105
75
45
15
CPM x 10⁻²
25
45
65
85
Fraction Number

Figure 35. Densimetric scans of SDS-PAGE autoradiograph of high molecular weight serum subpopulations at 0, 6 and 142 hours post-injection of radiolabeled high molecular weight serum immunoglobulin.

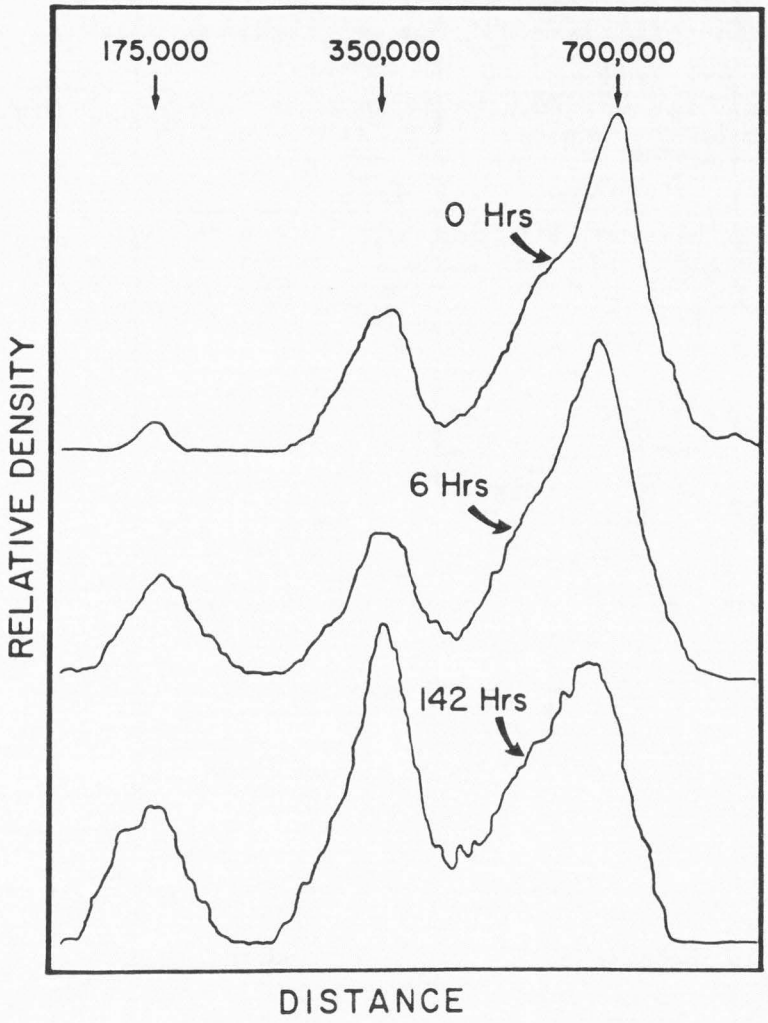
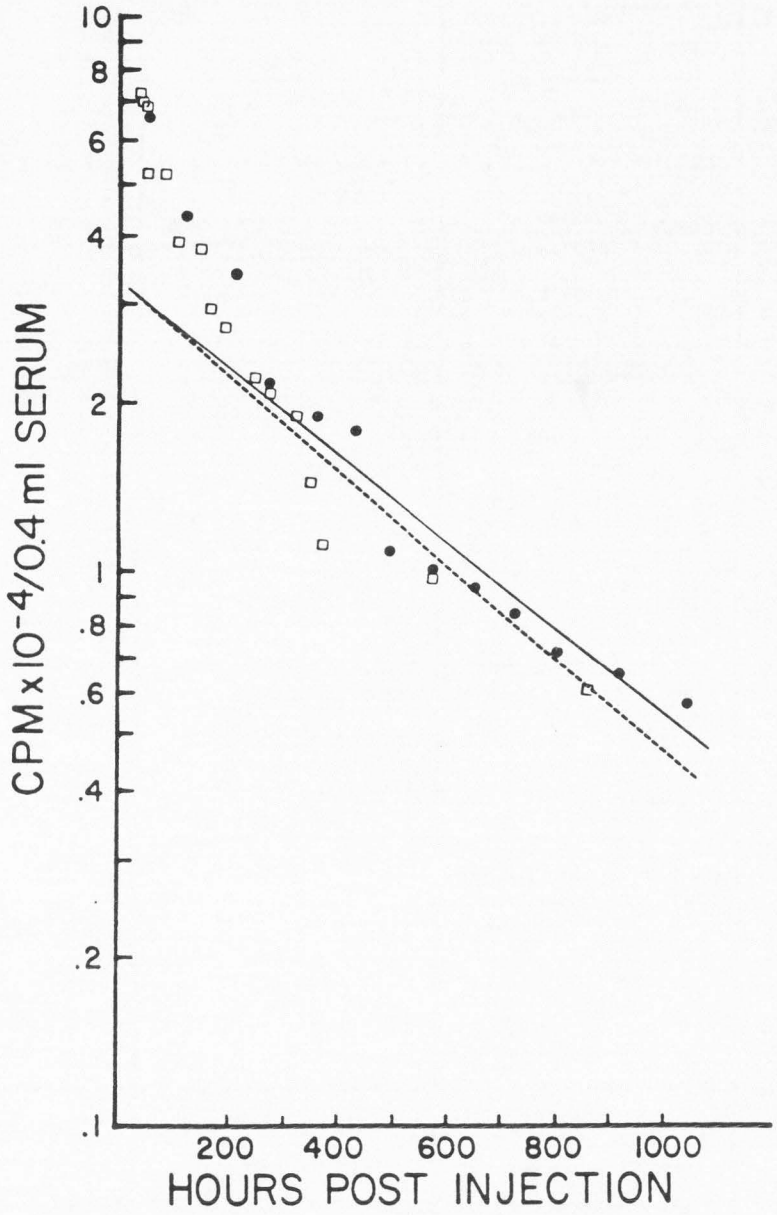

175,000
350,000
700,000
0 Hrs
6 Hrs
142 Hrs
RELATIVE DENSITY
DISTANCE

Figure 36. *In vivo* clearance curve of chloramine-T radiolabeled high molecular weight serum immunoglobulin from two sheepshead. Linear regression analysis was performed on bleeds post-200 hours. The regression equation for fish 1 (open squares) was $\hat{Y} = 5.50815\ X\ -0.00085$. The half-life was 350 hours. The regression equation for fish 2 (closed circles) was $\hat{Y} = 5.51121\ X\ -0.00076$. The half-life was 375 hours.


