PHYLOGENY OF IMMUNOGLOBULIN STRUCTURE AND FUNCTION*

I. Immunoglobulins of the Lemon Shark

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At the present time mammalian antibodies have been demonstrated in at least three classes of serum proteins referred to as immunoglobulins. The immunoglobulin classes have been designated gamma M, gamma G, and gamma A (1). Each of these proteins is composed of multiple polypeptide chains and the classes are distinguished primarily on the basis of differences of the heavier chains (2).

The ability to produce humoral antibodies in response to antigenic stimulation appears to be limited to the vertebrate species (recently reviewed, see Reference 3). Results previously obtained have indicated that the elasmobranchs are phylogenetically among the most ancient vertebrates capable of undergoing an antibody response (4, 5). In order to gain some understanding of the nature of the immunoglobulins synthesized by elasmobranchs, studies were initiated with the lemon shark, *Negaprion brevirostris*. It was hoped, in light of the complexity and heterogeneity of mammalian immunoglobulins, that the shark immunoglobulin(s) would prove to be a simpler or a more homogeneous population of molecules.

This report presents the results of studies with the lemon shark involving the production of antibodies to bovine serum albumin, the association of these antibodies with two molecular species of serum proteins, and the purification and characterization of these immunoglobulins. Results are also presented regarding the nature of shark proteins with electrophoretic mobilities comparable to gamma G immunoglobulin of mammals.

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Materials And Methods

Immunization Procedures.—Lemon sharks, *Negaprion brevirostris*, of both sexes weighing 20–30 lb. were maintained in tidal sea water (26°–28°C) within a fenced area of Bimini Bay. These animals fared well in captivity and were fed to satiation at least twice weekly with fresh fish. All sharks were immobilized with MS-222 (Sandoz Inc. New York, N. Y.) prior to experimental manipulation. Bleedings were accomplished by hemal arch puncture (4) and sera were separated by centrifugation after allowing the blood to stand at room temperature for 1–2 hr and overnight at 5°C. Sera were stored at −20°C. The antigen used was bovine serum albumin (BSA) (Armour Pharmaceutical Co., Chicago, Ill., Cohn fraction V) either incorporated in complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, Mich.) or adsorbed onto alum (6), and it was administered subcutaneously to multiple sites on the ventral surface.

Detection of Antibody Activity.—Antibodies were detected by the hemagglutination (HA) of BSA-coated, tanned sheep erythrocytes (7). All sera were absorbed two times with washed, packed sheep erythrocytes to remove naturally occurring hemagglutinins prior to testing for HA antibody to BSA. Due to the presence of heat labile lytic activity, relatively fresh sera were heated to 50°C for 20 min prior to absorption; sera stored for more than 2 wk at −20°C were no longer hemolytic. The diluent used throughout was phosphate buffered saline, pH 7.2 (PBS) which was prepared by mixing equal volumes of 0.15 M NaCl and 0.15 M PO_4, pH 7.2.

Reduction of Antibody Activity.—Immune sera were diluted 1:5 with PBS and dialyzed against 20 volumes of 0.1 M 2-mercaptoethanol (2-ME) in PBS at room temperature for 16 hr (8). The 2-ME was then removed by dialysis against two changes (50 volumes each) of PBS for 20 hr at 5°C. Alkylation, when employed, was accomplished by dialysis against 20 volumes of 0.02 M iodoacetamide in PBS at 5°C immediately following 2-ME treatment.

Fractionation Procedures.—Sucrose gradient (10–37% sucrose in PBS, pH 7.2) ultracentrifugation was performed in a Spinco model L ultracentrifuge using a SW-39 rotor at 100,000 *g* for 16 hr (9). 0.2 ml samples of whole serum (absorbed with sheep erythrocytes and diluted 1:2 with 10% sucrose) were layered over the gradient prior to centrifugation. 11 fractions were collected, either from the top of the tube with a syringe attachment equipped with a blunted needle, or from the bottom by puncturing a hole and collecting drops. Each fraction was diluted 1:4 with PBS prior to testing for antibody.

DEAE-cellulose (Brown Co., New York, N. Y.) chromatography (10) was performed at 5°C with elution being accomplished with an increasing NaCl gradient (convex upward formed by flowing limit buffer into a closed mixing chamber containing starting buffer, at the same flow rate as that of the column). The starting buffer was 0.015 M Tris-HCl, pH 8.0; the limit buffer was 0.4 M NaCl, 0.015 M Tris-HCl, pH 8.0. The column dimensions and starting buffer volumes, respectively, for the DEAE-cellulose columns employed here areas follows: Fig. 3 *a*, 34 × 2.5 cm, 800 ml; Fig. 3 *b*, 32 × 2.5 cm, 1000 ml. Flow rates were about 0.5 ml/hr/cm^2. Carboxymethyl (CM) cellulose (Bio-Rad Laboratories, Richmond, Calif.) chromatography was performed at 5°C with an increasing acetate gradient (11). The gradient was formed as described above for the DEAE-cellulose columns. The starting buffer for the 2.8 × 50 cm column was 750 ml 0.01 M sodium acetate–acetic acid, pH 5.5. The limit buffer was 0.9 M acetate, pH 5.5. Sera or fractions to be chromatographed were first exhaustively dialyzed against the starting buffer; precipitates were removed by centrifugation.

Gel filtration on Sephadex G-200 (12) was performed on a 2.5 cm × 95 cm column (the same column was used throughout) at 5°C. 5 ml fractions were collected at a flow rate of about 10 ml/hr. The buffer was 0.14 M NaCl in 0.01 M Tris-HCl, pH 7.4.

All column effluents were monitored for protein by absorbancy at 280 mμ and concentration of desired fractions was accomplished by pressure dialysis (13). Individual fractions were absorbed with sheep erythrocytes prior to testing for anti-BSA HA antibody.

