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Title: A Partial Characterization of High and Low Molecular Weight Immunoglobulin in Rainbow Trout (Salmo gairdneri)

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Reports in the literature indicate that only one type of immunoglobulin can be detected in the serum of rainbow trout (Salmo gairdneri). This high molecular weight immunoglobulin (HMWIg) is a tetramer with a sedimentation coefficient of approximately 14 S and a molecular weight of 620,000 daltons. A lower molecular weight immunoglobulin (LMWIg) has never been observed, even after prolonged immunization. Using Sepharose-6B-TNP-BSA affinity chromatography for isolation, and molecular sieve chromatography for separation, we have purified both HMWIg and LMWIg. The LMWIg has a sedimentation coefficient of approximately 10 S and a molecular weight of 490,000 daltons. The LMWIg is not a catabolic product of the HMWIg, nor is the HMWIg an anabolic product of the LMWIg.

The amino acid composition of the HMWIg differs from that of the LMWIg, yet by SDS-PAGE the molecular weights of their H and L chains are the same. In immunoelectrophoresis the HMWIg migrates more towards

the anode than the LMWIg. The precipitin arcs indicate that the HMWIg and LMWIg cross-react but that the HMWIg is devoid of at least one antigenic determinant found on the LMWIg.

The isoelectric points for the HMWIg and LMWIg are 4.1-4.8 and 4.47-5.1, respectively. The binding constants of the HMWIg and LMWIg determined at various intervals after immunization were calculated to be approximately 1-2 x 10^5 M^{-1}. No maturation of the immune response was detected.

Based on the above data it would appear that the HMWIg and LMWIg are two different immunoglobulin populations. The isoelectric focusing data and the binding constant heterogeneity index indicate that the LMWIg is more heterogeneous than the HMWIg.

It has been shown previously that the HMWIg is an "IgM-like" molecule with a tetrameric instead of pentameric configuration. The configuration of the LMWIg is not presently known. Based on the molecular weight of the H and L chains and the molecular weight of the HMWIg and LMWIg, it would seem that the LMWIg may be a trimer. It is conceivable, however, that the LMWIg is a dimer since the molecular weight of the H and L chains and the sedimentation coefficient is about the same as dimeric IgA.

A Partial Characterization of High and Low Molecular Weight Immunoglobulin in Rainbow Trout (*Salmo gairdneri*)

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A PARTIAL CHARACTERIZATION OF HIGH AND LOW MOLECULAR WEIGHT IMMUNOGLOBULIN IN RAINBOW TROUT (SALMO GAIRDNERI)

INTRODUCTION

A major part of the response of vertebrates to antigenic stimulation is the production of specific antibodies. The higher vertebrates possess a diverse humoral immune system consisting of multiple classes of immunoglobulins. Higher vertebrates react to an antigen by the production of a pentameric high molecular weight immunoglobulin (HMWIg) followed later by a monomeric low molecular weight immunoglobulin (LMWIg) of another class (or classes) (1). Associated with this shift in immunoglobulin class is an increase in the average intrinsic binding constant and an increase in specific antibody concentration (1).

Lower vertebrates possess only one class of immunoglobulin as defined by the characteristics of the H chain, which is manifested in a HMWIg and LMWIg form. The humoral response of these animals is similar to that of the higher vertebrates in that the LMWIg is observed usually after the synthesis of the HMWIg (2). No appreciable increase in the average intrinsic binding constant is normally observed in lower vertebrates, but some investigators report an increase in the "functional avidity" of the antibody molecule (3). The functional avidity is the ability of the molecule to actually do work such as agglutinate cells, kill virus, etc.

The bony fish differ from elasmobranchs, amphibians, reptiles and the higher vertebrates in that they produce a tetrameric HMWIg

instead of a pentameric HMWIg. It should also be pointed out that the humoral response of a large number of species of bony fish have been studied and only 4 have been reported to manifest a LMWIg (4-7, 18,19,21-24,26,27,33-37). Rainbow trout (RBT, Salmo gairdneri), a teleost, is one of the species which has not been reported to possess a typical LMWIg (8,32-35). Immunoelectrophoretic analysis of RBT serum, however, has revealed the presence of two distinct populations of immunoglobulin (8). Up to this time, a biochemical analysis of RBT immunoglobulins has not been conducted.

The present studies were initiated to describe the immunoglobulins in RBT. Our studies show that a LMWIg does indeed occur in RBT. Based on molecular weight and sedimentation analysis, the LMWIg is not a normal monomer and may be a trimer. Biochemical characterization of the HMWIg and LMWIg has been conducted, and our data include amino acid analysis, sedimentation coefficients, electrophoretic mobilities, isoelectric points, molecular weights of the intact molecule and H and L chains, antigenic relatedness, and intrinsic binding constants and heterogeneity indices at various times after immunization.

LITERATURE REVIEW

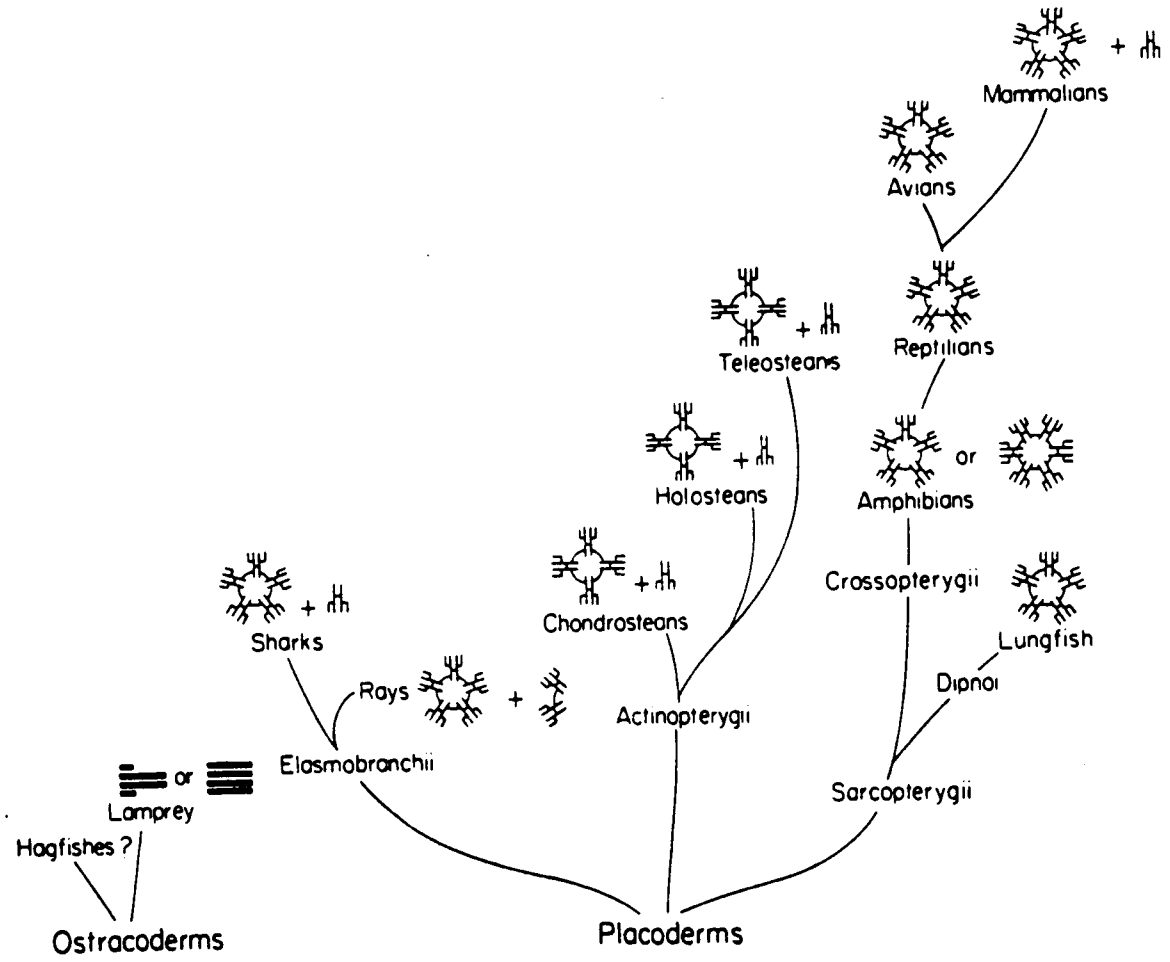
Characteristics of immunoglobulins and the classes of immunoglobulins in the vertebrate class

Immunoglobulins (Ig) of all vertebrates possess a stoichiometric relationship of two light (L) chains to two heavy (H) chains (38). The two H chains are joined to each other by disulfide bonds, and to each H chain one L chain is also connected by disulfide bonding (38). The relationship of L_2H_2 represents one monomeric Ig. The monomers can also be interconnected by disulfide bonds in order to form dimers, trimers, tetramers, pentamers, and hexamers (39).

The criteria for establishing a given Ig class is based on the chemical and physical characteristics of the H chain (reviewed in 9). These include such characteristics as molecular weight, sedimentation coefficient, antigenic relatedness, carbohydrate content, electrophoretic mobility, amino acid analysis, ability to fix complement, and ability to cross membranes. With the advent of more sophisticated technology the ultimate criterion for determining Ig class has become the amino acid sequence of the H chain. Each class of Ig may also be divided into subclasses based on slight variations in the amino acid sequences of the H chain constant region. Classes and subclasses of L chain have also been described. A class of L chain is not restricted to association with a particular class of H chain.

Immunoglobulins have been found in all vertebrates (Fig. 1). Immunoglobulins in the elasmobranchs and bony fish are represented by only one class of Ig (4,6,7,28,29). This class of Ig is found in both

Fig. 1. Immunoglobulins found in species representing the principal lines of vertebrate phylogenetic development and indicating the polymeric form of the immunoglobulins detected (taken from Litman, ref. 36).


Mammalians
Avians
Reptilians
Teleosteans
Holosteans
Amphibians
or
Crossopterygii
Lungfish
Dipnoi
Chondrosteans
Sharks
Rays
Actinopterygii
Elasmobranchii
Sarcopterygii
or
Lamprey
Hagfishes ?
Ostracoderms
Placoderms

a high molecular weight immunoglobulin (HMWIg) and low molecular weight immunoglobulin (LMWIg) form (4,6,7,28,29). A divergence in Ig class is first observed in amphibians where two subclasses of LMWIg have been idenfified (16). The higher the animal is phylogenetically, the greater the diversity in Ig class.

Immunoglobulins of higher vertebrates

Immunoglobulins of higher vertebrates have been studied and characterized far more extensively than Igs of lower vertebrates. Five different classes of Ig have been described in man: IgG, IgM, IgA, IgE, and IgD. Each class of Ig possesses properties to distinguish it from other classes of Ig (Table 1).

All Ig classes can be found as monomers (14,42,47). IgM is usually detected as a pentamer in serum (42). IgA is normally observed as a dimer in secretory fluids, but has also been detected as a trimer (41).

During the course of an immune response IgM is the initial Ig class detected (44,45). Because IgM is a pentameric molecule it has a greater avidity towards particulate antigens, though the average intrinsic binding constant does not mature appreciably during the immune response. Later in the immune response or after a second contact with the antigen, one can detect a shift in the predominant Ig class from IgM to IgG (44,45). IgG is the only class of Ig in which the average intrinsic binding constant of the Ig increases significantly (40,45). This shift in Ig class and increase in the binding affinity has been termed the "maturation of the immune response".

Table 1. Comparison of classes of immunoglobulins found in man[a].

	IgG	IgM	IgA[b]	IgE	IgD
H chain class	γ	μ	α	ε	δ
Molecular formula	$\gamma_2 L_2$	$(\mu_2 L_2)_5$	$(\alpha_2 L_2)_{1,2}$	$\varepsilon_2 L_2$	$\delta_2 L_2$
Sedimentation coefficient	6-7 S	19 S	7 S or 10 S	8 S	7-8 S
Molecular weight	150,000	900,000	160,000 or 400,000	190,000	180,000
Electrophoretic mobility	γ	fast γ to β	fast γ to β	fast γ	fast γ
Complement fixation	+	+++	-	-	-
Placental transfer	+	-	-	-	-
Reaginic activity	+	-	-	-	+++
Carbohydrate content of H chain (%)	4	15	10	18	18

[a]Compiled from ref. 1, 2, 9 and 10.

[b]Represents the monomeric or dimeric form of Ig.

IgE and IgD have not been characterized as extensively as the other Ig classes. IgE is found in the surfaces of certain leukocytes (12). It is known to play an important role in certain types of allergic reactions (13), as well as resistance to certain parasitic infections (46). IgD is found as an integral protein in the membrane of some, if not all, lymphocytes (14,47). The functions of IgD are still not known.