CPM x 10⁻⁴/0.4 ml SERUM
10
8
6
4
2
1
.8
.6
.4
.2
.1
200
400
600
800
1000
HOURS POST INJECTION

experiment. To investigate this possibility HMW Ig was labeled with Iodogen, which labels proteins in a different manner. Two fish were injected and serum was collected at various times. Results of SDS-PAGE autoradiography of the serum at various times were indistinguishable from Figure 33. Although densimetric scans were not made, there appeared to be a similar change in the ratio of the HMW Ig subpopulations to that observed in Figure 35. A-5M chromatography of the serum after 1040 hours *in vivo* (~43 days) showed that the Iodogen labeled ^{125}I-HMW still eluted in the same molecular weight elution volume (Figure 37). There was no evidence in either of these experiments to suggest that the serum LMW Ig was a degradation product of the HMW Ig.

A clearance curve for the Iodogen labeled ^{125}I-HMW Ig was constructed (Figure 38). Statistical analysis was made to determine the relationship of time post-injection to the cpm/0.4 ml serum of these injected fish (Sokal and Rohlf, 1969). As is shown in Table 1, the variation due to linear regression was significant at the 99.9 percent level. The half life of the HMW Ig was 400 hours (~17 days). The 95 percent confidence interval on the slope was ± .00007; the slope range was -0.00091 to -0.000056. This slope confidence interval included the slope values generated on the two chloramine-T ^{125}I-HMW injected fish. This suggested that there was no statistical difference between the

Figure 37. A-5M gel filtration of sheepshead serum 1040 hours post-injection of Iodogen radiolabeled high molecular weight serum immunoglobulin. Column size 1.5 X 58 cm; 1 ml sheepshead serum chromatographed; 1 ml fractions collected.

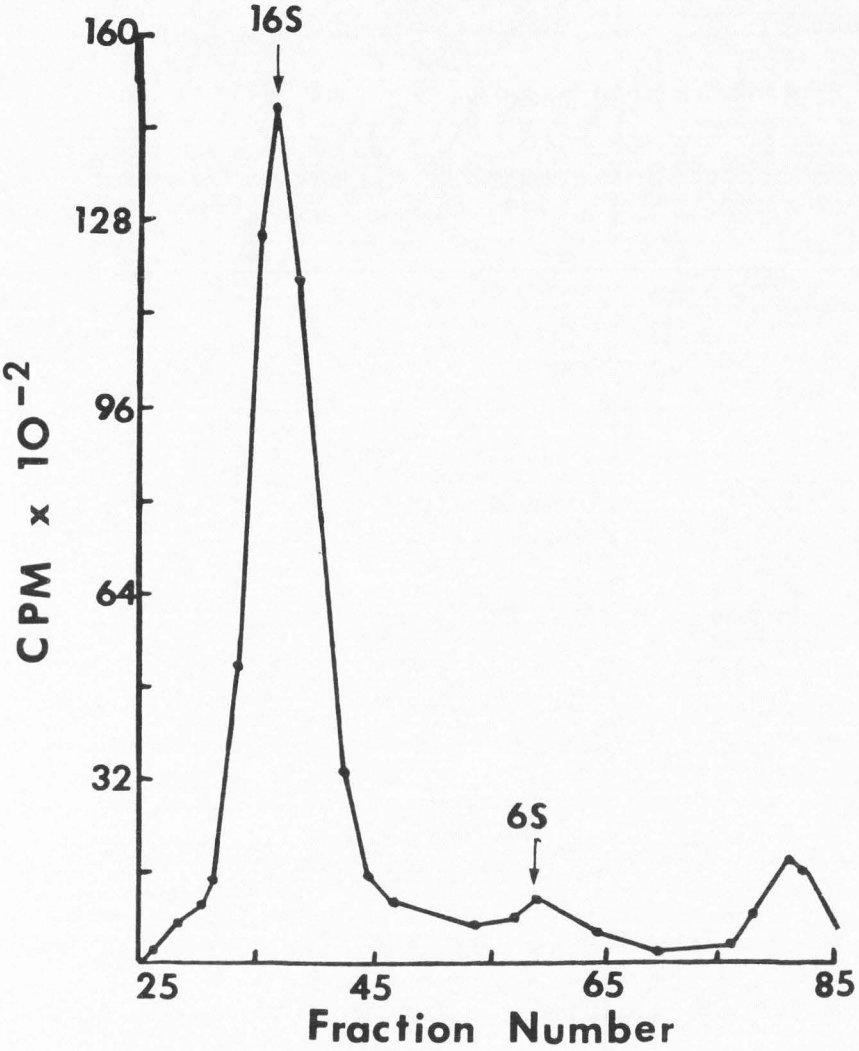

16S
6S
160
128
96
64
32
CPM x 10⁻²
25
45
65
85
Fraction Number

Figure 38. *In vivo* clearance curve of Iodogen radiolabeled high molecular weight serum immunoglobulin from two sheepshead. Linear regression analysis was performed on bleeds post-200 hours. The regression equation was $\bar{Y} = 5.38671\ X - .00074$. 95 percent confidence intervals on the regression line are indicated. The half-life was 400 hours.