Antigenic Analysis of Shark Proteins.—Rabbit antisera to normal lemon shark serum or to purified serum components were prepared according to the following immunization schedule. The antigens were emulsified with equal volumes of complete Freund's adjuvant (Difco). The initial immunization consisted of 0.1 ml of the emulsion injected into each hindfoot pad and two 0.4 ml aliquots administered subcutaneously on the back. (Each milliliter of antigen emulsion contained 0.5 ml of whole shark serum or 0.1 mg of the purified proteins or polypeptide chains.) Additional subcutaneous injections of 1 ml of emulsion were given at 1, 5, and 6 wk with bleedings at 3 and 8 wk. In each case the antiserum obtained at 8 wk was adjudged by immunodiffusion and immunoelectrophoresis to be the most potent and was used in the studies herein reported.

Ouchterlony analysis was performed in 1.5% Noble agar (Difco) in barbital buffer, $\Gamma/2 = 0.15$, pH 7.4 (14). Microimmunoelectrophoresis (15) was performed in 1% Agarose (Bausch and Lomb Inc., Rochester, N.Y.) using barbital acetate buffer, $\Gamma/2 = 0.05$, pH 8.6, on microscope slides. Approximately 2.5 v/cm were applied for 50–60 min at 5°C.

Preparation of Polypeptide Chains.—Extensive reduction with 0.1 M dithioerythritol (Cyclo Chemical Corp., Los Angeles, Calif.) and alkylation of shark immunoglobulins in 7 M guanidine—HCl followed by separation of the resultant polypeptide chains by gel filtration through an upward flow Sephadex G-200 column equilibrated with 5 M guanidine—HCl was performed as described in detail elsewhere (13). This column measured 120 × 2.5 cm and had previously been used to separate polypeptide chains from rabbit immunoglobulins.

Milder reduction and alkylation followed by gel filtration on Sephadex G-100 (column dimensions, 3.0 × 100 cm) equilibrated with 1 M propionic acid was performed essentially as originally described by Fleishman et al. (16). Protein solutions of 15–20 mg/ml in saline were buffered to pH 8.2 by the addition of 0.25 volumes of 4.4 M Tris-HCl and reduction was accomplished at room temperature for 1 hr by the addition of dithioerythritol to a final concentration of 0.1 M. After chilling to 0°C, alkylation for about 1 hr was accomplished with 0.25 M iodoacetamide. The reduced and alkylated protein was then dialyzed in the cold for about 15 hr against 300 volumes of saline prior to application to the G-100 column.

Analytical Ultracentrifugation.—Sedimentation velocity studies were performed in the Spinco model E ultracentrifuge under conditions described in the text. Molecular weights were determined by the high speed sedimentation equilibrium method of Yphantis (17). Calculations were performed with the aid of an H-800 computer and a Fortran program (PASOOIC) available upon request.

Determination of Partial Specific Volumes and Extinction Coefficients.—Extinction coefficients (1 cm and 1%) at 280 mμ were calculated for the purified proteins as follows: Concentrated solutions of protein were exhaustively dialyzed against 0.3 M KCl and then divided into aliquots: some of these were then dried in vacuo to constant dry weights and others diluted at least 1:200 in either 0.3 M KCl, 0.1 N NaOH, or 5 M guanidine–HCl and read for ultraviolet (280 mμ) absorbancy in a Beckman DU spectrophotometer. The dry weight of an equal volume of the 0.3 M KCl dialysate was subtracted from the dry weight of the protein-KCl mixture for the purpose of calculating the weight of protein. Because of the large concentration of salt required to solubilize the extensively reduced fractions, extinction coefficients were determined by dialyzing these fractions vs. water until all guanidine–HCl had been removed, then lyophilizing and allowing the protein to equilibrate with atmospheric moisture at room temperature. Aliquots were then weighed and dissolved in 5 M guanidine–HCl and the optical density read at 280 mμ. Other aliquots were dried to constant weight in vacuo at 95°C to allow estimation of moisture content.

Partial specific volumes were determined as suggested by Casassa and Eisenberg (18). Densities of protein solutions in 0.3 M KCl were determined in a density gradient column (mixture of *o*-dichlorobenzene and dodecane at 20°C) according to the Linderstrom-Lang

method (19). KCl solutions of known density were used as standards as described previously (13).

Disc Electrophoresis.—Disc electrophoresis was performed in polyacrylamide gel containing urea (20).

Fingerprints.—Fingerprinting of tryptic digests of polypeptide chains was performed by the method of Katz et al. (21) using a Gilson model D electrophorator (Gilson Medical Electronics, Middleton, Wis.). The trypsin employed (Worthington Biochemical Corp. Cleveland, Ohio, lot 591) had previously been shown to be low in chymotryptic activity and similarly to break all of the lysine and arginine peptide bonds in rabbit gamma G L chains (22).

Hexose Determination.—Protein bound hexose was measured by the orcinol method of Winzler (23) using a mannose standard.

EXPERIMENTAL RESULTS

Antibody Response of Lemon Sharks to Bovine Serum Albumin.—Lemon sharks injected with BSA responded with the production of antibodies detectable by passive hemagglutination (Fig. 1). The latent period with each immunization schedule was between 12 and 20 days with maximum titers being obtained at 25–40 days. These titers were in the range of 1:1280–1:5120 and no evidence of enhanced antibody synthesis was obtained in animals receiving additional injections of antigen. The specificity of the HA titers was established by a hemagglutination inhibition test performed by adding soluble BSA to each serum dilution tube prior to the addition of antigen-coated RBC; no inhibition was observed with human or horse serum albumins. None of these shark antisera formed visible immune precipitins with BSA when analyzed by the Ouchterlony or capillary precipitation methods in the presence of 0.14 or 2.0 M NaCl. The HA activity in whole serum or in separated fractions was stable to heating at 56°C for 30 min.