Immunoglobulins of birds, reptiles and amphibians

Even though birds and mammals have evolved along separate lines their immunoglobulins closely resemble one another. Birds have been shown to synthesize three classes of Ig: IgM, IgG, and IgA. The IgG H chain of birds contains approximately 100 amino acids more than the IgG H chain found in man (2,48,49).

The immunoglobulins of amphibians, reptiles, bony fish, and elasmobranchs differ from Igs of higher vertebrates because they possess only one class of Ig. This Ig class actually consists of a high molecular weight and a low molecular weight form, which are almost totally antigenically cross-reactive. These Igs are termed high molecular weight Ig (HMWIg) and low molecular weight Ig (LMWIg) instead of IgM-like or IgG-like immunoglobulins, in order to avoid any inference to the class designation used for Igs of higher vertebrates.

The amphibians represent the first emergence, phylogenetically, of different subclasses of LMWIg (16, reviewed in 15). These LMWIgs have been shown by immunoelectrophoresis to possess slightly different

electrophoretic mobilities and only partial identity (16, reviewed in 15,56). The LMWIg has been observed as a monomer in all species and the HMWIg has been identified as a pentamer in all species except Xenopus laevis in which the HMWIg is a hexamer (17). No appreciable maturation of either the HMWIg or LMWIg during the immune response has been observed.

Immunoglobulins in the bony fish

The Osteicthyes, or bony fish, is composed of three groups; Chondrostei, Holostei, and Teleostei. Immunoglobulins from fish of each group have been studied. Unlike the pentameric HMWIgs found in elasmobranchs, amphibians, reptiles, avians, and mammals, the HMWIg of bony fish is a tetrameric Ig (7,18, reviewed in 36). The LMWIg has been observed in only four species of bony fish and in each case has been detected as a 6-7 S monomer (Table 2).

Populations of immunoglobulin that do not appear to be typical HMWIg or LMWIg have been observed in bony fish. Lobb (5) has studied the sheepshead, Archosargus probatocephalus, which has been shown to possess a 16 S HMWIg and 6 S LMWIg. In addition, a second population of sheepshead HMWIg has been detected after treatment with 5 M guanidine HCl; it exists as 11 S dimers (5). Trump (37) has detected two HMWIg populations in the goldfish, Carassius auratus, which could be separated on the basis of charge. Both of the latter immunoglobulins were found to have sedimentation coefficients of approximately 16 S (37).

Table 2. Comparison of sedimentation coefficients of HMWIg and LMWIg reported in bony fish.

	HMWIg	LMWIg	Reference
Teleostei			
coho salmon (*Oncorhynchus kisutch*)	17S	-	18
brown trout (*Salmo trutta*)	16.7S	-	19
carp (*Cyprinus carpio*)	14.5S	-	20
channel catfish (*Ictalurus punctatus*)	14S	-	21
rainbow trout (*Salmo gairdneri*)	13.9S	-	22
margate (*Haemulon album*)	16S	7S	7
giant grouper (*Epinephelus itaira*)	16S	6.4S	6
pike (*Essox lucius*)	15S	-	23
gold fish (*Carassius auratus*)	14S	-	24
gray snapper (*Lutjanus griseus*)	14S	-	25
sheepshead (*Archosargus probatocephalus*)	16S	6.1S	5
Holostei			
bowfin (*Amia calva*)	13.6S	6.3S	4
gar (*Lepisosteus osseus*)	14S	-	26
Chondrostei			
paddle fish (*Polyodon spathula*)	14S	-	27

Analysis of the Igs of bony fish indicates that the HMWIg exists primarily as a 600,000 to 700,000 dalton tetramer, and the LMWIg has a molecular weight of 120,000 to 160,000 daltons (Table 3). In those bony fish which possess a LMWIg, it is interesting to note that the molecular weight of the H chain is smaller than the H chain of the HMWIg (4-7). This reduction in the size of the H chain is thought to be responsible for the slight antigenic differences found between the HMWIg and LMWIg (4-7). The paddlefish, a member of the most primitive bony fish group, Chondrostei, possesses a HMWIg with an H chain of approximately the same molecular weight of the H chain from the LMWIg of other bony fish (27). No LMWIg has been detected in the paddlefish and the significance of the H chain reduction is not known (27).

Immunoglobulins of the elasmobranchs

The HMWIg of the elasmobranchs is a pentameric molecule with a sedimentation coefficient of 18-19 S (28,29,31). It has been found to be antigenically identical to the 7 S monomeric LMWIg (28,29). The molecular weights of the H chain (70,000 daltons) and L chain (23,000 daltons) of the HMWIg and LMWIg are similar (28,29). The nurse shark is the only elasmobranch in which two LMWIgs have been reported (30). The H chain of the second LWMIg is only 50,000 daltons and this deletion of 20,000 daltons may be responsible for the slight antigenic differences that have been observed. It was originally thought that the LMWIg was a catabolic product of the HMWIg or that the HMWIg was an aggregate of the LMWIg, but evidence for this conversion has never been demonstrated (31).

Table 3. Comparison of immunoglobulins found in selected bony fish.

	Bowfin[a]		Sheepshead[b]		Giant Grouper[c]		Paddle fish[d]	Gar[e]
	HMWIg	LMWIg	HMWIg	LMWIg	HMWIg	LMWIg		
Molecular weight (daltons)	610,000	152,000	700,000	130,000 to 160,000	700,000	120,000	660,000	610,000
Sedimentation Coefficient (S)	13.9	6.3	16	6	16	6.4	14.2	14 S
H chain MW (daltons)	70,000	52,000	70,000	45,000	76,000	45,000	58,100	70,000
L chain MW (daltons)	24,000	24,000	25,000	25,000	22,000	22,000	21,000	23,000
Carbohydrate (%)	10.7	9.1	ND	ND	ND	ND	ND	ND

ND = not determined
a = Litman et al (4)
b = Lobb (5)
c = Clem (6)
d = Acton et al (27)
e = Acton et al (26)

The immune response of rainbow trout (*Salmo gairdneri*)

Hodgin *et al*. (8) has detected two electrophoretically distinct populations of Ig in rainbow trout (RBT) using immunoelectrophoresis. The two populations were shown to be antigenically related with the faster globulin migrating as a beta globulin while the slower globulin was described as a fast gamma or slow beta globulin (8). Post (32), in contrast to the results of Hodgins *et al*., found that all of the anti-*Aeromonas hydrophila* antibody in the serum of RBT could be isolated in one electrophoretic fraction. The antibody population described by Post was characterized by paper electrophoresis and was shown to be a beta globulin or slow alpha globulin. Hodgins *et al*. (22) have more recently reported evidence supporting the existence of 13.9, 10.5 and 4.2 S agglutinins in RBT. The 4.2 S agglutinin does not appear to be inducible, however, and is presently thought to be a lectin (33). The 10.5 S agglutinin was considered by Hodgins *et al*. to be a natural hemagglutinin and not an inducible Ig (22). Based on these results, and work reported by other investigators (34,35), RBT are considered to possess only a 14-16 S HMWIg.

REFERENCES

1. Fudenberg, H., D. Stites, J. Caldwell, and J. Wells. 1978. In Basic and Clinical Immunology. Edited by H. Fudenberg, D. Stites, J. Caldwell, and J. Wells. 2nd edition. Lange Medical Publications. p. 758.

2. Marchalonis, J. 1976. Comparative Immunology. Edited by J. Marchalonis. Halsted Press. p. 470.

3. Fiebig, H., R. Bruhn, and H. Ambrosius. 1977. Studies on the control of IgM antibody synthesis. III. Preferential formation of anti-DNA-antibodies of high functional affinity in the course of the immune response in carp. Immunochem. 14:721.

4. Litman, G., D. Frommel, J. Finstad, and R. Good. 1971. Evolution of the immune response. IX. Immunoglobulins of the bowfin : Purification and characterization. J. Immunol. 106:747.

5. Lobb, C. 1980. Humoral and secretory immunoglobulins of the sheepshead, Archosargus probactocephalus, a marine teleost. Utah State University, Ph.D. thesis. p. 153.

6. Clem, L. 1971. Phylogeny of immunoglobulin structure and function. IV. Immunoglobulin of the giant grouper (Epinephelus itaira). J. Biol. Chem. 246:9.

7. Clem, L., and W. McLean. 1975. Phylogeny of immunoglobulin structure and function. VII. Monomeric and tetrameric immunoglobulins of the margate; a marine teleost fish. Immunol. 29: 791.

8. Hodgins, H., G. Ridgeway, and F. Utter. 1965. Electrophoretic mobility of an immune globulin from rainbow trout serum. Nature (Lond.)208:1106.

9. Nisonoff, A., J. Hooper, and S. Spring. 1975. In The Antibody Molecule. Edited by A. Nisonoff, J. Hooper, and S. Spring. Academic Press. p. 542.

10. Katz, D. 1977. In Lymphocyte Differentation, Recognition, and Regulation. Academic Press. p. 749.

11. Putnam, F., M. Korzuru, and C. Easley. 1967. Structural studies of the immunoglobulins. IV. Heavy and light chains of the γM pathological macroglobulins. J. Biol. Chem. 242:2435.

12. Kanellopoulos, J., G. Rossi, and H. Metzger. 1979. Preparative isolation of the cell receptor for immunoglobulin E. J. Biol. Chem. 254:7691.

13. Prausnitz, C., and H. Küstner. 1921. Studien uber die Ueberempfindlichkeit. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. I. Orig. 86:160.

14. Mosier, D., I. Zitron, J. Monk, A. Ahmed, I. Scher, and W. Paul. 1977. Surface immunoglobulin D as a functional receptor for a subclass of B lymphocytes. Immunol. Rev. 37:89.

15. Hadji-Azimig, I. 1979. Anuran immunoglobulins: A review. Dev. Comp. Immunol. 3:223.

16. Green, C., and L. Steiner. 1976. Isolation and preliminary characterization of 2 varieties of low molecular weight immunoglobulins in the bull frog. J. Immunol. 117:364.

17. Hadji-Azimi, I., and M. Midrea-Hamzehpour. 1976. Xenopus laevis 19 S immunoglobulin ultrastructure and J chain isolation. Immunol. 30:587.

18. Cisar, J., and J. Fryer. 1974. Characterization of anti-Aeromonas salmonicida antibodies from coho salmon. Infect. Immun. 9:236.

19. Ingram, G., and J. Alexander. 1979. The immunoglobulin of the brown trout, Salmo trutta and its concentration in the serum of antigen-stimulated and non-stimulated fish. J. Fish Biol. 14:249.

20. Andreas, E., R. Richter, D. Hadge, and H. Ambrosius. 1975. Strukturelle und immunchemische Untersuchungen am Immunglobulin des Karpfen (Cyprinus carpio L.). II. Analyse der Untereinheiten. Acta Biol. Med. Germ. 34:1407.

21. Hall, S., E. Evans, H. Dupree, R. Acton, P. Weinheimer, and J. Bennett. 1973. Characterization of a teleost immunoglobulin: The immune macroglobulin from the channel catfish Ictalurus punctatus. Comp. Biochem. Physiol. 46B:187.

22. Hodgins, H., R. Weiser, and G. Ridgway. 1967. The nature of antibodies and the immune response in rainbow trout (Salmo gairdneri). J. Immunol. 99:534.

23. Clerx, J., A. Castel, J. Bol, and G. Garwig. 1980. Isolation and characterization of the immunoglobulin of pike (Esox lucius L). Vet. Immunol. Immunopath. 1:125.

24. Marchalonis, J. 1971. Isolation and partial characterization of gold fish (Carassius auratus) and carp (Cyprinus carpio). Immunol. 20:161.

25. Russell, W., E. Voss, and M. Sigel. 1970. Some characteristics of anti-dinitrophenyl antibody of the gray snapper. J. Immunol. 105:262.

26. Acton, R., P. Weinheimer, H. Dupree, E. Evans, and J. Bennett. 1971. Phylogeny of immunoglobulins. Characterization of a 14 S immunoglobulin from the gar, Lepisosteus osseus. Biochem. 10:2028.

27. Acton, R., P. Weinheimer, H. Dupree, T. Russell, M. Wilcott, E. Evans, R. Schronenloher, and J. Bennett. 1971. Isolation and characterization of the immune macroglobulin from the paddle fish, Polyodon spathula. J. Biol. Chem. 246:6760.

28. Suran, A., M. Tarail, and B. Papermaster. 1967. Immunoglobulins of the Leopard shark. I. Isolation and characterization of 17 S and 7 S immunoglobulins with precipitating activity. J. Immunol. 99:679.