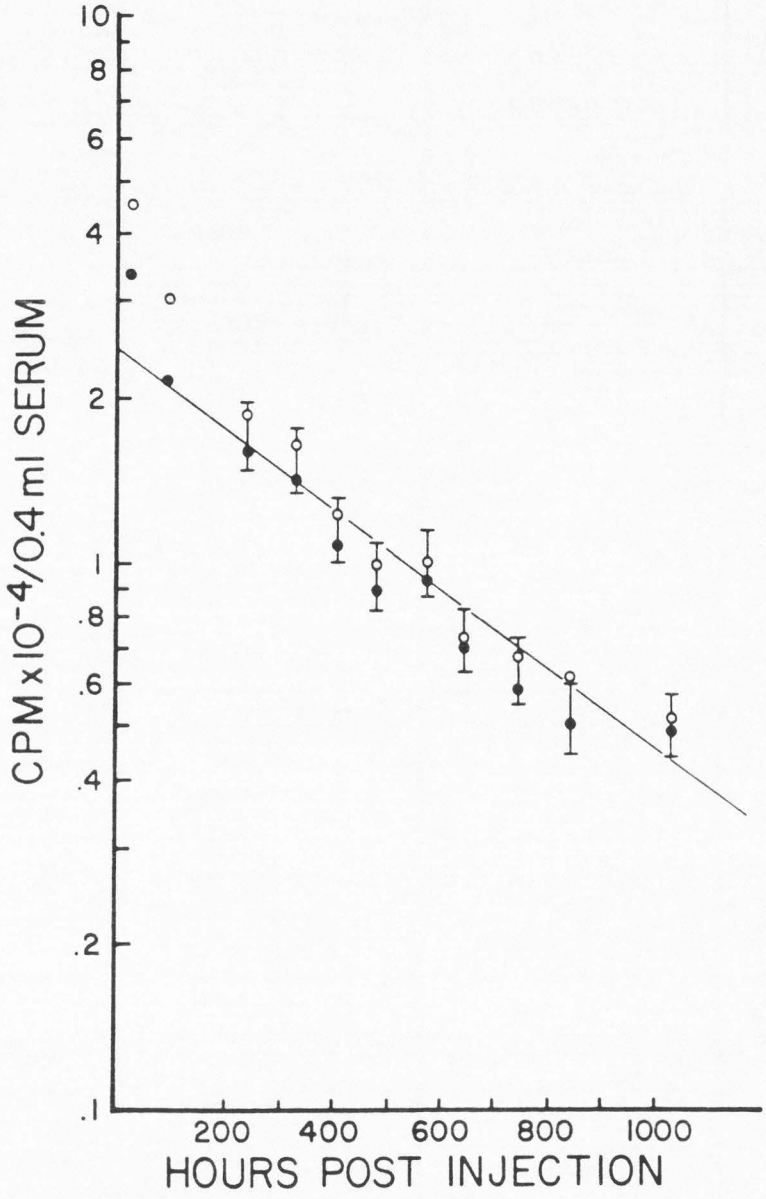

CPM x 10⁻⁴/0.4 ml SERUM
10
8
6
4
2
1
.8
.6
.4
.2
.1
200
400
600
800
1000
HOURS POST INJECTION

Table 1. Analysis of variance for linear regression of sheepshead serum for bleeds more than 200 hours post-injection with Iodogen radiolabeled ^{125}I high molecular weight serum immunoglobulin.

Source of Variation	df	SS	MS	F Ratio
Among Times	8	.6206	.0776	55.43***
Linear Regression	1	.5654	.5654	92.69***
Deviations from Regression	7	.0429	.0061	4.36
Within Times	9	.0123	.0014	
Total	17	.6329		

^{125}I-HMW Ig prepared by the chloramine-T or Iodogen methods.

In vivo clearance of sheepshead low molecular weight serum immunoglobulin. Three fish were injected with ^{125}I-LMW Ig prepared by the chloramine-T method. At various times the fish were bled and the serum collected. SDS-PAGE autoradiography was used to analyze the serum at the various time points. Autoradiographs from each fish showed that there was no *in vivo* aggregation to high molecular weight forms (Figure 39). The injected Ig remained virtually the same after 840 hours (35 days) *in vivo*.

A-5M chromatography of serum after 840 hours *in vivo* showed that the LMW Ig still eluted in the same column fractions (Figure 40). No activity was observed in the HMW Ig elution area.

Figure 39. SDS-PAGE autoradiograph of sheepshead serum at various times post-injection of radiolabeled low molecular weight serum immunoglobulin.

HOURS POST INJECTION

0 24 168 312 384 479 552 625 718 840

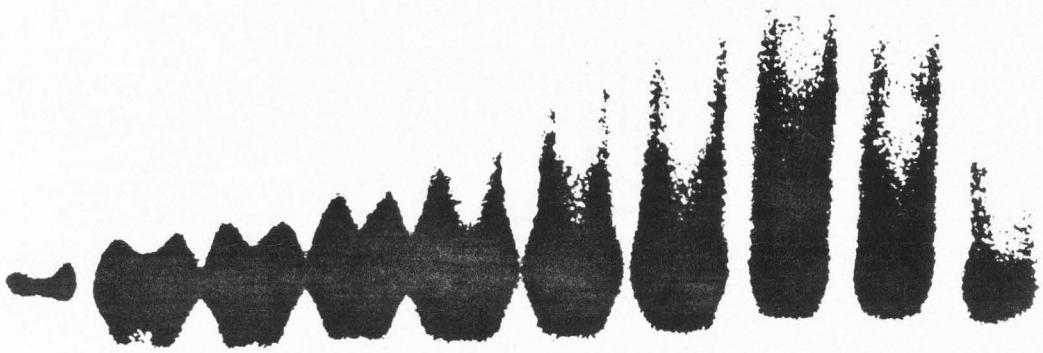
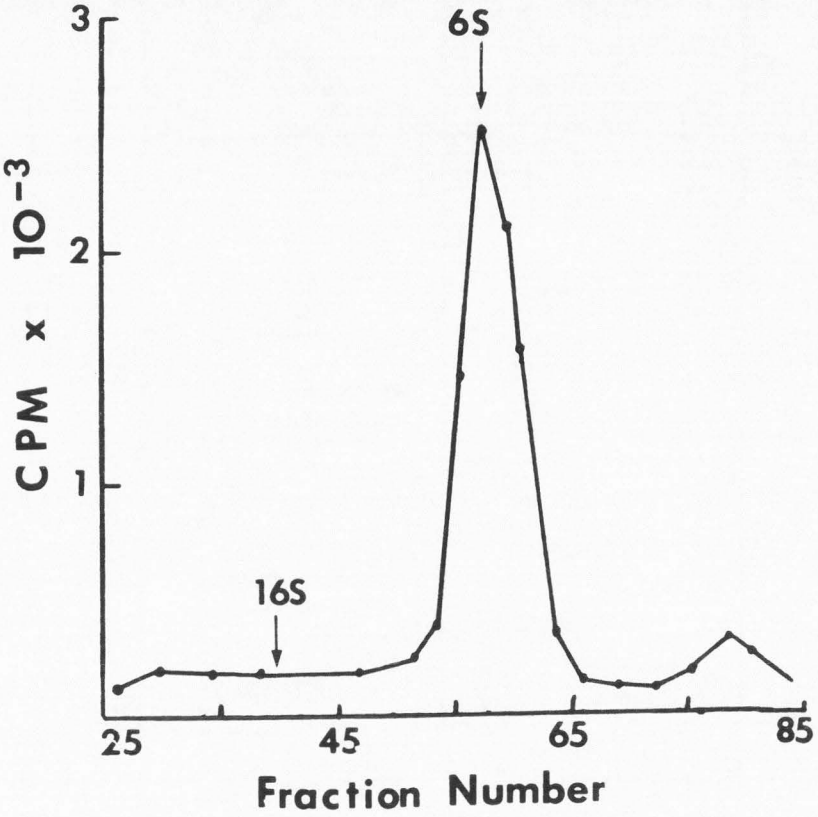