Demonstration of Two Molecular Species of Lemon Shark Antibody.—Various lemon shark anti-BSA sera were subjected to sucrose gradient untracentrifugation and to reduction with 2-ME. In the early stages (27–31 days) of the antibody response only 2-ME–sensitive, rapidly sedimenting HA antibody was detected (Table I). Immune sera obtained at progressively later times contained increasing amounts of a 2-ME–resistant, more slowly sedimenting form. Reduction of this lighter fraction after centrifugation indicated that it contained the resistant activity; serum reduced prior to centrifugation similarily yielded HA activity only in the light fractions. Alkylation of reduced lighter fractions usually resulted in a two to fourfold decrease in HA activity. Results comparable to these have been obtained with lemon shark antibodies to influenza virus assayed by the viral hemagglutination–inhibition test (24). It is also pertinent to mention that shark sera contain "natural" hemagglutinins for sheep and chicken erythrocytes and these have been found exclusively in the heavy, 2-ME–sensitive fractions.

Purification of Lemon Shark Serum Proteins.—In an initial attempt to purify immunoglobulins in amounts suitable for physicochemical studies, lemon shark

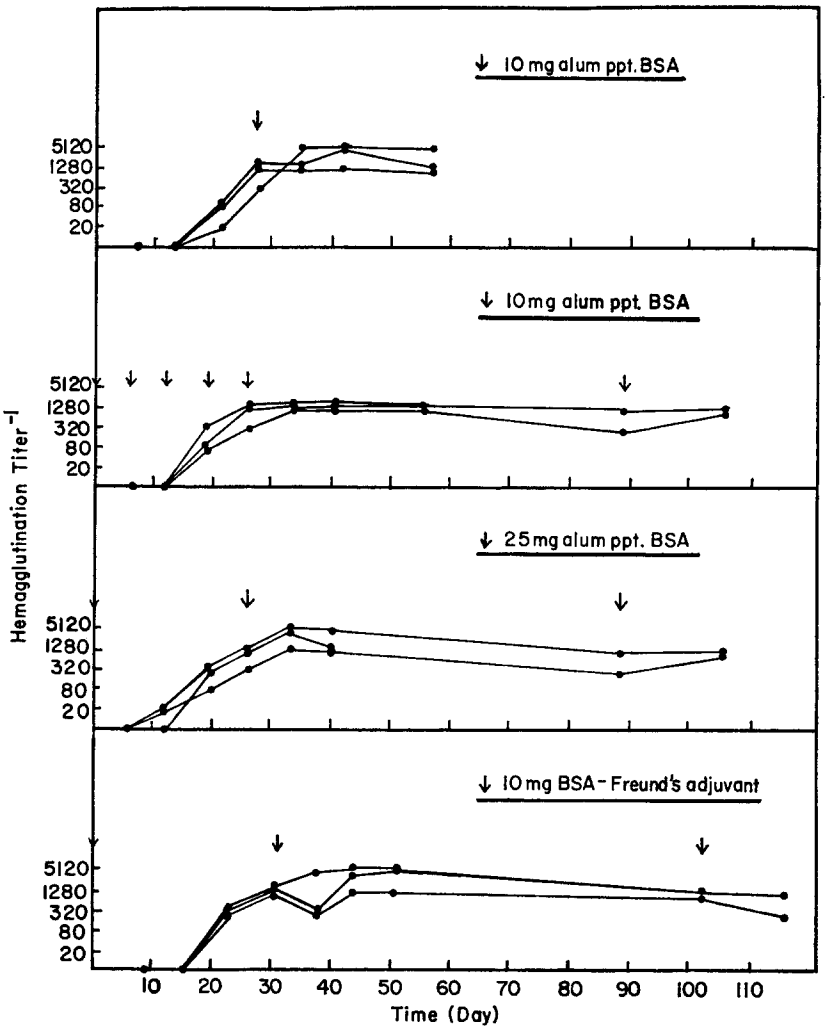

FIG. 1. Hemagglutination antibody response of lemon sharks to bovine serum albumin. Each line represents an individual animal; the points represent individual determinations.

anti-BSA sera were subjected to gel filtration on Sephadex G-200. An "early" (25–35 days) serum pool was found to contain only 2-ME–sensitive HA activity in the column exclusion volume (Fig. 2). A "late" (90–110 days) pool contained a mixture of 2-ME–sensitive macroglobulin and 2-ME–resistant activity associated with smaller molecules (Fig. 2). These column "profiles" showed relatively high amounts of macroglobulins (25–30% of the recovered optical density was in the column exclusion volume) and low amounts of smaller

molecules. Although this procedure readily separated the two molecular species of lemon shark antibody, Ouchterlony analysis (not shown) using rabbit antisera to lemon shark serum indicated each antibody-containing fraction to consist of multiple antigens, and immunoelectrophoresis (not shown) indicated considerable mobility differences between the proteins within each fraction; therefore, ion exchange chromatography was used as an initial purification step.

The previously mentioned "early" and "late" lemon shark anti-BSA serum pools were each subjected to DEAE-cellulose chromatography. The elution

TABLE I

Passive Hemagglutination Titers of 2-Mercaptoethanol Treated and Sucrose Gradient Ultracentrifuged Lemon Shark Antisera to Bovine Serum

Shark No.	Day of bleeding	Hemagglutination titer of whole serum		Hemagglutination titer of fractionated serum	
		Untreated	2-ME-treated	Heavy	Light
22	27	1:320	1:20	1:32	<1:4
	41	1:2560	1:80	1:256	1:16
	56	1:1280	1:320	1:256	1:64
25	26	1:1280	<1:20	1:256	<1:4
	40	1:1280	1:80	1:512	1:64
	105	1:1280	1:640	1:512	1:256
31	31	1:640	<1:20	1:64	<1:4
	51	1:2560	1:160	1:128	1:32
	116	1:2560	1:640	1:128	1:64
	116*	—	—	<1:8	1:64
	116‡	—	—	<1:4	1:64

* Separated fractions were 2-ME-treated after centrifugation.