29. Frommel, D., G. Litman, J. Finstad and R. Good. 1971. The evolution of the immune response. XI. The immunoglobulins of the horned shark, Heterodontus francisci: Purification, characterization, and structural requirements for antibody activity. J. Immunol. 106:1234.

30. Fuller, L., J. Murray, and J. Jensen. 1978. Isolation from nurse shark serum of immune 7 S antibodies with two different molecular weight H chains. Immunochem. 15:251.

31. Small, P., D. Klapper, and L. Clem. 1970. Half-lives, body distribution and lack of interconversion of serum 19 S and 7 S IgM of sharks. J. Immunol. 105:29.

32. Post, G. 1966. Serum proteins and antibody production in rainbow trout (Salmo gairdneri). J. Fish Res. Bd. Can. 23:1957.

33. Hodgins, H., F. Wendling, B. Braaten, and R. Weiser. 1973. Two molecular species of agglutinins in rainbow trout (Salmo gairdneri) serum and their relation to antigenic exposure. Comp. Biochem. Physiol. 45B:975.

34. Ikeda, K. 1978. Gel chromatographic characteristics of agglutinating antibody of rainbow trout to Aeromonas salmonicida. Bull. Freshwater Fish. Res. Lab. Tokyo 28:55.

35. Dorson, M. 1972. Some characteristics of antibodies in the primary immune response of rainbow trout, Salmo gairdneri. Symp. Zool. Soc. London. 30:129.

36. Litman, G. 1976. Physical properties of immunoglobulins of lower species; a comparison of immunoglobulins of mammals. In Comparative Immunology. Edited by J. Marchalonis. Halsted Press. p. 248.

37. Trump, G. 1970. Goldfish immunoglobulins and antibodies to bovine serum albumin. J. Immunol. 104:1267.

38. Porter, R. 1973. Structural studies of immunoglobulins. Science. 180:713.

39. Edelman, G. 1973. Antibody structure and molecular immunology. Science. 180:830.

40. Voss, E., and R. Wah. 1977. Comparison of the microenvironment of chicken and rabbit active sites. In Avian Immunology. Edited by A. Benedict. p. 391-401.

41. Tomasi, T., and J. Bienenstock. 1968. Secretory immunoglobulin (IgA). Adv. Immunol. 9:2.

42. Green, N. 1969. Electron microscopy of immunoglobulins. Adv. Immunol. 11:1.

43. Siskind, G., and B. Benacerraf. 1969. Cell selection by antigen in the immune response. Adv. Immunol. 10:1.

44. Nossal, G., A. Szenberg, G. Ada, and C. Austin. 1964. Single cell studies on 19 S antibody production. J. Exp. Med. 119:485.

45. Cosenza, H., and A. Nordin. 1970. Immunoglobulin classes of antibody-forming cells in mice. III. Immunoglobulin antibody restriction of plaque-forming cells demonstrated by the double immunofluorescent technique. J. Immunol. 104:976.

46. Orr, T., and A. Blair. 1969. Potentiated reagin response to egg albumin and conalbumin in Nippostrongylus brasiliensis infected rats. Life Sci. 8:1073.

47. Van Boxel, J., W. Paul, D. Terry, and I. Green. 1972. IgD-bearing human lymphocytes. J. Immunol. 109:648.

48. Travis, J., and B. Sanders. 1973. Structural comparisons between chicken low molecular weight immunoglobulin heavy chains and human γ chains. Biochem. Gen. 8:391.

49. Benedict, A., O. Roholt, K. Yamaga, and D. Pressman. 1972. Peptides from the site of chicken anti-DNP antibodies. Immunol. Comm. 1:279.

50. Marchalonis, J., and G. Edelman. 1966. Phylogenetic origins of antibody structure. II. Immunoglobulins in the primary immune response of the bullfrog, Rana catesbeiana. J. Exp. Med. 124:901.

51. Gitlin, D., A. Perricelli, and J. Gitlin. 1973. Multiple immunoglobulin classes among shark and their evolution. Comp. Biochem. Physiol. 44:225.

52. Coe, J. 1972. Immune response in the turtle (Chrysemys picta). Immunol. 23:45.

53. Coe, J., D. Leong, J. Portis, and L. Thomas. 1976. Immune response in the garter snake (Thamnophis ordinoides). Immunol. 3:417.

54. Clem, L., and P. Small. 1967. Phylogeny of immunoglobulin structure and function. I. Immunoglobulin of the Lemon shark. J. Exp. Med. 125:893.

55. Clem, L., F. Boutand, and M. Sigel. 1967. Phylogeny of immunoglobulin structure and function. II. Immunoglobulins of Nurse shark. J. Immunol. 99:1226.

56. Marchalonis, J. 1971. Immunoglobulins and antibody production in amphibians. Am. Zool. 11:171.

II. A Characterization of Immunoglobulins in Rainbow Trout (Salmo gairdneri) I. Isolation and Characterization of High and Low Molecular Weight Immunoglobulin

INTRODUCTION

One method by which vertebrates respond to antigenic stimulation is through the production of immunoglobulins. In higher vertebrates this is associated with the production of immunoglobulins of various classes differing in molecular weight, configuration, and biological function (1-3,39,40,51). The lower vertebrates possess only one class of antibody, which is usually manifested in both a high molecular weight (HMWIg) and lower molecular weight form (LMWIg) (4-6,9-12). With the exception of a hexameric immunoglobulin found in Xenopus laevis (7) and tetrameric immunoglobulins observed in the bony fishes (6,8-20), the HMWIg is characteristically a pentamer. The LMWIg is usually a monomer, but has been observed in only four bony fish: the margate (9), giant grouper (10), bowfin (11) and sheepshead (12). Efforts up to this time to detect a LMWIg in rainbow trout (RBT, Salmo gairdneri) have not been successful (21-26). We report data here, however, which indicate that a LMWIg does indeed exist in RBT, and our findings suggest that it may be a trimer or dimer.

MATERIALS AND METHODS

Animals and holding conditions

Two-year-old adult male rainbow trout (RBT) weighing 2-3 kg were obtained from Roaring River Hatchery, Oregon. The fish were maintained in circular tanks with flowing 12 C, fish pathogen-free, well water.

Antigen Preparation

2,4,6-trinitrobenzene sulfonic acid (TNP, Sigma, St. Louis, Mo) was conjugated with bovine serum albumin (BSA, Sigma) and ovalbumin (OVA, Sigma), by the method of Garvey et al. (27). The concentration of TNP was varied stoichiometrically to produce a high (TNP_{25}-BSA), and low (TNP_5-BSA) conjugation ratio of TNP to BSA.

Production and storage of antisera

The RBT were injected intraperitoneally with 0.3 ml of Freund's complete adjuvant containing 1-2 mg TNP-BSA. The primary injections were carried out with TNP_{30}-BSA, and all subsequent injections employed TNP_5-BSA. Fish were injected and the serum harvested at selected intervals thereafter.

The fish were anesthetized with benzocaine and bled from the duct of Cuvier as described by Lied et al. (28). Serum was harvested, sodium azide (0.02%) was added, and the serum plus preservative mixture was stored at -20 C.

Preparation of affinity column

Sepharose 6B was conjugated with TNP_5-OVA (a low conjugation ratio) according to a modification of the methods of Robins et al. (29) and

March et al. (30). To a slurry of Sepharose 6B and water, an equal volume of 2 M sodium carbonate was added and stirred with a glass rod in an ice bath. To this, 0.05 vol of a cyanogen bromide-acetonitrile solution (2 g CNBr/ml acetonitrile) was added dropwise. The solution was swirled for 5 min and poured over a coarse scintered glass filter. It was then washed sequentially with: 5-10 volumes of 0.1 M sodium bicarbonate, pH 9.5; 5-10 volumes of water; and finally with 5-10 volumes of 0.2 M sodium bicarbonate, pH 9.5. One volume of 0.2 M sodium bicarbonate, pH 9.5, and TNP_5-OVA was added to the moist cake and the coupling reaction was allowed to proceed on a rotating stirrer at 4 C for 12-18 hr. The unreacted Sepharose groups were blocked by the addition of serine (1 M). The mixture was allowed to react for an additional 2 hr, followed by sequential washing with 20 volumes each of: 1 M acetic acid, 0.5 M sodium chloride, 2 M urea, 1 M acetic acid, water and finally 0.1 M phosphate buffer (PB), pH 8.0. The TNP-OVA-Sepharose-6B with 0.02% sodium azide was then stored in PB at 4 C until needed.

Purification of specific anti-TNP immunoglobulin

Serum (20 ml) was initially treated with 1.0 ml of a 5% solution of sodium dextransulfate (Pharmacia, Upsala, Sweden) in order to remove lipoprotein (Fig. 1). The solution was stirred in an ice bath for 10 min, and then 1.8 ml of 1 M calcium chloride was added. After 10 min of further stirring, the lipid-rich precipitate was pelleted at 5,000 x g for 20 min. The supernatant was harvested and saturated ammonium sulfate was added dropwise until 50% saturation was attained.

The solution was allowed to stir overnight at 4 C and then was centrifuged at 5,000 x g for 20 min. The pellet was resuspended in 0.1 M PB and dialysed extensively against the same buffer.

The dialysed sample was mixed on a rotator with 20 ml of a packed volume of TNP-OVA-Sepharose 6B at 4 C for 12-18 hr. After packing this mixture into a column, 0.15 M PB, pH 8.0 was added to elute any non-specifically adherent material. The specifically bound RBT antibody was eluted with 3 M potassium thiocyanate (31,32) or with 0.1 M free hapten where indicated. The eluant was assayed spectrophotometrically on a Beckman Model 35 spectrophotometer at 280 nm. The fractions containing an absorbance greater than 0.1 were pooled and dialysed at 4 C against 0.1 M PB, pH 8.0. Where antibody levels were low, protein was concentrated by precipitation with an equal volume of saturated ammonium sulfate. The specific antibody was applied to a 2.6 x 90 cm column containing Sephacryl S-300 (Pharmacia) equilibrated with 0.1 M PB, pH 8.0 containing 0.02% sodium azide. Proteins were eluted from the column at 4 C in an ascending manner with a flow rate of 15 ml/hr.

Protein A purification of RBT Ig

Whole RBT serum and purified RBT immunoglobulin was applied to a 7 x 50 mm column of Protein-A-Sepharose CL-4B (Pharmacia) equilibrated with 0.1 M PB. The column was washed with the same buffer and bound materials were eluted with 3 M potassium thiocyanate.

Disaggregation of immunoglobulin

Purified HMWIg and LMWIg were dialysed against 6 M urea for 24 hr at 4 C to disaggregate any proteins that may have aggregated. The proteins were then applied to a 2.6 x 90 cm column containing Sephacryl S-300 equilibrated with 0.1 M PB.

Immunoelectrophoresis

Immunoelectrophoresis was performed in a Gelman deluxe electrophoresis chamber using 1% agarose in a barbital buffer, pH 8.8. The plates were run at 220 V for 2 hr at 4 C. Antiserum was added and allowed to react at 4 C.

Anion exchange chromatography

Whatman DE-52 (diethylaminoethyl cellulose) was prepared by equilibration in 0.02 M PB, pH 8.0 for 12-18 hr at 4 C followed by deaeration. Purified RBT immunoglobulin to be used for ion-exhange chromatography was extensively dialysed against the same buffer and applied to the column (1.2 x 10 cm). A gradient from 0.02-2.0 M PB, pH 8.0 was applied, and the antibody was collected and assayed spectrophotometrically.

Determination of the intrinsic binding constant of specific RBT immunoglobulin in whole serum

A modified Farr technique (33,34) was employed to measure the binding constant of hyperimmune RBT whole serum to DNP. Briefly, 90 µl of either hyperimmune whole RBT anti-TNP antisera or normal control serum were mixed with 10 µl of 10^{-4} to 10^{-7} M ^{3}H-DNP-L-lysine and

allowed to stand for 2 hr at 4 C. Tritiated DNP-L-lysine at the appropriate concentration was prepared by the addition of ^{3}H-N-ε-DNP-L-lysine (New England Nuclear; 3.90 Ci/mmole) to cold N-ε-L-lysine (Sigma) at the appropriate concentration to provide approximately 10^{5} cpm/10 µl as measured in a Beckman Model LS8000 Liquid Scintillation System. The specific activity for each concentration of hapten was determined. One hundred µl of saturated ammonium sulfate was added, mixed, and left for an additional hour at 4 C. The precipitates were pelleted at 8000 g for 5 min and the concentration of both free and bound DNP-L-lysine was determined. A Scatchard plot was constructed in order to determine the molar concentration of antibody binding sites, and with this information the data were analyzed by a Sips plot to determine the intrinsic binding constant (K_a) and heterogeneity index (a) (33,34).