Figure 40. A-5M gel filtration of sheepshead serum 840 hours post-injection with radiolabeled low molecular weight serum immunoglobulin. Column size 1.5 X 58 cm; 1 ml of sheepshead serum chromatographed; 1 ml fractions collected.


3
2
1
CPM x 10⁻³
6S
16S
25
45
65
85
Fraction Number

A clearance curve was made for the ^{125}I-LMW injected sheepshead. Statistical analysis showed that the majority of the variability was due to linear regression (Table 2).

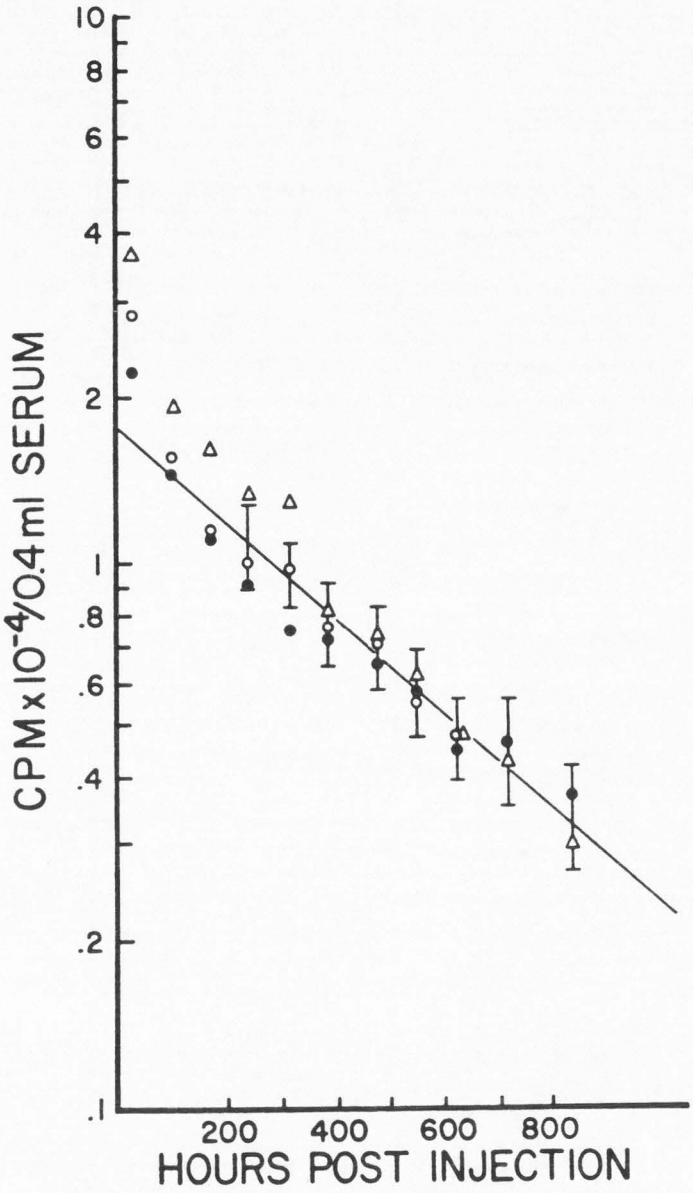
Table 2. Analysis of variance for linear regression of sheepshead serum for bleeds more than 200 hours post-injection with chloramine-T radiolabeled ^{125}I-LMW serum immunoglobulin.

Source of Variation	df	SS	MS	F Ratio
Among Times	7	.5488	.0784	19.60***
Linear Regression	1	.5402	.5402	385.86***
Deviations from Regression	6	.0086	.0014	<1 ns
Within Times	14	.0554	.0040	
Total	21	.6042		

Linear regression was done, and the slope and regression line are shown in Figure 41. The half life for the LMW Ig in serum was 360 hours ($\sim$ 15 days). The slope's 95 percent confidence interval was $-.00085 \pm .00013$. This slope range was interesting because all of the slopes for the HMW clearance curves fell within these limits.

In vivo relationship of the humoral immunoglobulins to the immunoglobulins of mucus and bile. During the in vivo clearance experiments where ^{125}I-HMW or ^{125}I-LMW Igs were followed, cutaneous mucus was collected at various times during the course of the experiments.

Figure 41. *In vivo* clearance curve of chloramine-T radiolabeled low molecular weight serum immunoglobulin from three sheepshead. Linear regression analysis was performed on bleeds post-200 hours. The regression equation was $\bar{Y} = 5.24380\ X\ -0.00086$. 95 percent confidence intervals are indicated. The half-life was 360 hours.