‡ Whole serum was 2-ME-treated prior to centrifugation.

profile of this early pool, containing only macroglobulin HA activity, showed the antibody activity to be associated with the first major protein fraction to elute after the gradient had started (Fig. 3a). Both the 2-ME-sensitive (macroglobulin) and 2-ME-resistant (lighter) antibody activities present in the late serum pool were found in this first major fraction after the start of the gradient (Fig. 3b). This large fraction accounted for about 50% of the recovered optical density and contained about 10–12 mg protein/ml serum using $\epsilon(^{1\%}_{280m\mu}) = 13.7$ (see below). Only one precipitin line was seen by Ouchterlony analysis with the rabbit anti-serum to whole shark serum (see below), and thus this fraction will hereafter be referred to as the immunoglobulin mixture. The naturally occurring macroglobulin hemagglutinins for sheep erythrocytes were found to elute in a comparable fraction when normal serum was chromatographed.

The antibody-containing immunoglobulin mixtures obtained from the DEAE-cellulose columns of the early and late serum pools were each concentrated and individually subjected to gel filtration on Sephadex G-200. This

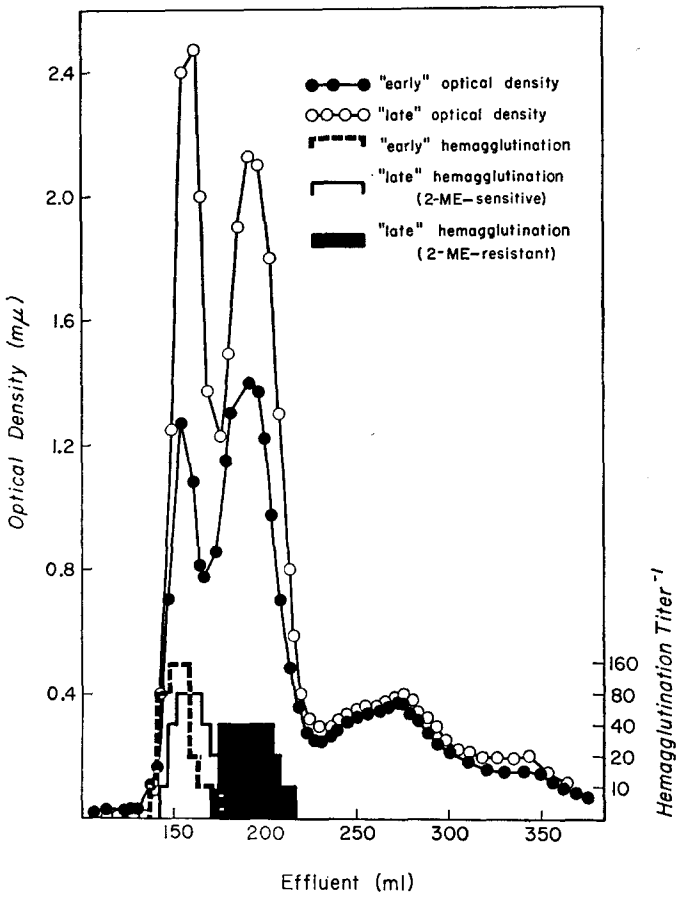

FIG. 2. Sephadex G-200 gel filtration of an "early" and a "late" lemon shark anti-BSA serum pool including the effect of reduction with 2-mercaptoethanol (2-ME) on the separated fractions.

procedure further resolved each of these preparations into fractions of differing molecular weights. The heavier fraction contained the 2-ME-sensitive HA activity and the second fraction (from the late pool) contained the 2-ME-resistant activity as expected from the above gel filtration experiments with whole serum. The ratio of the heavier to the lighter component was between 1:1.6 to 1:1.9 for the various immunoglobulin preparations studied. There was