Determination of the intrinsic binding constant of specifically purified RBT immunoglobulin by fluorescence quenching

An analysis of fluorescence quenching was carried out according to established procedures (35,36). The fluorescence of 40 µg of purified anti-TNP antibody in 1 ml of 0.05 M PB, pH 8.0 was determined with excitation and emission wavelengths of 280 nm and 350 nm. Increments of 0.05 ml of a 1.2 x 10^{-5} M solution of N-ε-2,4-dinitrophenyl-L-lysine HCl (Sigma) in the same buffer was added, and the fluorescence was determined. Background fluorescence of PB was deducted from each measurement. After the addition of free hapten to the antibody, a dilution factor was taken into account in order to determine the percentage of real fluorescence which was quenched. Q_{max} was taken to be

0.75, and a Sips plot was constructed in order to obtain the binding constant and heterogeneity index.

SDS-PAGE of RBT immunoglobulins

The H and L chains were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with an acrylamide concentration of 4% in the stacking gel, and 10% in the running gel according to the method of Laemli (37). In order to estimate the molecular weights of the sample, proteins of known molecular weight which were run concurrently included Waldenstrom macroglobulinemia IgM (H chain 72,000 daltons, L chain 30,000 daltons), phosphorylase B (94,000 daltons), BSA (68,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), soybean trypsin inhibitor (21,000 daltons), and lysozyme (14,300 daltons).

Preparation of anti-RBT Ig and anti-whole RBT serum

New Zealand white rabbits were injected intrascapularly with 1-2 mg of specifically purified RBT Ig or RBT serum in Freund's complete adjuvant. The rabbits were then bled at 21 and 30 days post injection to obtain the desired antiserum. Antiserum containing 0.02% sodium azide was stored at -20 C until required.

RESULTS

Purification of anti-TNP specific immunoglobulins

Pooled RBT anti-TNP-BSA antisera were applied to a TNP-OVA-Sepharose-6B affinity column as a means of purifying the anti-TNP specific antibody (Fig. 1). The purified immunoglobulins were applied to a Sephacryl S-300 molecular sieve column, and two protein peaks referred to as HMWIg and LMWIg, respectively, were observed (Fig. 2). Based on the gel filtration chromatography, approximate molecular weights of the HMWIg and LMWIg were calculated to be 620,000 and 490,000 daltons, respectively. It is interesting to note that virtually no protein was detected in the molecular weight range of a monomeric immunoglobulin (150,000 daltons).

The Ig was eluted from the affinity column by 0.1 M free hapten in PB to ascertain if the LMWIg could be produced as an artifact of the high salt (3 M KSCN) elution. The eluted material was concentrated by positive pressure using an Amicon XM-100 filter and applied to the Sephacryl S-300 column equilibrated with PB (Fig. 1 top). The molecular weights of HMWIg and LMWIg were identical to those observed previously with the thiocyanate-eluted material.

In order to determine if either the HMWIg or LMWIg were aggregates of a lower molecular weight form, both populations were equilibrated with 6 M urea and then applied to the Sephacryl S-300 column previously described. The proteins eluted identically as without the urea (Fig. 2 bottom). It was concluded that neither the HMWIg or LMWIg was an aggregate of a lower molecular weight form.

Figure 1. Isolation of high and low molecular weight immunoglobulin from rainbow trout.

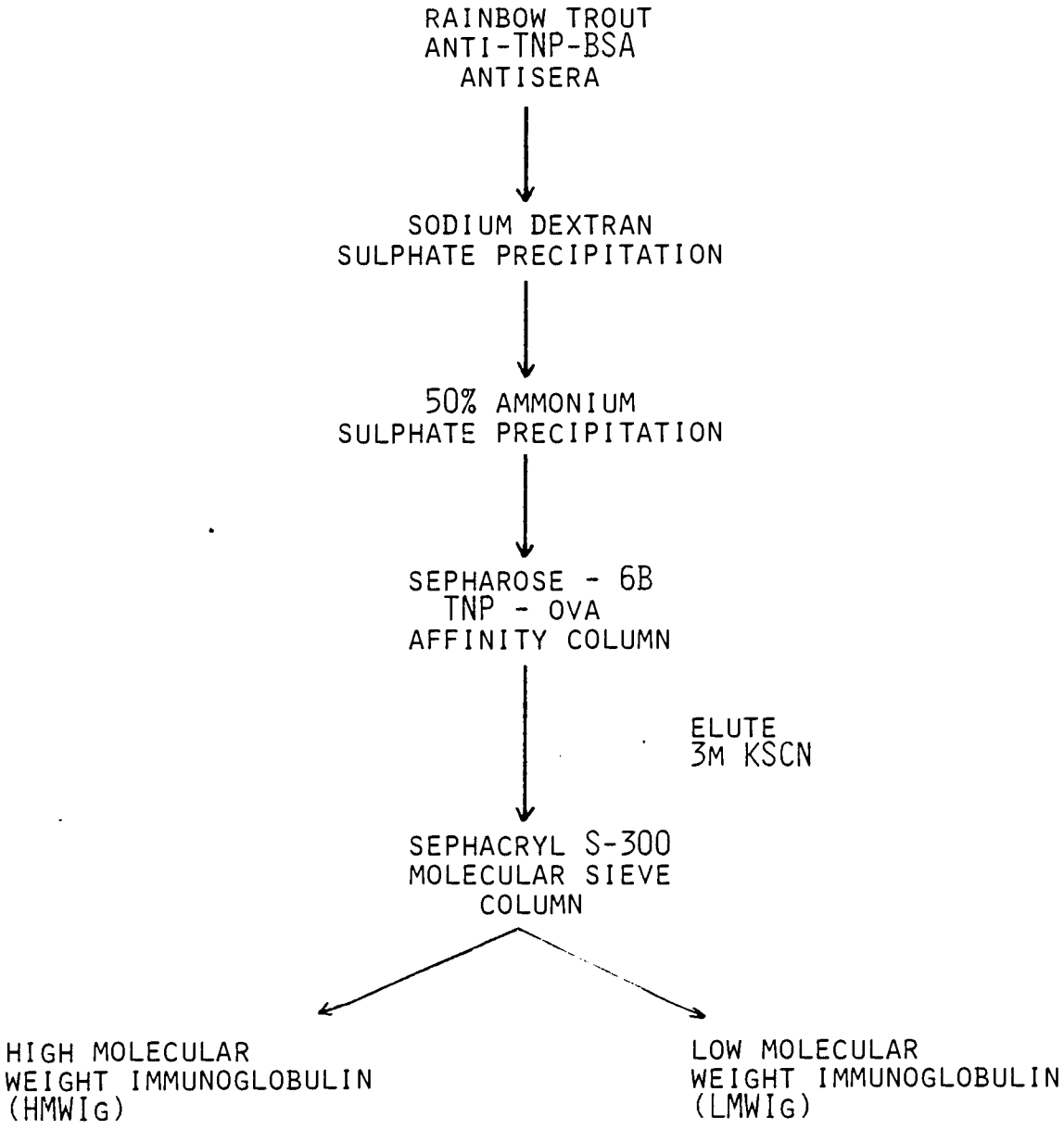
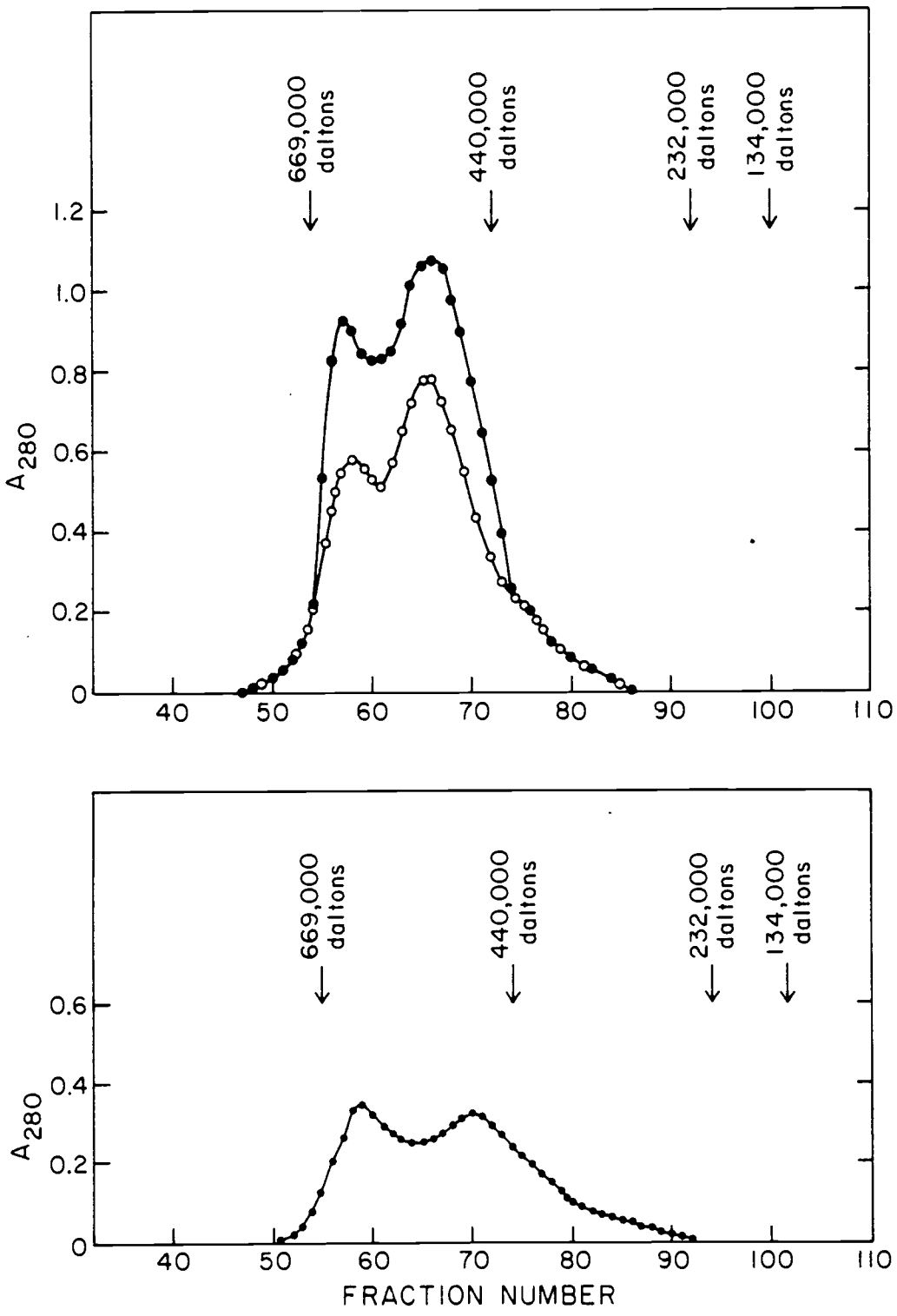

RAINBOW TROUT
ANTI-TNP-BSA
ANTISERA
SODIUM DEXTRAN
SULPHATE PRECIPITATION
50% AMMONIUM
SULPHATE PRECIPITATION
SEPHAROSE - 6B
TNP - OVA
AFFINITY COLUMN
ELUTE
3M KSCN
SEPHACRYL S-300
MOLECULAR SIEVE
COLUMN
HIGH MOLECULAR
WEIGHT IMMUNOGLOBULIN
(HMWIG)
LOW MOLECULAR
WEIGHT IMMUNOGLOBULIN
(LMWIG)

Figure 2 Top. Isolation of purified RBT HMWIg and LMWIg using a 2.6 x 90 cm Sephacryl S-300 column equilibrated with 0.1 M PB, pH 8.0. Closed circles represent Ig eluted from the affinity column with 3 M KSCN. Open circles represent Ig eluted from the affinity column with 0.1 M DNP.

Bottom. Separation of RBT immunoglobulins equilibrated with 6 M urea by chromatography in a Sephacryl S-300 column as described above.


669,000 daltons
440,000 daltons
232,000 daltons
134,000 daltons
A280
1.2
1.0
0.8
0.6
0.4
0.2
0
40
50
60
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90
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110
669,000 daltons
440,000 daltons
232,000 daltons
134,000 daltons
A280
0.6
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40
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60
70
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90
100
110
FRACTION NUMBER

SDS-PAGE analysis of RBT HMWIg and LMWIg

Both the HMWIg and LMWIg were subjected to SDS-PAGE in an effort to analyze the molecular weight of the H and L chains in each of these Ig populations (Fig. 3). Both the HMWIg and LMWIg were found to possess H and L chains of 72,000 and 27,000 daltons, respectively.