10
8
6
4
2
1
.8
.6
.4
.2
.1
CPM x 10⁻⁴/0.4 ml SERUM
200
400
600
800
HOURS POST INJECTION

At the termination of each experiment, bile was also collected. Radioactivity levels were monitored, and the amounts of Igs in serum, bile and mucus were quantitated by radial immunodiffusion.

The quantities (mg/ml) of Igs in serum, mucus and bile are summarized in Table 3. These values are the means from 26 serum samples, 23 mucus samples and 10 bile samples. The HMW Ig was quantitatively the predominant Ig of the sheepshead serum. There was approximately 1/20th the amount of LMW Ig as HMW Ig in the serum.

Table 3. A summary of the quantities of immunoglobulins in serum, bile and mucus as determined by radialimmunodiffusion.

Body Fluid		Ig Quantity (mg/ml)	Standard Deviation (mg/ml)
Serum	HMW[1]	2.90	±.78
	LMW[2]	0.17	±.07
Bile	HMW	.09	±.01
	LMW	None Detected	
Mucus[3]	HMW	.09	±.03
	LMW	.024	±.002

[1]HMW = Detected by anti-HMW specific antiserum

[2]LMW = Detected by limited pore size diffusion (did not allow detection for 200,000 or greater molecular weight)

[3]After concentration to constant volume of 0.75 ml

The bile contained only HMW Ig by these analyses and was approximately 1/30th the concentration of the HMW Ig in the serum. HMW Ig was present in all but one of 26 mucus samples collected. The concentration of mucus HMW Ig was approximately the same as found in bile. In four mucus samples with the highest HMW Ig concentrations, LMW Ig was also found (although in very small amounts of ~.024 mg/ml). The ratio of LMW to HMW Ig (in matched samplings of serum and mucus) was considerably higher in the mucus than in serum (Table 4).

Table 4. Ratio of low molecular weight immunoglobulin to high molecular weight immunoglobulin in four serum and mucus samples in which low molecular weight immunoglobulin was detected in mucus.

Ratio: LMW/HMW	
Serum	Mucus
.0307	.1385
.0681	.2790
.1791	.1917
.0670	.2000
x = 6.12	x = 20.23

Since the radial immunoassay values for LMW Ig do not establish exact identity to serum LMW Ig (the technique detects Ig less than ~200,000 daltons), the values could include LMW Ig as well as monomeric HMW Ig.

Because of this observation the pools of Figure 15 were reexamined by radial immunoassay. Pool 4 had .037 mg Ig and Pool 5 had .027 mg Ig for a total of .064 mg of Ig (less than 200,000 daltons). Pool 2 had .21 mg and Pool 3 had .09 mg HMW Ig for a total of .30 mg (greater than 200,000 daltons). The ratio of LMW Ig to HMW Ig was 21.3 percent (.064/.30 mg) and was in accord with similar ratios of Table 4. Pools 4 and 5 were combined and further concentrated, and the Ouchterlony reaction of identity with LMW serum Ig was observed. This shows that LMW Ig is present in the mucus.

The amount of immunoglobulin in mucus did not appear to change significantly during consecutive mucus samplings from the same fish. This finding suggested that the replacement of Ig in mucus is a dynamic process.

Radioactivity levels in all the body fluids were carefully measured. Trichloroacetic acid (TCA) precipitations were done on bile and serum. Due to the low radioactivity levels of concentrated mucus samples, cpm were read directly from the samples. (Thus, these values represent the highest cpm possible.)

TCA precipitations of serum, even after the longest times of sampling, were usually in excess of 90 percent (range 86 to 94 percent). The bile always had on a unit basis more cpm than the serum. However, after TCA precipitation the bile cpm were approximately 3 percent

(range 1 to 6 percent). Many of the TCA precipitated counts were associated with the lipids of bile. After alcohol-ether extraction only about 30 percent of the TCA-precipitated cpm remained (range 23 to 65 percent), or less than 1 percent of the original cpm. Alcohol-ether extraction had no effect on the TCA precipitable cpm of serum.

With the radioactivity levels monitored and the amounts of Ig quantitated, it was possible to compare the Igs in serum and secretions on a cpm/mg basis. By this method it was shown that the Igs in bile and mucus were not derived from the serum in the ^{125}I-HMW injected fish (Table 5). This table shows that in every case of the ^{125}I-HMW injected fish the cpm/mg values were higher in serum than in secretions. Thus, the secretory Igs could not have been derived by simple transudation or active transport of the HMW serum Ig.

Work with IgA suggested that clearance to secretions occurred very rapidly. To test this question, three fish were injected with Iodogenated ^{125}I-HMW Ig and sacrificed three days later. The Ig levels in serum, mucus and bile were quantitated and radioactivity levels monitored. The results showed that there was no rapid clearance of HMW Ig to these secretions (Table 6). It was again observed that on a cpm/mg basis the secretory Igs of mucus and bile were not derived from serum HMW Ig. The amounts of secretory Igs were approximately the same

Table 5. Comparison of the cpm/mg of immunoglobulins in serum, bile and mucus from radiolabeled high molecular weight serum immunoglobulin injected sheepshead.

Chloramine-T HMW #1[1]				
	Hours Post-Injection			
	56	116	292	796
Serum	37453	32261	15064	6930
Mucus	3478	1117	666	1379
Bile	-	-	-	5364

Chloramine-T HMW #2				
	Hours Post-Injection			
	171	317	602	1104
Serum	74817	57573	36390	13147
Mucus	No Ig Detected	384	471	1081
Bile	-	-	-	1641

Iodogen HMW #1[2]			
	Hours Post-Injection		
	336	576	1034
Serum	58155	42364	31656
Mucus	815	733	766
Bile	-	-	5523

Iodogen HMW #2			
	Hours Post-Injection		
	336	576	1034
Serum	63171	44270	32564
Mucus	2183	546	209
Bile	-	-	3500

[1]Refers to HMW Ig labeled by the Chloramine-T method

[2]Refers to HMW Ig labeled by the Iodogen method

Table 6. Comparison of the cpm/mg of immunoglobulins in serum, bile and mucus from radiolabeled high molecular weight immunoglobulin injected sheepshead three days post-injection.