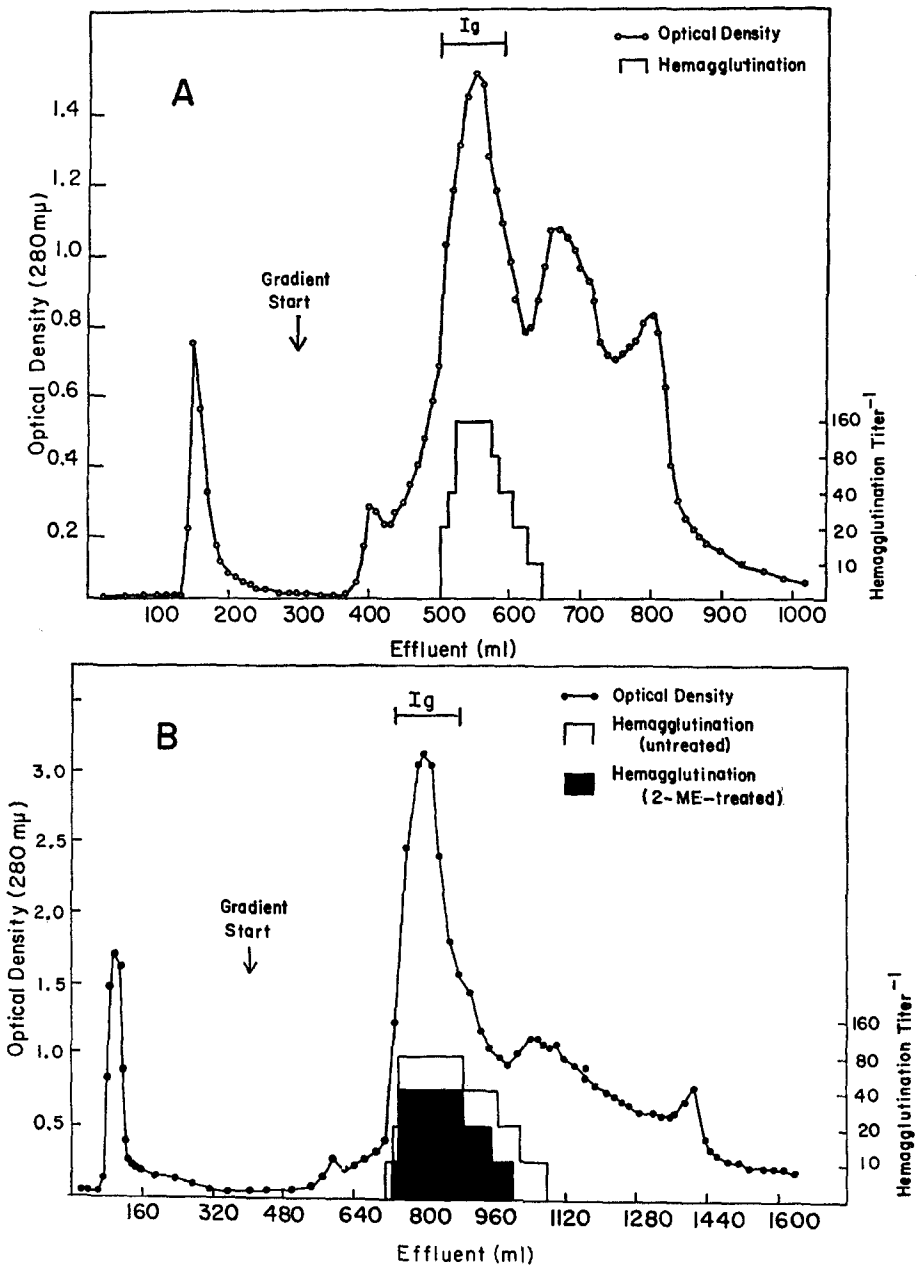

FIG. 3. Ion exchange chromatography on DEAE-cellulose of (A) "early" and (B) "late" lemon shark anti-BSA serum showing hemagglutinating activity.

no apparent difference in this ratio between the four normal sera and the three immune sera examined.

An alternative method for the purification of these two immunoglobulin fractions from whole serum involved an initial fractionation of the late pool by sucrose density gradient ultracentrifugation in the zonal centrifuge (kindly performed by Dr. William Fisher, Oak Ridge National Laboratory, Oak Ridge, Tenn.). The heavy and light components were then further purified by DEAE-

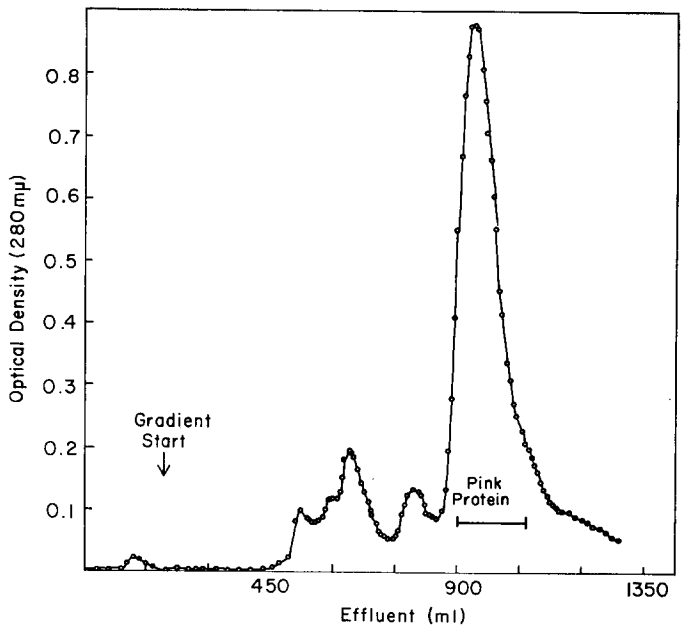

FIG. 4. Ion exchange chromatography on carboxymethyl cellulose of the pink mixture obtained by DEAE-cellulose chromatography of lemon shark serum.

cellulose chromatography. Stepwise elution indicated the antibody activity in each of these fractions to elute between 0.08 and 0.15 M NaCl (0.015 M Tris, pH 8.0).

The conditions for the DEAE-cellulose chromatography employed would be expected to yield relatively pure rabbit gamma G immunoglobulin in the void column fraction. Therefore it was significant that no shark antibody was present in this fraction although it contained at least two proteins as determined by Ouchterlony analysis and was readily observed to be pink when concentrated to 5–10 mg/ml. Attempted fractionation of this pink mixture by gel filtration on Sephadex G-200 yielded late eluting material consisting of one major peak with a skewed leading edge. Fractionation of another preparation of CM-cellulose

yielded multiple peaks (Fig. 4) with the major component containing the pink color. This protein (hereinafter called the pink protein as opposed to pink mixture) showed an extended, cathodic moving, immunoelectrophoretic precipitin

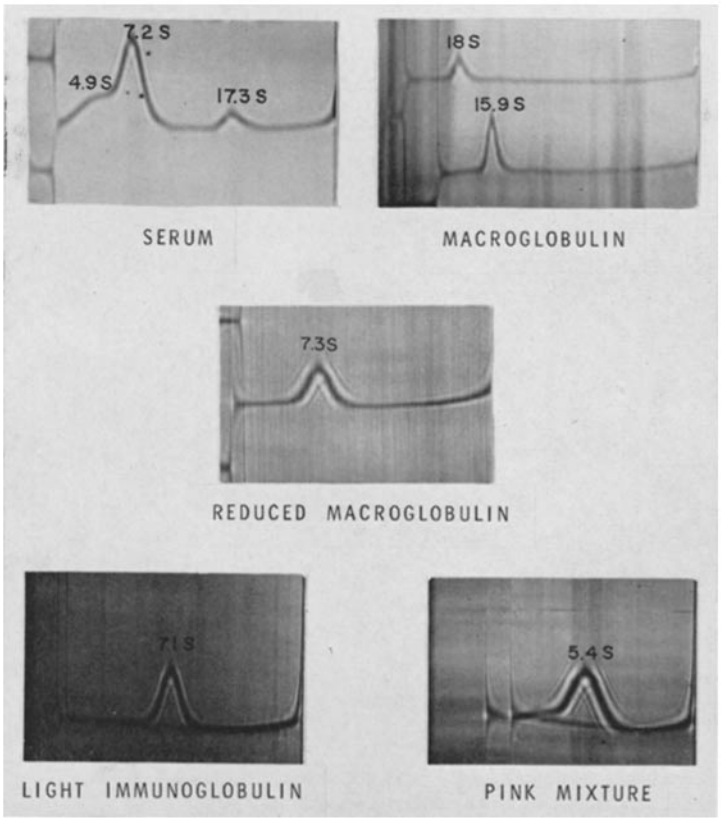

FIG. 5. Schlieren patterns of whole serum and of purified proteins from the lemon shark. $s_{20,w}$ values are as indicated. Solvent for the upper two patterns was 0.14 M NaCl, 0.01 M Tris-HCl, pH 7.4, and for the lower patterns the Tris was replaced by phosphate of the same concentration and pH. Sample concentrations were: serum diluted to 8 mg/ml (assuming ϵ 280 mμ $^{1cm}_{1\%}$ = 14); macroglobulin (upper) 1.0 mg/ml and (lower) 2.5 mg/ml; light immunogloublin and pink mixture 4 mg/ml; reduced macroglobulin 6 mg/ml. The $s_{20,w}$ extrapolated from these two concentrations of the macroglobulin was 19.2.

arc reminiscent of gamma G immunoglobulin. However, as will be shown below, pink protein does not appear to be an immunoglobulin.