Protein A purification of RBT immunoglobulins

Chromatography employing Protein A- Sepharose CL-4B was attempted as a method to purify RBT immunoglobulins. We were unsuccessful in isolating Ig by this method, indicating that RBT Ig does not behave in the same manner as human IgG C_H2 domains (38).

Anion exchange chromatography

Anion exchange chromatography is a method routinely employed to isolate immunoglobulins. This method was attempted to separate the HMWIg from the LMWIg or to purify RBT Ig from whole sera. Purified RBT Ig was applied to a DE-52 column and eluted with an increasing PB gradient. Both the HMWIg and LMWIg were eluted together at a PB concentration corresponding to 0.05 M.

Determination of the intrinsic binding constant and heterogeneity index of RBT anti-TNP immunoglobulins

Purified anti-TNP HMWIg and LMWIg were isolated from sera collected at various times after antigenic stimulation, and were then analyzed by the technique of fluorescence quenching to determine the intrinsic binding constant (K_a) and heterogeneity index (a) of the respective samples (Table 1). The results of these experiments indicate that

Fig. 3. Determination of H and L chain molecular weight by SDS-PAGE. Protein standards are: phosphorylase B (94,000 daltons), BSA (68,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), soybean trypsin inhibitor (21,000 daltons), lysozyme (14,000 daltons), and human Waldenstrom macroglobulinemia IgM (H chain 72,000 daltons and L chain 27,000 daltons).

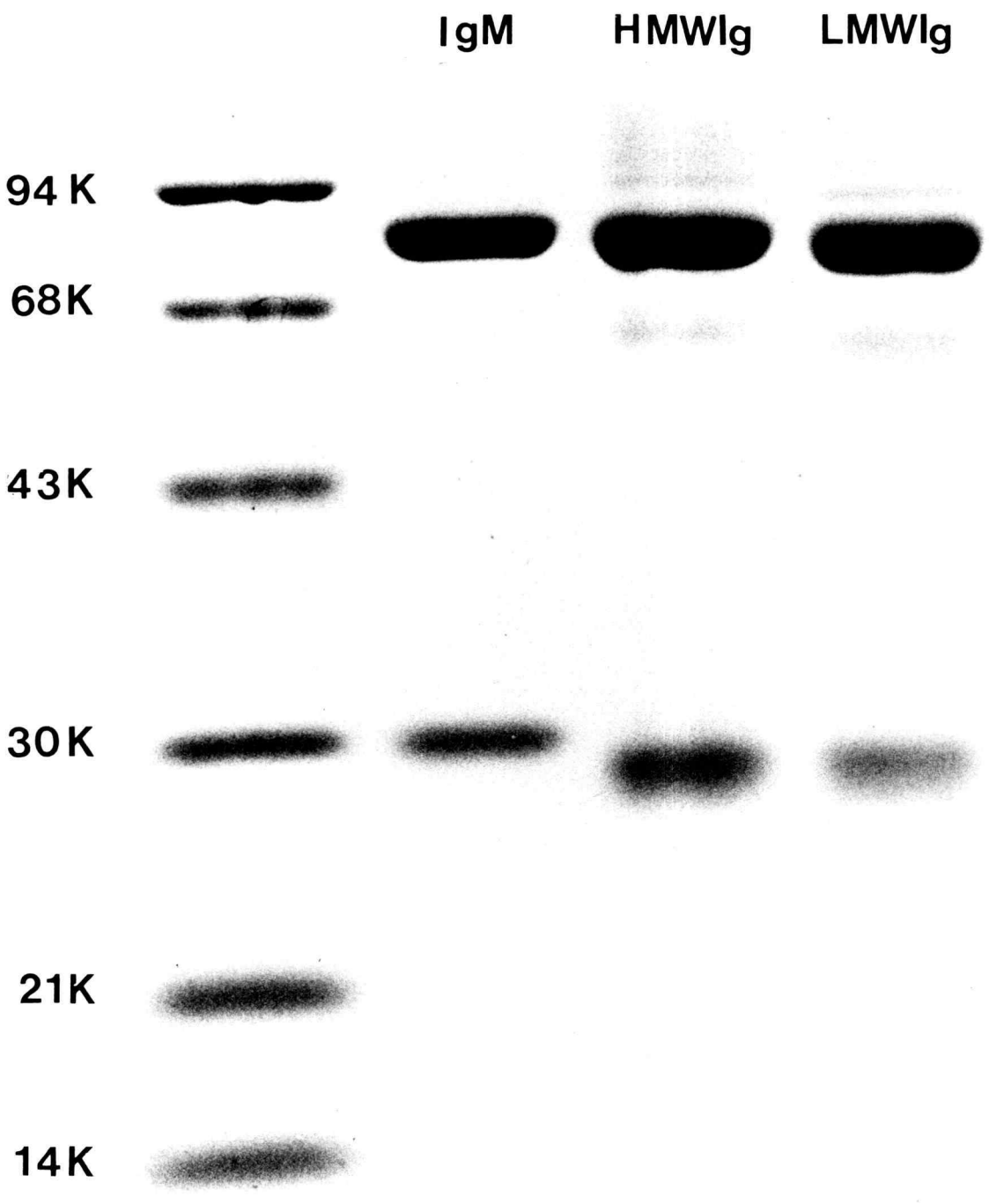

IgM
HMWIg
LMWIg
94 K
68K
43K
30K
21K
14K

Table 1. Comparison of specifically purified RBT anti-TNP HMWIg and LMWIg average intrinsic association constant and heterogeneity index as determined by fluorescence quenching.

Day serum taken post primary immunization[a]	HMWIg K_a[b] (M^{-1})	Heterogeneity Index	LMWIg K_a (M^{-1})	Heterogeneity Index
56	1.16×10^5	0.60	1.00×10^5	0.54
73	1.09×10^5	0.63	9.08×10^4	0.60
87	9.32×10^4	0.67	1.06×10^5	0.63
167	1.37×10^5	0.59	8.73×10^4	0.61
257	2.09×10^5	0.79	2.26×10^5	0.69

[a]Sera were collected from fish injected on days 0, 21, 56, 73, and 187.

[b]K_a = average intrinsic binding constant.

the K_a and a values for the HMWIg and LMWIg were approximately the same. The data also show that the binding affinity of neither the HMWIg and LMWIg changed with time after immunization.

It has been previously shown that the scheme employed for eluting antibody from an affinity column can be used to select for immunoglobulin with a discrete average intrinsic binding constant (31). In order to determine if the affinity purification scheme selected for only a discrete population of Ig, the binding constant and heterogeneity index were determined with non-purified hyperimmune anti-TNP RBT serum. A modified Farr assay was employed which has the advantage of determining the K_a of specific Ig in whole serum (35,36) (Table 2). It appears that these data agree quite well with those obtained by the method of fluorescence quenching.

Immunoelectrophoresis of RBT immunoglobulins

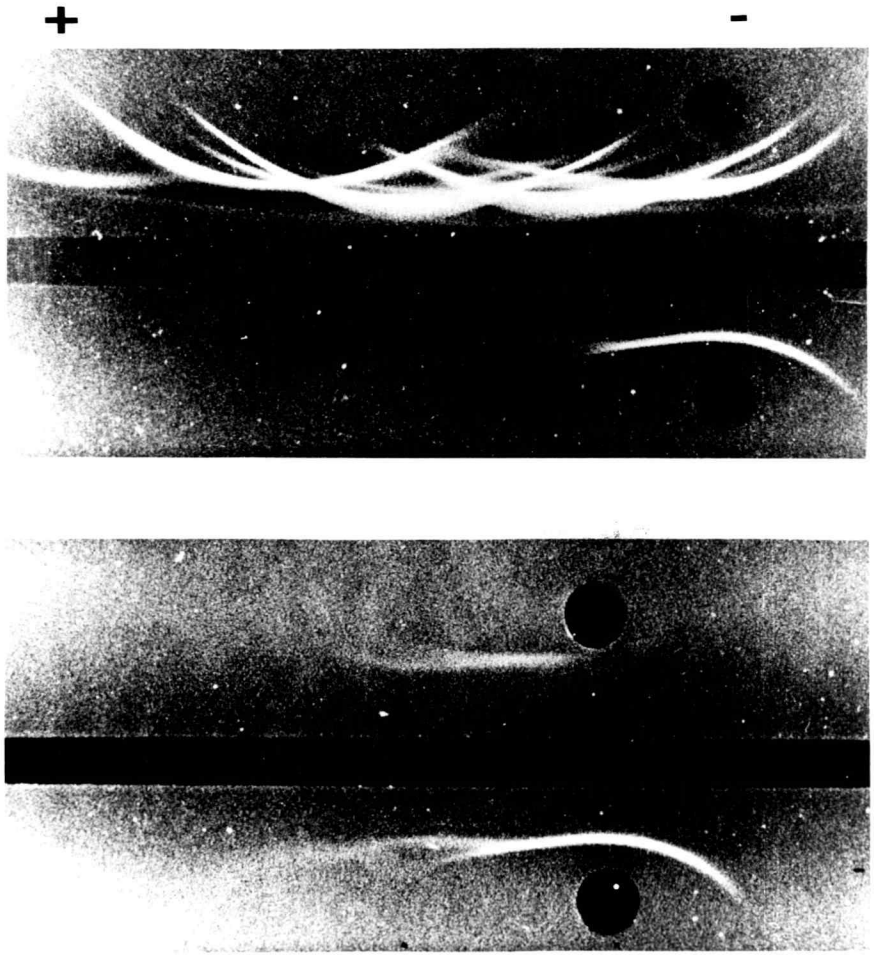
Purified RBT immunoglobulins and normal RBT serum were analyzed by immunoelectrophoresis (Fig. 4). Two populations with different mobilities were observed. The mobility of the HMWIg is more anodic in comparison to the LMWIg which tends to have little if any migration. When anti-RBT Ig antiserum is used a spur of purified identity occurs. This indicates that the HMWIg shares antigenic determinants with the LMWIg, but the LMWIg possesses at least one unique antigenic determinant.

Table 2. Determination of the average intrinsic binding constant and heterogeneity index of specific anti-TNP RBT immunoglobulin in whole serum by a modified Farr assay.

Day serum taken post immunization[a]	Average intrinsic binding constant (K_a)	Heterogeneity Index
56	1.9×10^5 M^{-1}	0.35
208	1.2×10^5 M^{-1}	0.54

[a]Serum was collected from fish injected on days 0, 21, 56, 73, and 187.

Figure 4. Immunoelectrophoresis of RBT Ig and whole serum.


+
-
RBT serum
anti-RBT serum
RBT Ig
HMWIg
anti-RBT Ig
RBT Ig
HMWIg
LMWIg

DISCUSSION

The immunoglobulins of bony fish differ from all other vertebrates by the presence of a tetrameric HMWIg as opposed to a pentameric HMWIg seen in most other species (1-3, 39-44). The HMWIg has been reported to have a molecular weight between 600,000 and 800,000 daltons and possess a sedimentation constant between 14 and 18 S (6,8-13,15-20). Bony fish also differ in the appearance of a low molecular weight immunoglobulin (LMWIg), which has been characteristically observed in most other vertebrates, but has not always been detected in bony fish. In lower vertebrates the LMWIg is antigenically indistinguishable from the HMWIg (4,9-12), and for this reason, they are considered to be sterically different immunoglobulins of the same class.

Studies reported in the literature on 14 different species of bony fish indicate that only 4, the margate (9), giant grouper (10), bowfin (11), and sheepshead (12), possess a LMWIg. The rainbow trout, a teleost, has been reported to possess the characteristic 14S tetrameric immunoglobulin, but a monomeric LMWIg has never been observed (21-26). Hodgins _et al_. (23) have reported the finding of 13.5S, 10.5S and 4.2S molecules with agglutinating activity. The 4.2S population is presently thought not to be Ig in nature and probably is a lectin (24). Little attention has been directed to the relevance of the 10.5S molecule. It has never been reproducibly isolated nor characterized and many thought that it was an artifact or degradation product of the 14S tetramer.

Techniques that assess particulate aggregation, such as titering antisera against red blood cells, are normally used for measuring immunoglobulin in lower vertebrates. In this study more sensitive techniques were utilized to elucidate the presence of a LMWIg. Specific anti-TNP antibody was isolated and applied to a calibrated Sephacryl S300 column and two Ig populations were observed. The molecular weights of these immunoglobulins, 620,000 and 490,000 daltons, suggest the possibility that the HMWIg is a tetramer, and the LMWIg is a trimer. No monomeric antibody was detected, even after chromatography in 6 M urea. Our data suggest, moreover, that the HMWIg and LMWIg do not exist as _in vitro_ aggregation or degradation products. The possibility still exists, however, that the LMWIg is a product of _in vivo_ catabolism of the HMWIg.