	Fish 1	Fish 2	Fish 3
Serum	132,358	171,458	236,779
Mucus	666	Not collected	625
Bile	24,318	5,000	No Ig detectable

as observed for other fish. The relationship of the cutaneous mucus Ig to the ^{125}I-LMW serum injected fish was also examined. By radial immunodiffusion assay only four of 26 mucus samples were positive for LMW Ig (as discussed above). Of these four, only one was from a LMW injected animal. In this one positive sample, the cpm/mg of LMW Ig in serum was 42,933, whereas the mucus was 6,000 cpm/mg. This would be in accord with the ^{125}I-HMW injected fish results which showed that high molecular weight mucus Ig was not derived by transudation.

Analysis of bile Ig (presented in the previous section) showed that bile Ig was not composed of aggregated LMW monomers. Radial immunodiffusion also found no evidence for low molecular weight Ig in bile. Thus any attempt to attribute cpm/mg LMW serum Ig to cpm/mg bile seemed unsupportable. (If such comparisons were to be presented, they also showed that transudation

of LMW aggregate could not be the mechanism to explain Ig presence in bile.) However, the possibility remains that there may be extremely small amounts of a low molecular weight immunoglobulin in bile that were below the sensitivity of the assays used in this study.

Thus, it can be concluded that:

1) HMW Ig is quantitatively the predominant Ig in serum.
2) LMW Ig is present in serum at ~1/20th the concentration of HMW Ig.
3) There is both HMW Ig and a LMW Ig in mucus.
4) Bile apparently does not contain LMW Ig.
5) Bile Igs and mucus Igs are not derived by simple transudation or active transport of the serum Igs.

DISCUSSION

The sheepshead has two predominant serum Igs. Quantitatively the high molecular weight Ig is the most abundant (~2.9 mg/ml). The HMW Ig has a sedimentation coefficient of ~16S in physiological buffer which is in agreement with other values for teleosts' HMW Ig (Clem, 1971; Cisar and Fryer, 1974; and Clem and McLean, 1975). The present study is the first reported work to analyze intact teleost HMW Ig by SDS-polyacrylamide gel electrophoresis. This analysis disclosed that the purified HMW Ig was composed of two immunoglobulin subpopulations: one being a disulfide-linked form (~700,000 daltons) and a second population of predominantly disulfide-linked dimers (~350,000 daltons). However, upon reduction with 2-mercaptoethanol, only two bands were observed. The H (heavy) chain was ~70,000 daltons, and the L (light) chain was ~25,000 daltons. The molecular weights of the H and L chains were similar to those reported for other HMW teleost Igs (Clem, 1971; Clem and McLean, 1975; Marchalonis, 1971), as well as mammalian IgM (Nisonoff, et al., 1975).

The existence of two subpopulations of sheepshead HMW Ig was also demonstrated by sedimentation velocity studies in 5 M guanidine-HCl. When the HMW Ig was equilibrated in guanidine and analytical ultracentrifuge

studies were made, the HMW Ig dissociated into two boundaries (at ~16S and at ~11S). These sedimentation coefficients are indicative of tetrameric and dimeric populations. The two HMW Ig subpopulations did not appear to be in equilibrium. The tetrameric and dimeric Ig populations remained essentially the same when eluted from SDS gels. The tetrameric population did not further dissociate to yield the dimeric form. Both reanalyzed subpopulations did yield monomeric forms, which may be due to the periodate digesting the associated Ig carbohydrate. Ig carbohydrate has been suggested as a method for stabilization of polymeric immunoglobulins (Melchers, 1973).

J chain is also suspected to be important in the intracellular polymerization of polymeric Igs (Koshland, 1975). J chain is found on both mammalian IgM and IgA. In addition J chain appears to be a phylogenetically "conserved" polypeptide in polymeric immunoglobulins (McCumber and Clem, 1976). Although J chain has not been found in all teleost HMW Igs (Weinheimer et al., 1971), this may be due to the methods used. J chain was observed in the HMW sheepshead Ig. Alkaline-urea gel analysis demonstrated that the sheepshead J chain had the characteristic fast anodal mobility which is typical of all J chain polypeptides. Although stoichiometric measurements were not made on the sheepshead J chain, it is interesting to speculate that only one of

the HMW subpopulations may contain this polypeptide.

The discovery of two subpopulations of sheepshead HMW Ig supports work with other teleosts' HMW Igs. The rainbow trout, Salmo gairdneri, showed immunoglobulin electrophoretic heterogeneity (Hodgins et al., 1965). Trout antibodies partially purified by G-200 chromatography showed two boundaries at 13.9S and 11S when subjected to analytical ultracentrifugation. Unfortunately, the antibody fraction was not critically analyzed to determine purity. However, by immunoelectrophoresis the antibody fraction showed no contaminants (Hodgins et al., 1967). The goldfish, Carassius auratus, HMW Ig was also observed to have two distinct immunoelectrophoretic populations which had similar sedimentation coefficients of ~16S (Trump, 1970).

The possibility exists that the two HMW subpopulations may also have different biological properties. Goldfish immunized with bovine serum albumin (BSA) or BSA-hapten showed that although only one size of antibody was observed (13.2S), there were two immunoelectrophoretically detectable populations (Everhart, 1972). Both electrophoretic populations reacted with BSA in fish receiving BSA alone. However, in fish receiving BSA-hapten, the faster moving antibody reacted with the hapten, the other reacted with BSA.

Investigations into the affinity of hapten binding sites of the 16S giant grouper Ig also showed definite

heterogeneity (Clem and Small, 1970). Half of the binding sites measured had a higher affinity for the hapten than did the other half. These investigators questioned whether or not the high and low binding sites were on the same or different Ig molecules.