Characterization of Lemon Shark Immunoglobulins.—The two lemon shark immunoglobulins, obtained by DEAE-cellulose chromatography followed by Sephadex G-200 gel filtration, were subjected to sedimentation velocity examination in the analytical ultracentrifuge (Fig. 5). The macroglobulin fraction

contained predominantly homogeneous material with a sedimentation coefficient ($S^{\circ}_{20,w}$.) of about 19S. The lighter immunoglobulin was similarly relatively pure and had an observed sedimentation of about 7. These two fractions will therefore be referred to as the 19S and 7S immunoglobulins, respectively. Molecular species with comparable sedimentation characteristics are evident in whole serum as shown here and the slight differences presumably relate to the concentration dependence of the observed sedimentation values. A shoulder on the 7S peak in whole serum is at least in part accounted for by the pink protein which is the major component of the pink mixture and was found to have a sedimentation coefficient of about 5S. Partial reduction of the 19S immunoglobulin resulted in it dissociating into 7S material.

TABLE II

Characteristics of Lemon Shark Serum Proteins

Proteins	Solvent	Extinction coefficient	Partial specific volume	Hexose content
		$\epsilon_{280\ m\mu}^{1cm\ 1\%}$ ± SD	$\bar{v}$	% ± SD
19S	0.3 M KCl	13.39 ± 0.67	0.709	3.67 ± 0.19
	0.1 N NaOH	13.75 ± 0.03	—	
	5 M guanidine	12.79 ± 0.01	—	
7S	0.3 M KCl	13.85 ± 0.23	0.707	3.51 ± 0.11
	0.1 N NaOH	14.04 ± 0.26	—	
	5 M guanidine	12.82 ± 0.18	—	
Pink protein	0.3 M KCl	12.39 ± 0.12	0.716	—

The extinction coefficients in different solvents and partial specific volumes of the 19S and 7S immunoglobulins as well as the pink protein are given in Table II. Hexose content of these immunoglobulins are also given. The similarity between the 19S and 7S proteins is evident.

The molecular weights of the three shark serum proteins are presented in Table III. In light of the above similarities of the 19S and 7S proteins, the partial specific volumes ($\bar{v}$) in saline were averaged and a value of 0.708 used for the molecular weight calculations. Since in saline there was no obvious concentration of rpm dependence of the molecular weights the different values were averaged. The 19S weight average determinations represent a possible exception which could represent some high molecular weight contamination (see Reference 13 for discussion). However, the linearity of plots of the logarithm (Ln) of the concentration of material as a function of the radius squared (Fig. 6) indicated that heterogeneity was minimal. Thus, the molecular weight of the 19S immunoglobulin is 800,000–900,000 and that of the 7S immuno-

globulin is approximately 160,000. The pink protein mixture was homogeneous and had a molecular weight of ~75,000.

Antigenic analysis of the lemon shark immunoglobulins, using rabbit antisera to whole shark serum, indicated the 19S and 7S proteins to be identical

TABLE III

*Molecular Weights × 10^{-3} of Lemon Shark Proteins**

	rpm	Initial concentration			Average ± SD
		0.25	0.50	0.75	
		mg/ml	*mg/ml*	*mg/ml*	
19S					
Weight Average					
	6,166	920	910	900	869 ± 61
	8,225	775	840		
Z Average					
	6,166	920	990	990	983 ± 64
	8,225	930	1085		
7S					
Weight Average					
	13,410	148	156	160	161 ± 9
	16,200	172	165	150	
Z Average					
	13,410	168	184	187	187 ± 12
	16,200	206	188	182	
Pink Protein					
Weight Average					
	16,200	78	73	77	75 ± 3
	17,980		71	78	
Z Average					
	16,200	74	72	83	75 ± 3
	17,980		73	73	

* Solvent was 0.14 M saline, 0.01 M phosphate, pH 7.4.

(Fig. 7). No evidence for cross reactivity with the pink 5S protein was observed in this or other experiments. Also depicted here is the single precipitin line obtained with the immunoglobulin mixture (containing both 19S and 7S proteins) thus further substantiating the antigenic similarity of these two proteins. Rabbit antisera to both the 19S and 7S immunoglobulins were prepared and likewise showed these two components to be antigenically identical. These antisera did not react with the pink 5S protein nor with any of the other concentrated proteins of the pink mixture obtained by the CM-cellulose chromatograph