An alternate explanation for the existence of a HMWIg and LMWIg may have been a deletion in the H chain structure. By SDS-PAGE it appears that the heavy and light chain of the HMWIg and LMWIg are identical at 72,000 and 27,000 daltons, respectively. It would appear then that the difference between the HMWIg and LMWIg must be due to some configurational difference of the intact Ig molecule.

Two techniques commonly employed to isolate and purify the Ig species in mammals are anion-exchange chromatography and protein-affinity chromatography. Attempts to isolate or separate the HMWIg from LMWIg by Protein A or ion exchange chromatography were unsuccessful. The Protein A did not bind either Ig under the conditions employed, and each immunoglobulin eluted from DE-52 at approximately the same

ionic strength. These methods are not useful for carrying out the purification of RBT Ig populations.

It was of interest to determine whether maturation occurs in either population of Ig. The fluorescence quenching assay showed that the K_a of specifically purified HMWIg and LMWIg was 1-2 x 10^5 M^{-1} and there appeared to be little maturation in the binding constant of either the HMWIg or LMWIg. Various schemes have been employed for eluting antibody from affinity columns (45,46), and depending on the conditions, only a portion of the applied antibody (with a restricted binding affinity) may be selected (31). To be certain that our elution was not selective, the K_a of specific TNP Ig in whole serum prior to any antibody purification was determined. Using a modified Farr assay it was shown that the combined binding affinity of the RBT antibody was in the range of 1-2 x 10^5 M^{-1}, indicating good agreement with data obtained by the method of fluorescence quenching on isolated HMWIg and LMWIg. These data are also in good agreement with published data for other bony fish (50).

Hodgins *et al.* (22) have recently discovered two electrophoretically distinct populations of RBT immunoglobulins. Our observations have confirmed this report. We have prepared anti-HMWIg and anti-LMWIg and have shown that the Ig population described by Hodgins *et al.* with slow beta or gamma mobility is the LMWIg and the Ig with beta mobility is the HMWIg. These two populations possess a band of partial identity but the LMWIg appears to have at least one unique antigenic

determinant. This pattern of immunoelectrophoresis has been observed in several other species of fish (8,9,22,47-50) in which an actual low molecular weight Ig species has never been detected. One could hypothesize that these fish may also have a LMWIg similar to that of RBT, but an adequate analysis of the immunoglobulins of other fish species has not yet been carried out.

Lobb (12) has reported the occurrence of three species of Ig (16S, 11S, and 6.1 S) in the sheepshead (Archosargus probatocephalus). The 6.1S Ig was shown to be a monomer and the 16S a tetramer. The 11S Ig was generated from the 16S Ig after equilibration in 5 M guanidine HCl. It appears that the 11S Ig exists normally in serum as a 16S tetramer, but when subjected to reducing conditions it breaks down from the tetramer to form dimers. To ascertain if this possibility existed in RBT, antibody was eluted from the affinity column with 0.1 M free hapten and in this way the high salt and reducing conditions were avoided. The chromatographic characteristics of the HMWIg and LMWIg prepared in this manner were identical to those observed in the presence of 3 M thiocyanate. These results, along with the data obtained from chromatography in 6 M urea indicate that the HMWIg is highly stable, and suggest that the HMWIg is not a simple aggregate of the LMWIg or any smaller molecular weight structure.

Our results indicate that RBT possess two stable species of immunoglobulin. The HMWIg appears to be a tetramer, based not only on our evidence, but also on data which have been previously published (23,25,26). We have also detected a LMWIg in RBT, and the chromato-

graphic behavior suggests that it may be a trimer. Further experimentation is necessary in order to conclusively establish the polymeric structure of this species. The presence of a trimer in other vertebrates is not common, but has been observed in isolated circumstances (51).

REFERENCES

1. Larsen, B. 1972. Antibody activity in various classes of hedgehog immunoglobulins. Comp. Biochem. Physiol. 42B:357.

2. Coe, J., and R. Race. 1978. Ontogeny of mink IgG, IgA, and IgM. Proc. Soc. Exp. Biol. Med. 157:289.

3. Hadji-Azimi, I. 1979. Anuran immunoglobulins: A review. Dev. Comp. Immunol. 3:223.

4. Clem, L., and P. Small. 1967. Phylogeny of immunoglobulin structure and function. I. Immunoglobulin of the Lemon shark. J. Exp. Med. 125:893.

5. Sigel, M. 1974. Primitive immunoglobulins and other proteins with binding functions in the shark. Ann. N.Y. Acad. Sci. 234:198.

6. Acton, R. 1971. Tetrameric immune macroglobulins in three orders of bony fishes. Proc. Natl. Acad. Sci. 68:107.

7. Hadji-Azimi, I., and M. Michea-Hamzhpour. 1976. Xenopus laevis 19S immunoglobulin ultrastructure and J chain isolation. Immunol. 30:587.

8. Cizar, J., and J. Fryer. 1974. Characterization of anti-Aeromonas salmonicida antibodies from coho salmon. Infect. Immun. 9:236.

9. Clem, L., and W. McLean. 1975. Phylogeny of immunoglobulin structure and function. VII. Monomeric and tetrameric immunoglobulins of the margate; a marine teleost fish. Immunol. 29:791.

10. Clem, L. 1971. Phylogeny of immunoglobulin structure and function. IV. Immunoglobulins of the giant grouper (Epinephelus itaira). J. Biol. Chem. 246:9.

11. Litman, G., D. Frommel, J. Finstad, and R. Good. 1971. Evolution of the immune response. IX. Immunoglobulins of the bowfin: Purification and characterization. J. Immunol. 106:747.

12. Lobb, C. 1980. Humoral and secretory immunoglubolins of the sheepshead, Archosargus probatocephalus, a marine teleost. Utah State University, Ph.D. thesis. p. 153.

13. Hall, S., E. Evans, H. Dupree, R. Acton, P. Weinheimer, and J. Bennett. 1973. Characterization of a teleost immunoglobulin: The immune macroglobulin from the channel catfish, Ictalurus punctatus. Comp. Biochem. Physiol. 46B:187.

14. Shelton, E. and M. Smith. 1970. The ultrastructure of carp (Cyprinus carpio) immunoglobulin: a tetrameric macroglobulin. J. Mol. Biol. 54:615-617.

15. Acton, R., P. Weinheimer, H. Dupree, T. Russell, M. Wilcott, E. Evans, R. Schronenloher, and J. Bennett. 1971. Isolation and characterization of the immune macroglobulin from the paddlefish, Polyodon spathula. J. Biol. Chem. 246:6760.

16. Acton, R., P. Weinheimer, H. Dupree, E. Evans, and J. Bennett. 1971. Phylogeny of immunoglobulins. Characterization of a 14S immunoglobulin from the gar, Lepisosteus osseus. Biochem. 10:2028.

17. Bradshaw, L., L. Clem, and M. Sigel. 1971. Immunologic and immunochemical studies on the gar, Lepisosteus platyrhincus. II. Purification and characterization of immunoglobulin. J. Immunol. 106:1480.

18. Bradshaw, C., L. Clem, and M. Sigel. 1969. Immunological and immunochemical studies in the gar (Lepisosteus platyrhincus). I. Immune responses and characterization of antibody. J. Immunol. 103:496.

19. Bradshaw, C., L. Clem, and M. Sigel. 1971. Immunological and immunochemical studies on the gar, (Lepisosteus platyrhincus). II. Immune responses and characterization of antibody. J. Immunol. 106:1480.

20. Andreas, E., R. Richter, D. Hadge, and H. Ambrosius. 1975. Structurelle und immunchemische untersuchungen am Immunoglobulin des karpfens (Cyprinus carpio L.). II. Analyse der Untereinheiten. Acta. Biol. Med. Germ. 34:1407.

21. Post, G. 1966. Serum proteins and antibody production in rainbow trout (Salmo gairdneri). J. Fish. Res. Bd. Can. 23:1957.

22. Hodgins, H., G. Ridgeway, and F. Utter. 1965. Electrophoretic mobility of an immune globulin from rainbow trout serum. Nature (Lond)208:1106.

23. Hodgins, H., R. Weiser, and G. Ridgeway. 1967. The nature of antibodies and the immune response in rainbow trout (Salmo gairdneri). J. Immunol. 19:534.

24. Hodgins, H., F. Wendling, B. Braaten, and R. Weiser. 1973. Two molecular species of agglutinins in rainbow trout (Salmo gairdneri) serum and their relation to antigenic exposure. Comp. Biochem. Physiol. 45B:975.

25. Dorson, M. 1972. Some characteristics of antibodies in the primary immune response of rainbow trout, Salmo gairdneri. Symp. Zool. Soc. London 30:129.

26. Ikeda, K. 1978. Gel chromatographic characteristics of agglutinating antibody of rainbow trout to Aeromonas salmonicida. Bull. Freshwater Fish. Res. Lab. 28:55.

27. Garvey, J., N. Cremer, and D. Sussdorf. 1977. In Methods in Immunology. Third edition. W. A. Benjamin, Inc. pp. 153-158.

28. Lied, E., J. Gjerde, and O. Brackkan. 1975. Simple and rapid technique for repeated blood sampling in rainbow trout (Salmo gairdneri). J. Fish. Res. Bd. Can. 32:699.

29. Robbins, J., J. Haimorich, and M. Sela. 1967. Purification of antibodies with immunoadsorbents prepared using bromoacetyl cellulose. Immunochem. 4:11.

30. March, S., I. Parikh, and P. Cuatrecasus. 1974. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. Anal. Biochem. 60:149.

31. Shimizu, F., H. Mossmann, K. Himmelspach, H. Takamiya, and A. Vogt. 1978. Separation of anti-dinitrophenyl antibodies according to affinity by fractionated elution from immunoadsorbent using thiocyanate. Res. Exp. Med. 173:165.

32. Stone, S., J. Patterson, and M. Phillips. 1979. Isolation and purification of bovine IgM by dissociating immunoglobulin-Brucella complexes. J. Immunol. Meth. 31:379.

33. Steward, M., and R. Petty. 1972. The use of ammonium sulphate globulin precipitation for determination of affinity of antiprotein antibodies in mouse serum. Immunol. 22:747.

34. Stupp, Y., T. Yoshida, and W. Paul. 1969. Determination of antibody-hapten equilibrium constants by an ammonium sulphate precipitation technique. J. Immunol. 103:625.

35. Eisen, H. 1964. Determination of antibody affinity for haptens and antigens by means of fluorescence quenching. In Methods in Medical Research. Edited by H. Eison. Vol. X., p. 115. Chicago Year Book Medical Publishers.

36. Parker, C. 1978. Spectrofluorometric methods. In Handbook of Experimental immunology Vol. 1 Immunochemistry. Edited by D. M. Weir. Third edition. Blackwell Scientific Publication pp. 18.1-18.25.

37. Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 222:680.

38. Endresson, J. and A. Grov. 1976. Further characterization of Protein A reactive and non-reactive subfragments of Fc from human IgG. Acta. Path. Microbiol. Scand. 84C:397.

39. Cavagnolo, R. 1979. The immunology of marine mammals. Dev. Comp. Immunol. 3:245.

40. Bruce, D., and P. Murtagh. 1978. Studies on immunoglobulins of Richardson's ground squirrel. I. Characterization of ground squirrel IgG. Immunochem. 15:193.

41. Clem, L., F. Boutaud, and M. Sigel. 1967. Phylogeny of Immunoglobulin structure and function. II. Immunoglobulins of nurse shark. J. Immunol. 99:1226.

42. Frommel, D., G. Litman, J. Finstad, and R. Good. 1971. The evolution of the immune response. XI. The immunoglobulins of the horned shark (Heterodontus francisei) : Purification characterization, and structural requirement for antibody activity. J. Immunol. 106:1234-1243.

43. Hadji-Azimi, I., and M. Michea-Hamzehpour. 1976. Xenopus laevis 19S immunoglobulin ultrastructure and J chain isolation. Immunol. 39:587.

44. Hadji-Azimi, I., D. Brandt, A. Bossus., and M. Michea-Hamzchpour. 1976. Studies on immunoglobulins of Xenopus boredis, Xenopus clivil and Xenopus muelleri. J. Exp. Zool. 195:107.

45. Hoyer, L., W. Vannier, and L. Renter. 1968. Antibody elution from hapten-cellulose immunoadsorbents : The effect of hapten structure, pH and concentration. Immunochem. 5:277.

46. Hill, R. 1972. Elution of antibodies from immunoadsorbants : Effect of dioxane in promoting release of antibody. J. Immunol. Meth. 1:231.