Thus, an important question yet to be answered is if the heterogeneity observed with the sheepshead HMW Ig is typical of most teleosts. Future work is required to show whether other teleosts have two types of 16S HMW Ig: one a disulfide-linked form and the other a noncovalently-linked population of predominantly disulfide-linked dimers. Future studies are needed to determine whether or not the biological functions of the subpopulations are different.

In addition to the HMW Ig, the sheepshead also has a low molecular weight (LMW) Ig in the serum. The LMW Ig was found in much lower quantities ($\sim$.17 mg/ml), approximately 1/20th the amount of the HMW Ig. LMW Ig was antigenically deficient to the HMW Ig by Ouchterlony analysis (using antisera prepared against the HMW Ig). The reduced LMW Ig on SDS-gels was composed of two polypeptide chains: a heavy chain of $\sim$45,000 daltons and a light chain of $\sim$25,000 daltons. Thus, the LMW Ig H chain is $\sim$25,000 daltons lighter than the HMW Ig H chain. This deficiency is presumed to be the reason for the antigenic reaction of partial identity.

Sedimentation velocity studies with the LMW Ig indicated a symetrical boundary with a sedimentation

coefficient of 6.1S in physiological buffer. However, when the 6S protein was analyzed in 4 M guanidine-HCl, the protein dissociated into a single boundary at 4S, indicative of halfmers or H-L chain pairs. This observation was also shown on SDS-gels. The unreduced 6S LMW Ig migrated in a single band at 70,000 daltons.

Low molecular weight immunoglobulins have been observed in other fish. The initial report on goldfish immunoglobulins described both HMW and LMW Igs (Uhr et al., 1962). (Uhr's report even suggested that the LMW Ig was a maturation product which was enhanced at high temperatures.) Yet in a later study, no LMW Ig was observed in goldfish (Trump, 1970). When the goldfish Igs were reinvestigated, a "LMW Ig" was found, but the component polypeptide chains did not match the HMW Ig H and L chains (Marchalonis, 1971). It was suggested that this LMW form represents a degradation product of the HMW Ig.

A similar controversy surrounds the Igs of the trout, Salmo gairdneri. LMW Ig was observed in the initial report (Hodgins et al., 1967) yet could not be found in a subsequent report (Cisar and Fryer, 1974). In an attempt to shed light onto this controversy, Clem and McLean (1975) reinvestigated the margate to determine if a low molecular weight Ig existed. (An earlier report stated that no LMW Ig was present in the margate [Clem and Sigel, 1966]). Using sensitive binding assays, a

LMW Ig was found which had H and L chains identical to the HMW Ig, suggestive of a tetramer-monomer relationship.

A LMW Ig was also described in the giant grouper. The LMW Ig described was remarkably similar to the sheepshead LMW Ig (ie. it was antigenically deficient to the HMW Ig, and the H chain was "missing" a 30,000 dalton segment found on the HMW H chain.) Unfortunately, the margate and giant grouper LMW Igs were not analyzed to determine if the intact Ig was associated by means of noncovalent forces (eg. using SDS-gels, analytical ultracentrifugation in guanidine, gel filtration in proprionic acid, etc.).

A low molecular weight immunoglobulin (6.6S) of the lamprey was shown to be composed of H and L chains which were noncovalently attached (Marchalonis and Edelman, 1968). The hagfish was also shown to have a low molecular weight immunoglobulin (~160,000 daltons) which was weakly stabilized by covalent interactions and aggregated noncovalently to higher molecular weight forms (Raison et al., 1978). To my knowledge there is no reference to any Igs from fish or other animals which dissociate into halfmers or heavy-light pairs in denaturing solvents.

The question as to the origin of the LMW Ig has also been controversial. Marchalonis (1971) suggested that the LMW Ig of fish came from degradation of the higher molecular weight immunoglobulin. Increased amounts of low molecular weight Ig were observed with increasing

length of storage at 4 C. However, this reported LMW Ig did not have H or L chains similar to the HMW form. Clem and McLean (1975) reported that margate HMW Ig did not dissociate to yield LMW Ig *in vitro* after 3 weeks at 2.5 C, at room temperature for 5 days, at 36 C for 48 hours or at -20 C for 3 months (analyzed by comparing gel filtrations of whole margate sera under the various conditions).

A similar question was also asked regarding shark LMW Ig which is apparently a monomer of the high molecular weight form. There was no evidence of interconversion of the radioactively tagged HMW or LMW Igs after 140 hours *in vivo* (Small et al., 1970).

The sheepshead purified HMW Ig did not dissociate into lower molecular weight forms after one year at -20 C. However, studies were made to determine if *in vivo* degradation of the HMW Ig resulted in the LMW Ig, or alternatively if the LMW was somehow aggregated *in vivo* to form the HMW Ig. Sheepshead HMW Ig labeled by chloramine-T methodology and injected intravenously showed no evidence of being degraded into LMW Ig after 796 hours *in vivo*.

Further experiments were conducted to insure that the labeling procedure did not mask a protease site on the HMW Ig, thus being an inaccurate reflection of true *in vivo* metabolism. HMW Ig was labeled with Iodogen and injected intravenously into other sheepshead.

Again, no *in vivo* degradation into LMW Ig was observed after 1040 hours. Radioactive LMW Ig was also injected intravenously into sheepshead to determine if the LMW Ig could be aggregated *in vivo* to "yield" the HMW Ig.

These experiments also showed that the half-lives of the HMW and LMW Igs were identical, both ~16 days. It is interesting that the half-lives observed for the sheepshead Igs are considerably higher than IgM of mammals. Thus, these studies suggest that the sheepshead HMW and LMW Igs are not metabolic products of one another, but instead are either the products of separate genes or reflect differences in transcription or translation.

Avascular or secretory immunoglobulins were found in sheepshead cutaneous mucus. Prior to this report cutaneous mucus had been observed to contain Ig in three species of fish: the gar, *Lepisosteuus platyrhincus* (Bradshaw et al., 1971); Australian catfish, *Tachysurus australis* (Diconza and Halliday, 1971); and the plaice, *Pleuronectes platessa* (Fletcher and Grant, 1969 and Fletcher and White, 1973). These reports did not characterize the mucus Ig except to show antigenic identity with serum Ig, and to show that the Ig was probably greater than 200,000 daltons (the Ig voided a G-200 column).