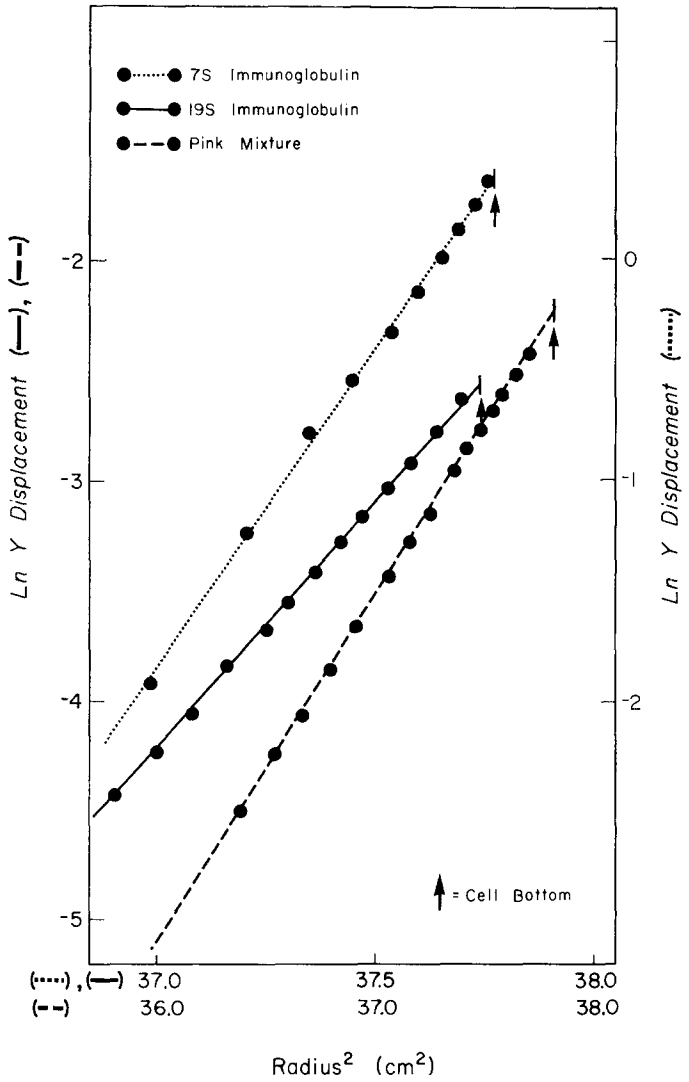

FIG. 6. Plots of the logarithm of the concentration (in vertical displacement from the baseline of a fringe in cm) versus distance from the center of rotation squared for lemon shark 19S and 7S immunoglobulins and the pink mixture. The latter material was obtained from the peak tube of the Sephadex G-200 chromatograph shown in Fig. 8. All three determinations were obtained at protein concentrations of 0.25 mg/ml in 0.14 M NaCl, 0.01 M PO_4, pH 7.4. Centrifugation was performed at 20°C for 48 hr; a speed of 16,200 rpm was used for both the 7S immunoglobulin and the pink mixture while 6,166 rpm was used for the 19S immunoglobulin.

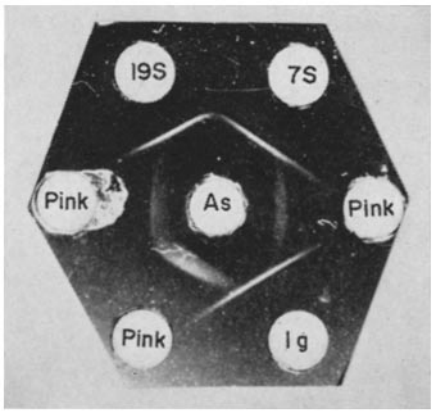

FIG. 7. Ouchterlony analysis of purified lemon shark serum proteins. *As*, rabbit antiserum to whole lemon shark serum; *19S* and *7S*, approximately 2.5 mg/ml; *Pink* (mixture), approximately 3 mg/ml; *Ig*, immunoglobulin fraction of DEAE-cellulose (contains both 19S and 7S proteins), approximately 3 mg/ml.

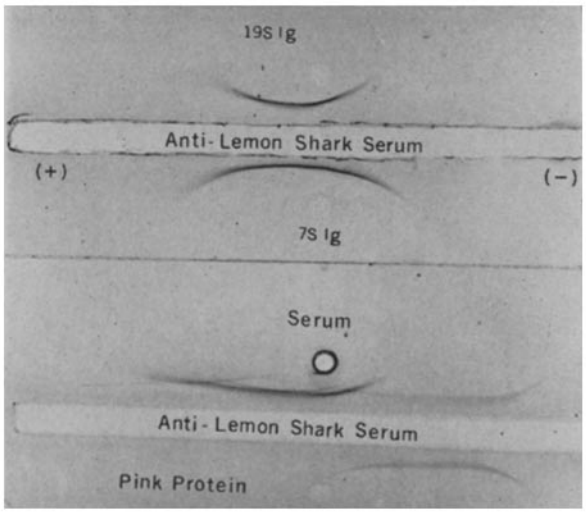

FIG. 8. Immunoelectrophoretic analysis of lemon shark serum and purified proteins. Concentration of purified proteins is about 2.5 mg/ml and the lemon shark and rabbit anti-lemon shark sera were undiluted.

previously shown in Fig. 4. Immunoelectrophoresis showed the 19S and 7S immunoglobulins to have comparable mobilities when tested using rabbit antiserum to whole shark serum (Fig. 8); similar results were obtained with rabbit antisera to the purified proteins. Also depicted in Fig. 8 are the patterns of whole lemon shark serum and of the pink 5S protein showing the extended cathodic

arc reminiscent of that of the gamma G immunoglobulin of many higher animals.

Although the above antigenic analysis indicated that the 19S and 7S proteins were identical, the possibility remained that the antibody activity present in these fractions may not be associated with the antigen being detected by the immunodiffusion tests. Therefore several experiments were performed to demonstrate that the antibody activity was associated with the shark antigen(s) precipitated by the rabbit antisera. Table IV presents the results of one such experiment and shows that antibody activity in the 19S fraction was precipitated by rabbit antisera to both 19S and 7S proteins. Similarly 7S antibody was precipitated by both antisera.

Fractionation and Characterization of Polypeptide Chains from Lemon Shark

TABLE IV

Precipitation of Lemon Shark 19S and 7S Anti-BSA Antibody Activity with Rabbit Antisera to the 19S and 7S Proteins

Shark antibody	Rabbit antiserum added	Reciprocal of hemagglutination titer
19S	Normal	64
"	Anti-19S	<4
"	Anti- 7S	<4
7S	Normal	32
"	Anti-19S	<4
"	Anti- 7S	<4

Shark proteins were precipitated at 1 mg/ml.