47. Heartwell, C. 1975. Immune response and antibody characterization of the channel catfish (Ictalurus punctatus) to a naturally pathogenic bacterium and virus. U.S. Fish Wildl. Serv. Tech. Paper 85 pp. 1-34.

48. Trump, G. 1970. Goldfish immunoglobulins and antibodies to bovine serum albumin. J. Immunol. 104:1267.

49. Ingram, G., and J. Alexander. 1979. The immunoglobulin of the brown trout, Salmo trutta and its concentration in the serum of antigen-stimulated and non-stimulated fish. J. Fish. Biol. 14:249.

50. Clem, L., and P. Small. 1970. Phylogeny of immunoglobulin structure and function. VI. Valences and association constants of teleost antibodies to a haptenic determinant. J. Exp. Med. 132:385.

51. Kobayashi, K. and H. Hirai. 1980. Studies on subunit components of chicken polymeric immunoglobulins. J. Immunol. 124:1695.

III. A Characterization of Immunoglobulins in Rainbow Trout (Salmo gairdneri) II. A Further Biochemical Analysis

INTRODUCTION

High molecular weight immunoglobulin (HMWIg) and low molecular weight immunoglobulin (LMWIg) have been difficult to detect in most bony fish (1-20). Reports from this laboratory have demonstrated the presence of a LMWIg in rainbow trout (RBT), a teleost previously considered to possess only a tetrameric HMWIg (O'Leary et al, manuscript in preparation). The tetrameric HMWIg has a molecular weight of approximately 620,000 daltons whereas the size of the LMWIg is 490,000 daltons. The H and L chains of the HMWIg and LMWIg have the same molecular weight (72,000 and 27,000 daltons respectively). Neither population appears to be a degradation or aggregation product of the other. The two immunoglobulins have been shown by immunoelectrophoresis to have different electrophoretic mobilities and are antigenically related. Our previous studies have strongly suggested that the difference between the HMWIg and LMWIg is of a conformational nature and is not due to a reduction in H chain mass.

This study was initiated to determine if the HMWIg and LMWIg are truly "identical" molecules with different conformations or if the two populations are actually distinct molecules. A physiochemical analysis of the HMWIg and LMWIg was carried out, including determination of the sedimentation rate and amino acid composition. The isoelectric points

(pI) for the immunoglobulins were also determined, and the evidence indicates that the HMWIg and LMWIg are indeed two distinct molecular species of immunoglobulin.

MATERIALS AND METHODS

Preparation of pure HMWIg and LMWIg

Preparation of pure HMWIg and LMWIg has been described in detail (O'Leary et al, manuscript in preparation). Briefly, adult rainbow trout (RBT, Salmo gairdneri) were injected at various intervals with TNP-BSA. The serum was harvested and applied to a TNP-Ovalbumin affinity column to select for TNP specific Ig. The Ig was eluted with 3 M KSCN, dialysed and applied to a Sephacryl S-300 molecular sieve column. Two proteins were obtained which correspond to the HMWIg and LMWIg.

Preparation of ^{125}I labeled proteins

Purified proteins were iodinated by the chloramine-T method (21) using Na ^{125}I (New England Nuclear). Conditions were established so that approximately 10 iodine groups were conjugated per mole of protein (22). Unreacted ^{125}I was removed by extensive dialysis against 0.05 M phosphate buffer, pH 8.0.

Determination of the pI for the HMWIg and LMWIg

The isoelectric point (pI) for purified HMWIg and LMWIg was determined in an LKB Ampholine electrofocusing column, type 110 ml. Ampholyte (BioLyte 3/10 or BioLyte 4/6) was incorporated at 1% in a sucrose gradient. The protein to be analysed, after labeling with ^{125}I, was applied to the column and run at 1600 V for 20 hr at 4 C. After electrofocusing both the pH and the concentration of iodinated material in each fraction were determined.

Determination of the sedimentation constant for the HMWIg and LMWIg

The sedimentation constants of the HMWIg and LMWIg were determined by two separate procedures. One method utilized radioiodinated RBT immunoglobulins, and external standards of BSA and human IgM which were centrifuged in a Beckman Model L5-65 preparative ultracentrifuge using a swinging bucket SW-41 rotor. The samples were run in a 10-30% glycerol gradient containing 0.15 M NaCl, 0.05 M TRIS, pH 7.4 and centrifuged at 40,000 rpm for 17 hr at 4 C. Fractions were collected and the radioactivity was determined with a Beckman Model LS-8000 Series Liquid Scintillation System.

Each sample, approximately 0.5 mg/ml, was also centrifuged in a Beckman Model E analytical ultracentrifuge. Samples were equilibrated with either 0.1 M phosphate buffer, pH 8.0 or 3 M KSCN and centrifuged at 36,000 rpm at 20 C. Protein concentrations were determined by UV optics and plotted at 4 min intervals.

Amino acid analysis

Samples of purified HMWIg and LMWIg were prepared for analysis by hydrolysis in 6 N HCl for 22 hr at 110 C. The sample was dried and diluted in 0.2 N sodium citrate, pH 2.2 and filtered. It was applied to a Beckman Model 120B Amino Acid Analyzer fitted with a 6 mm single column. The sample was eluted stepwise using: 0.2 N sodium citrate, pH 3.21, 0.2 N sodium chloride, pH 4.12, and 1.0 N sodium as sodium citrate and sodium chloride, pH 6.17. Fractions corresponding to different amino acids were detected by the ninhydrin method (23),

and the data were analyzed with a Spectrophysics Autolab System IVB integrator. Results were expressed as numbers of residues of the amino acid per 1000 residues of the peptide. Tryptophan was not determined.

RESULTS

Determination of sedimentation values of the HMWIg and LMWIg

Sedimentation constants for the HMWIg and LMWIg using the Beckman Model L5-65 preparative ultracentrifuge and external standards were calculated to be 14 S and 10 S, respectively (Figure 1). Values obtained by centrifugation with the Beckman Model E analytical ultracentrifuge were more difficult to obtain due to aggregation of the molecules upon standing, therefore samples had to be processed quickly to reduce aggregation. The HMWIg was analysed in PB and determined to be 14 S. The LMWIg was dialysed into 3 M KSCN to prevent aggregation and a sedimentation coefficient of 10 S was determined (data presented in Appendix A).

Amino acid analysis

The amino acid sequence is the definitive assay to determine relatedness among proteins. However, when this is not possible an amino acid analysis is often sufficient to establish whether two proteins are unique. The amino acid content of HMWIg and LMWIg was determined and the two proteins were found to be significantly different (Table 1).

Determination of the pI for HMWIg and LMWIg

Analysis of the isoelectric points for intact HMWIg and LMWIg was initially conducted using unlabeled samples. The immunoglobulins were found to be insoluble at their isoelectric points at concentrations

Fig. 1. Preparative ultracentrifuge determination of the sedimentation coefficients of RBT immunoglobulins. Radioiodinated proteins were centrifuged in a 10-30% glycerol gradient at 40,000 rpm for 17 hrs at 4 C.

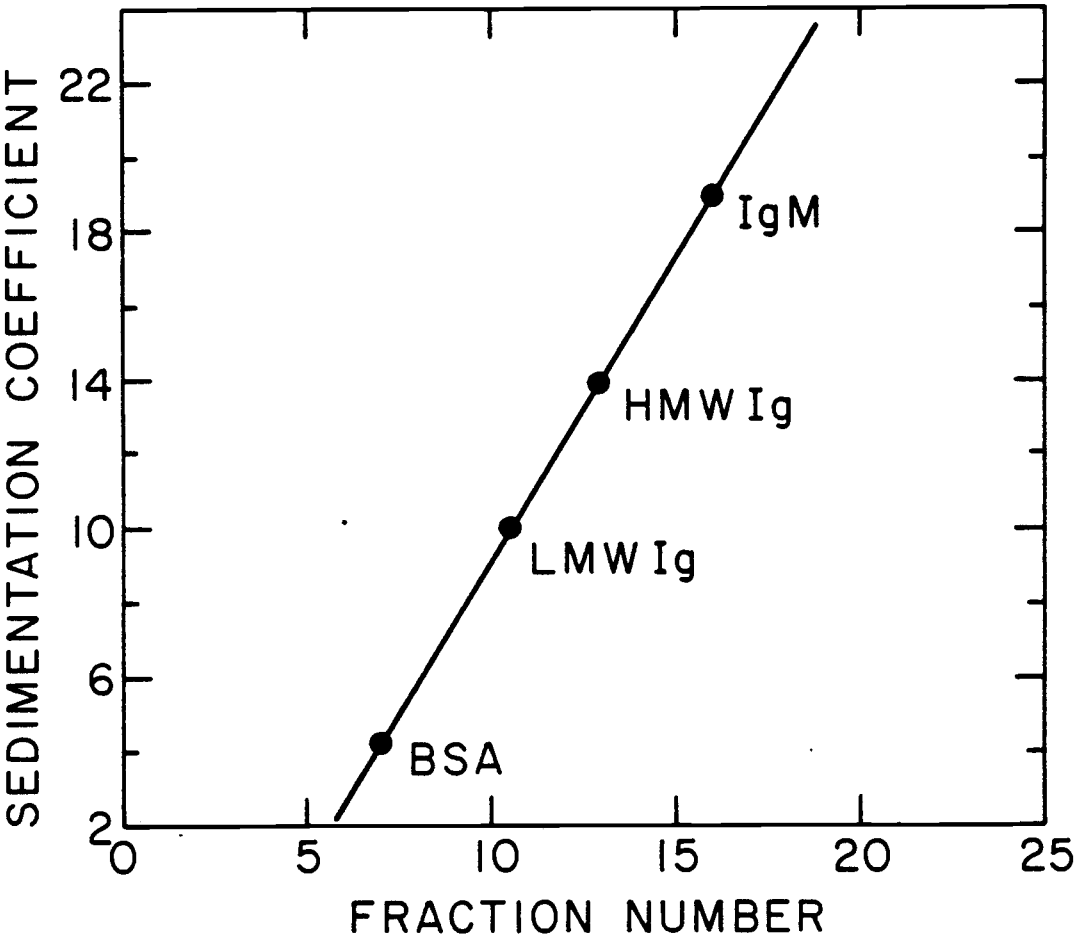

SEDIMENTATION COEFFICIENT
22
18
14
10
6
2
0
5
10
15
20
25
FRACTION NUMBER
IgM
HMW Ig
LMW Ig
BSA

Table 1. Amino acid analysis of high and low molecular weight immunoglobulin[a].

Amino Acid[b]	HMWIg	LMWIg	Δ[c]
Lys	60.4	48.1	12.3
His	14.4	13.8	0.6
Arg	32.8	25.7	7.1
Cys	19.4	12.8	6.6
Asx	94.6	124.5	29.9
Thr	88.5	79.6	8.9
Ser	101.7	142.0	40.3
Glx	118.4	160.9	42.5
Pro	44.5	37.1	7.4
Gly	75.2	54.4	20.8
Ala	61.4	44.9	16.5
Val	81.0	64.4	16.6
Met	27.9	22.2	5.7
Ile	29.0	58.5	29.5
Leu	73.8	56.9	16.9
Tyr	40.9	29.4	11.5
Phe	36.1	24.8	11.3