The sheepshead cutaneous mucus had two types of immunoglobulins greater than 200,000 daltons. The first

gel filtered identically to the HMW serum Ig. Upon reduction, its component chains were found to be identical to the HMW serum Ig, with H chains ~70,000 daltons and L chains ~25,000 daltons. However, interpretation of the unreduced form was difficult. This mucus tetrameric Ig apparently dissociated in a similar pattern to the HMW serum Ig. However, upon reduction the "dimeric" band did not reduce to the H and L chains. This "dimeric" form is either a contaminant or represents a 2-mercaptoethanol (2-ME) resistant form of Ig. Since all of the previously identified sheepshead Igs were 2-ME sensitive, this band is probably a contaminant. If one assumes the band to be a contaminant, then the unreduced mucus Ig resembles the HMW serum "tetrameric subpopulation" that was eluted from SDS-gels and reanalyzed. This suggests even more strongly that the HMW serum Ig is heterogeneous, and that the subpopulations are separable in vivo.

The second form of cutaneous mucus Ig greater than 200,000 daltons gel filtered at ~350,000 in the range of an Ig dimer. By ion exchange chromatography two dimeric Ig populations were observed. These unreduced dimeric populations were different on SDS-gels. One population dissociated into monomeric forms; the other remained essentially dimeric, although its molecular weight was slightly greater than the dimeric subpopulation of the HMW serum Ig. Both populations had identical

H and L chains of ~70,000 and ~25,000 daltons, very similar to the HMW serum H and L chains. However, the dimeric population which was slightly greater in molecular weight also yielded another polypeptide of ~95,000 daltons. This polypeptide may well be the equivalent of the secretory piece in higher vertebrates. The mobility of this "secretory" polypeptide is consistent with mammalian secretory piece (Tomasi, 1976). At this time, however, no strong suggestions can be made as to whether the extra polypeptide of the mucus dimer has secretory functions similar to the functions of the secretory piece of mammals.

The bile was also shown to contain Ig. There was only one form of bile Ig in physiological buffers: dimeric. The dimer, however, did dissociate into monomers on SDS-gels, indicating that the dimer was not covalently linked. The molecular weight of the monomer units was ~320,000 daltons on SDS-gels. When the bile Ig was reduced, two components were observed: a heavy chain ~55,000 daltons and a light chain ~25,000 daltons. Thus, the heavy chain was different from either of the serum Igs H chains and the mucus Ig H chains. The bile H chain, however, did not appear to be a different class of Ig, since antisera to the HMW serum Ig immunoprecipitated the bile Ig. It was interesting that absorbed antisera specific for the HMW serum Ig (which did not precipitate LMW serum Ig) immunoprecipitated the bile Ig.

This suggests that the bile H chain contains a portion of the 25,000 dalton segment of the HMW serum H chain which is absent in the LMW serum H chain.

A secretory piece was not observed in bile Ig similar to the extra polypeptide chain seen with the dimeric mucus Ig. This finding is consistent with known facts about secretory piece association. It has been shown that only covalently associated Ig polymers with J chain bind secretory component (Tomasi, 1976). Since the bile Ig dissociated into monomeric forms on SDS-gels, this observation suggests that J chain is probably not present and therefore neither is the secretory piece.

It is interesting though to speculate that the gene(s) which may have led to IgA may have been an IgM precursor. Evidence is presented here that the molecular weight of the sheepshead bile Ig H chain is similar to the mammalian "α" heavy chain (52,000 daltons). Chuang et al. (1973) have speculated that IgA is more closely related to IgM than to IgG. These investigators showed the presence of an additional 19 residues in both μ and α chains that extend beyond the C-terminus of the γ_1 chain. The degree of homology between α and μ in the 40-residue sequence is 55 percent and, of the 18 positions at which α and μ differ, 14 can be explained by a single base change in a codon (as reviewed by Nisonoff et al., 1975). Thus it seems very possible that teleosts may have the "early" gene for IgA.

Another major finding of this study was that the mucus and bile Igs of sheepshead were not derived from either the serum HMW or LMW Igs. During the *in vivo* clearance experiments where ^{125}I-HMW or ^{125}I-LMW serum Igs were followed, cutaneous mucus and bile were collected. Radioactivity levels in all the body fluids were carefully measured. By measuring the Ig levels in the serum, mucus and bile by radial immunodiffusion and comparing the Ig quantity to the radioactivity, it was possible to express the results in terms of cpm/mg of Ig. By this measurement the bile averaged 29 percent and the mucus 4 percent of the TCA precipitable cpm/mg found in the serum. Thus, the secretory immunoglobulins could not have been derived by simple transudation or active transport of the serum Igs. This finding suggests that the Ig in the secretions is locally synthesized.

It is interesting to note that in orally immunized fish, humoral antibodies were not observed, yet the fish were apparently protected against the pathogens for which they were immunized (Anderson and Nelson, 1974; Fryer et al., 1972). These observations are reminiscent of the observations of Besredka who argued that local immunity could be established independently of circulating antibody and systemic immunity (Besredka, 1927). It was not until Tomasi in the early sixties established the role of IgA in secretions that Besredka's hypothesis was shown to be correct.

Tomasi (1976) defines one of the hallmarks of a true secretory immune system to be local synthesis. The sheepshead has been shown to have immunoglobulins in secretions which were not derived by active transport or by transudation of serum HMW or LMW Igs. Further work is necessary to determine if plasma cells can be found in the interstial tissues of the fish's skin and intestinal mucosa.

In conclusion it seems appropriate to point out several directions for future work in this area that may result in practical applications. The first direction would be to establish the site(s) of synthesis of the secretory immunoglobulins in fish. Once this is accomplished subsequent studies must focus on methods to preferentially elicit the production and secretion of antibodies in fish external secretions. Finally, the question of whether such antibodies can exhibit protective functions can be approached. Optimistically, completion of the above projected studies will result in practical methods for the mass immunization of fish against a variety of infectious diseases.

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