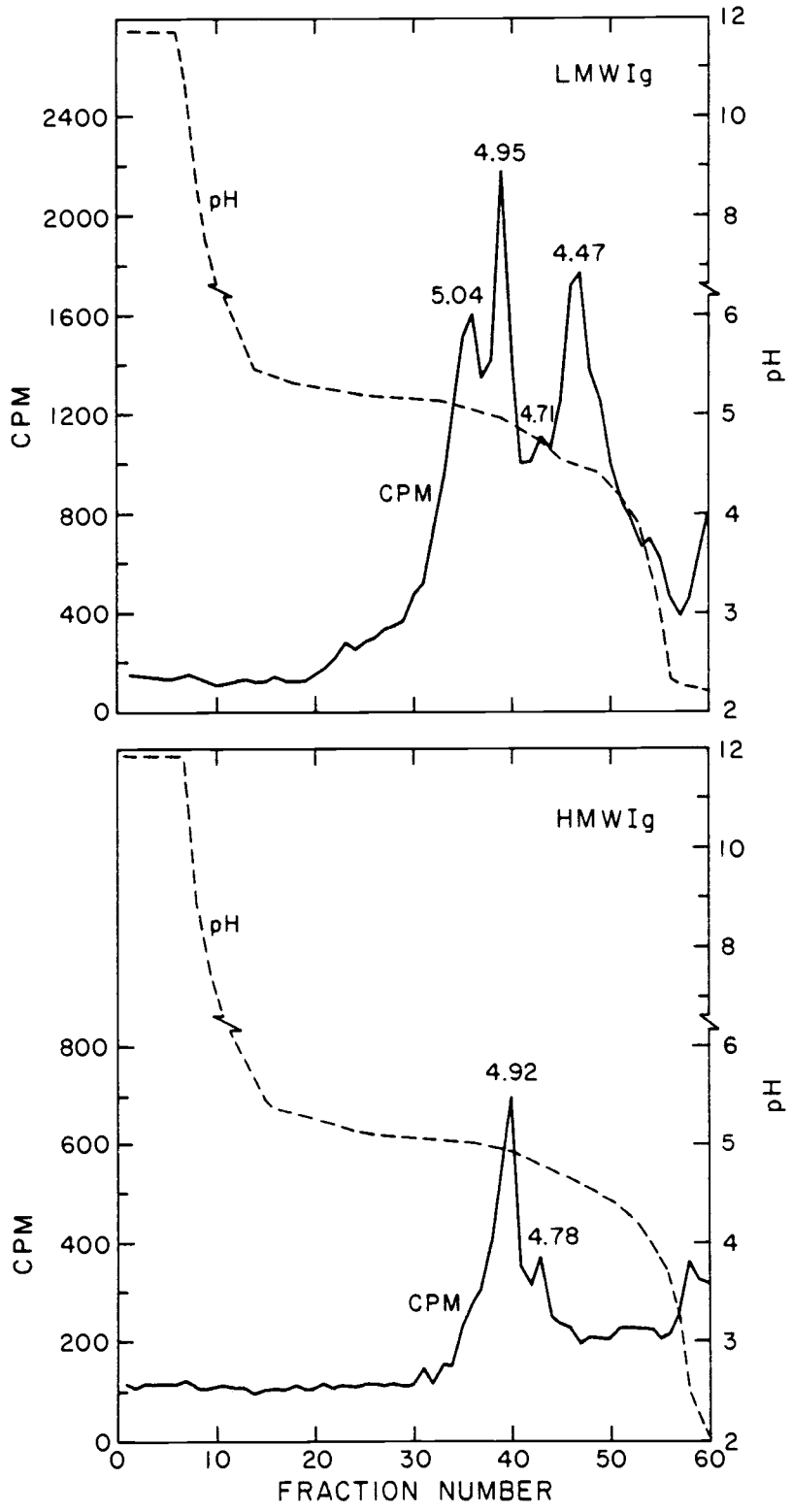
[a]Expressed as number of amino acids per 1000 residues (± 5%).

[b]Tryptophan was not determined.

[c]Difference in amino acid content between the HMWIg and the LMWIg.

required to detect the protein. The immunoglobulin samples were radioiodinated to avoid this problem. Each sample was initially run in a 3-10 pH gradient and the pI for both the HMWIg and the LMWIg were determined to be approximately 5. The pI of each was then analysed using a pH 4-6 gradient (Fig. 2). The results show that HMWIg was less heterogeneous than the LMWIg. Two populations of HMWIg, with pI values of 4.92 and 4.78, were observed. Four populations of LMWIg were observed in the range of 4.47 to 5.14. Each immunoglobulin sample was analysed separately in three experiments with concentrations of protein varying over a 100 fold range. The profile of each sample remained identical regardless of the specific activity or concentration of the protein up to the point of insolubility.

Fig. 2. Determination of the isoelectric point for the LMWIg (a) and HMWIg (b) in RBT. Proteins were labeled with $Na^{125}I$ and run in a Biotype 4/6 pH gradient.


LMWIg
HMWIg
pH
CPM
4.95
5.04
4.47
4.71
4.92
4.78
2400
2000
1600
1200
800
400
0
600
200
12
10
8
6
5
4
3
2
0
10
20
30
40
50
60
FRACTION NUMBER

DISCUSSION

Rainbow trout is a representative of the order Teleostei in the subclass Actinopterygii. These fish represent a group which is divergent from the mainline of mammalian evolutionary development. They possess a tetrameric high molecular weight immunoglobulin (HMWIg) instead of a pentameric immunoglobulin (Ig) normally found in other vertebrates (1-9). A monomeric low molecular weight immunoglobulin (LMWIg) has only been detected in four species of bony fish (1-4). We have previously reported data which indicate that RBT possess a trimeric or dimeric LMWIg (O'Leary et al, manuscript in preparation). The present study describes other physical characteristics of both the LMWIg and HMWIg.

The isoelectric point (pI) of the HMWIg and the LMWIg were determined, and the pI of both populations of Ig were found to be in the range of 4.4 to 5.0. The LMWIg was far more heterogeneous than the HMWIg. The variability of the pI in the molecules may be due in part to the variable region of the immunoglobulin and not the constant region. Whether the heterogeneity in pI of the LMWIg represents activation of more clones of anti-TNP cells than are found for the HMWIg is not known.

The pI for RBT Ig is low in comparison with that of higher vertebrates (22). The pI for immunoglobulins of other lower vertebrates has not been recorded. The low pI indicates a fairly charged molecule at pH values normally found in RBT serum.

Hodgins et al. (12) have reported that RBT possess three populations of immunoglobulin, with sedimentation coefficients of 13.9, 10.5 and 4.2, and all with agglutinating activity. The 4.2 S population is presently thought to be a lectin (24). The 10.5 S molecular species has never been isolated, and up to this time subsequent investigations have not been successful in confirming the existence of this material (24). For this reason RBT were thought to possess only a 14 S HMWIg. Sedimentation coefficients of HMWIg from other bony fish have been reported to be 14 to 17 S (1-9,11,12,14,16, 18). The LMWIg has been reported to be approximately 7 S in the four species of bony fish in which it has been detected (1-4). The HMWIg of the sheepshead was found to disassociate in 4 M guanidine HCl into 11 S dimers (4). The relationship of this 11 S molecule to the RBT 10 S molecule is uncertain. The sheepshead 11 S molecule can only be detected under certain conditions (14), whereas the 10 S RBT Ig that we report has been shown to be present naturally.

The most striking evidence to indicate that the HMWIg and LMWIg are not identical molecules with different configurations is the amino acid analysis which demonstrates a different amino acid content. The amino acid analysis of RBT immunoglobulins resembles that of immunoglobulins of other bony fish such as the catfish (18), gar (6,18), giant grouper (2), and paddlefish (18). Relatedness to human immunoglobulins, especially IgM, can also be observed but to a lesser degree (26). The relatively high amount of acidic amino acids would account for the relatively low pI found in RBT.

It is clear from this study that RBT possess two distinct molecular species of Ig: a tetrameric HMWIg which is normally observed in all bony fish, and a LMWIg which possesses a unique molecular weight, sedimentation coefficient, and amino acid composition. The LMWIg of RBT is unlike other LMWIg of other lower vertebrates due to a larger molecular weight and sedimentation coefficient. Unlike other lower vertebrates, the LMWIg in RBT is not a monomer. Further work is required to determine the significance of this distinctly different immunoglobulin.

REFERENCES

1. Clem, L., and W. McLean. 1975. Phylogeny of immunoglobulin structure and function. VII. Monomeric and tetrameric immunoglobulins of the margate; a marine teleost fish. Immunol. 29:791.

2. Clem, L. 1971. Phylogeny of immunoglobulin structure and function. IV. Immunoglobulins of the giant grouper (Epinephelus itaira). J. Biol. Chem. 246:9.

3. Litman, G., D. Frommel, J. Finstad, and R. Good. 1971. Evolution of the immune response. IX. Immunoglobulins of the bowfin: Purification and characterization. J. Immunol. 106:747.

4. Lobb, C. 1980. Humoral and secretory immunoglobulins of the sheepshead, Archosargus probatocephalus, a marine teleost. Utah State University, Ph.D. thesis. p. 153.

5. Acton, R., P. Weinheimer, H. Dupree, T. Russell, M. Wilcott, E. Evans, R. Schronanloher, and J. Bennett. 1971. Isolation and characterization of the immune macroglobulin from the paddlefish, Polyodon spathula. J. Biol. Chem. 246:6760.

6. Acton, R., P. Weinheimer, H. Dupree, E. Evans, and J. Bennett. 1971. Phylogeny of immunoglobulins. Characterization of a 14 S immunoglobulin from the gar, Lepisosteus osseus. Biochem. 10:2028.

7. Andreas, E., R. Richter, D. Hadge, and H. Ambrosins. 1975. Strukturelle und immunchemische untersuchungen am immunglobulin des karpfens (Cyprinus carpio L.) II. Analyse der untereinheiten. Acta Biol. Med. Germ. 34:1407.

8. Cisar, J., and J. Fryer. 1974. Characterization of anti-Aeromonas salmonicida antibodies from coho salmon. Infect. Immun. 9:236.

9. Clerx, J., A. Castel, J. Bol, and G. Gerwig. 1980. Isolation and characterization of the immunoglobulin of pike (Essox luceus L.). Vet. Immunol. Immunopath. 1:125.

10. Dorson, M. 1972. Some characteristics of antibodies in the primary immune response of rainbow trout, Salmo gairdneri. Symp. Zool. Soc. London. 30:129.

11. Hall, S., E. Evans, H. Dupree, R. Acton, P. Weinheimer, and J. Bennett. 1973. Characterization of a teleost immunoglobulin: The immune macroglobulin from the channel catfish Ictalurus punctatus. Comp. Biochem. Physiol. 46B:187.

12. Hodgins, H., R. Weiser, and G. Ridgway. 1967. The nature of antibodies and the immune response in rainbow trout (Salmo gairdneri). J. Immunol. 99:534.

13. Ikeda, K. 1978. Gel chromatographic characteristics of agglutinating antibody of rainbow trout to Aeromonas salmonicida. Bull. Freshwater Fish Res. Lab. Tokyo. 28:55.

14. Ingram, G., and J. Alexander. 1979. The immunoglobulin of the brown trout, Salmo trutta and its concentration in the serum of antigen-stimulated and non-stimulated fish. J. Fish. Biol. 14:249.

15. Marchalonis, J. 1971. Isolation and partial characterization of goldfish (Carassius auratus) and carp (Cyprinus carpio). Immunol. 20:161.

16. Russell, W., E. Voss, and M. Sigel. 1970. Some characteristics of anti-dinitrophenyl antibody of the gray snapper. J. Immunol. 105:262.

17. Trump, G. 1970. Goldfish immunoglobulins and antibodies to bovine serum albumin. J. Immunol. 104:1267.

18. Acton, R. 1971. Tetrameric immune macroglobulins in three orders of bony fishes. Proc. Natl. Acad. Sci. 68:107.

19. Bradshaw, C., L. Clem, and M. Sigel. 1971. Immunological and immunochemical studies on the gar, (Lepisosteus platyrhincus). II. Immune responses and characterization of antibody. J. Immunol. 106:1480.

20. Bradshaw, C., L. Clem, and M. Sigel. 1969. Immunological and immunochemical studies in the gar (Lepisosteus platyrhincus). I. Immune responses and characterization of antibody. J. Immunol. 103:496.

21. Sonoda, S., and M. Schlamowitz. 1970. Studies of ^{125}I trace labeling of immunoglobulin G by chloramine-T. Immunochem. 7:885.

22. Gallagher, J., and E. Voss. 1970. Immune precipitation of purified chicken antibody at low pH. Immunochem. 7:771.

23. Moore, S., and W. Stein. 1954. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J. Biol. Chem. 211:907.

24. Hodgins, H., F. Wendling, B. Braaten, and R. Weiser. 1973. Two molecular species of agglutinins in rainbow trout (Salmo gairdneri) serum and their relation to antigenic exposure. Comp. Biochem. Physiol. 45B:975.

25. Post, G. 1966. Serum proteins and antibody production in rainbow trout (Salmo gairdneri). J. Fish Res. Bd. Can. 23:1957.

26. Putnam, F., M. Korzuru, and C. Easley. 1967. Structural studies of the immunoglobulins. IV. Heavy and light chains of the γM pathological macroglobulins. J. Biol. Chem. 242:2435.

Appendix A: Calculation of the sedimentation coefficients of RBT HMWIg and LMWIg from data generated by analytical ultracentrifugation.

	Radius (cm)	
Time (sec)	LMWIg[a]	HMWIg[b]
0	6.0950	6.0750
720	6.1600	6.1550
1440	6.2250	6.2500
2160	6.2900	6.3450

[a]Sample was equilibrated in 3M KSCN, pH 8.0, and centrifuged at 36,000 rpm, at 20 C, in a Beckman Model E analytical ultracentrifuge.

[b]Sample was equilibrated in 0.1M PB, pH 8.0, and centrifuged as described above.