Immunoglobulins.—Extensive reduction and alkylation in 7M guanidine–HCl of the 19S and 7S lemon shark immunoglobulins followed by gel filtration on a Sephadex G-200 column equilibrated with 5 M guanidine–HCl (13) yielded heavy (H) and light (L) polypeptide chains eluting as single well separated peaks. The quantitative aspects of these experiments are summarized in Table V. The yield of H chains from each immunoglobulin was approximately 72% of the recovered optical density. Gel filtration in 5 M guanidine–HCl of unreduced immunoglobulins showed no evidence of dissociation into smaller units. These later experiments were performed with alkylated protein to prevent disulfide exchange (25).

The extinction coefficients at 280 mμ for a 1 cm path length and at 1% protein concentration in 5 M guanidine of the 7S immunoglobulin H and L chains were found to be 11.74 $\pm$ 0.05 and 13.1 $\pm$ 0.7 respectively. Due to the lack of material the moisture content of the L chain preparation was assumed to be the same as that of the H chains (i.e., 3.3%) for the calculation of the extinction

coefficient. Using these extinction coefficients, and the fact that the H chains make up approximately 72% of the optical density of the molecule, it can be calculated that the H chains make up 74% of the mass of the 7S molecule with the L chains making up the remaining 26%. This is probably also true for the 19S immunoglobulin since, as will be shown below, the H chains or L chains from these two immunoglobulins are indistinguishable.

It is possible to calculate the molecular weight of the chains from their elution volumes off the guanidine–HCl equilibrated Sephadex column. Since all the materials were extensively reduced, alkylated, and fractioned in the presence

TABLE V

Summary of Gel Filtration Experiments with Extensively Reduced and Alkylated Lemon Shark Immunoglobulins

Material applied	Amount	Recovery	K_D* of H	K_D* of L	$\frac{\text{H}‡}{\text{H \& L}}$
	mg	%			
19S	13.1	110	0.194	0.456	0.72
19S	12.3	110	0.182	0.453	0.73
			0.188	0.454	
7S	48.0	94	0.200	0.462	0.71
7S	4.7	81	0.197	0.456	0.68§
			0.198	0.459	

* $K_D = \frac{V_E - V_0}{V_i}$ where V_E is the peak elution volume, V_0 excluded volume (130 ml for this column) and V_i the included volume. $V_0 + V_i$ = the elution volume for small molecules (~470 ml for this column). (See text and Reference 25.)

‡ Based on optical density at 280 mμ.

§ Less reliable figure due to small amount of material.

of guanidine–HCl, their configurations should approach those of random coils. Thus, when shape factors are minimized, the elution position relative to known markers should be a good estimate of mass. Following the suggestion of Andrews (26) a plot of $\sqrt{K_D}$ vs $\sqrt{\text{mol wt}}$ with known markers (Fig. 9) shows the shark L chains (both 19S and 7S) to be indistinguishable from those of the rabbit, thereby suggesting their molecular weight to be 22,000–23,000 (13, 27). The H chains from both the 19S and 7S shark immunoglobulins eluted in a volume corresponding to a molecular weight of 71,000 ± 3000.

Fingerprints of tryptic digests of both 19S and 7S H chains were compared and found to be quite similar (Fig. 10). The L chain fingerprints from each immunoglobulin were similar to each other but distinct from those of the H chains. Unfortunately an insufficient amount of 7S light chains was used to obtain the

fingerprint shown here (Fig. 10) but no differences were observed on the original maps. A repeat of this 7S L chain fingerprint is shown in Fig. 14, and the pattern is very similar to that of the 19S L chain fingerprint, except for variations attributable to chromatography and probably resulting from lack of adequate temperature control.

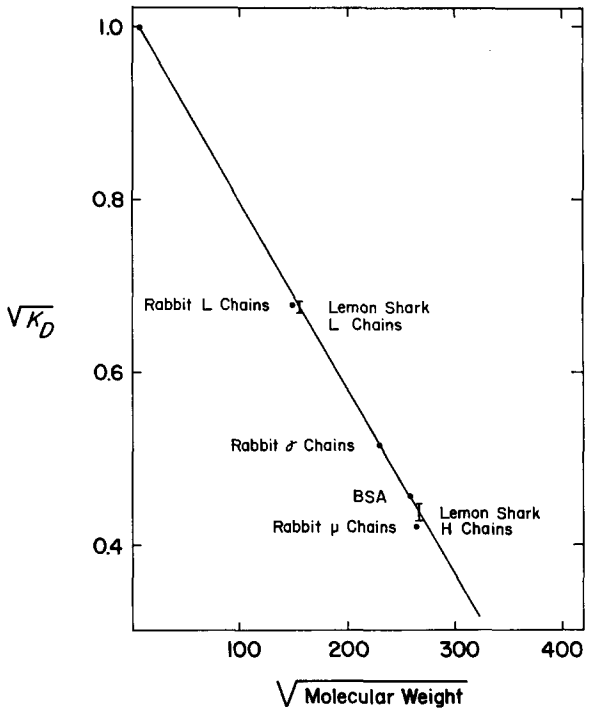

FIG. 9. Plot of $\sqrt{K_D}$ (see footnote to Table V) vs. $\sqrt{\text{mol}}$ wt for extensively reduced and alkylated materials filtered through a Sephadex G-200 column equilibrated with 5 M guanidine–HCl. Molecular weights of the marker materials used in these experiments were: rabbit light chains 22,500 (13), rabbit gamma chains 53,000 (13), BSA 67,000 (28), rabbit μ chains 70,000 (27). The vertical bars represent standard deviations of the mean K_D's given in Table V.

Disc electrophoretic patterns of the 19S and 7S H chains were similar and exhibited primarily a broad, relatively fast moving band. The disc patterns of the L chains, while similar to each other, were quite different from those of the H chains. The shark L chains showed considerable evidence of heterogeneity by exhibiting multiple discrete bands as illustrated for the 19S L chain in the right hand portion of Fig. 15.

In order to obtain polypeptide chains with the configuration, and resultant antigenic properties, comparable to that existing in the native molecules,

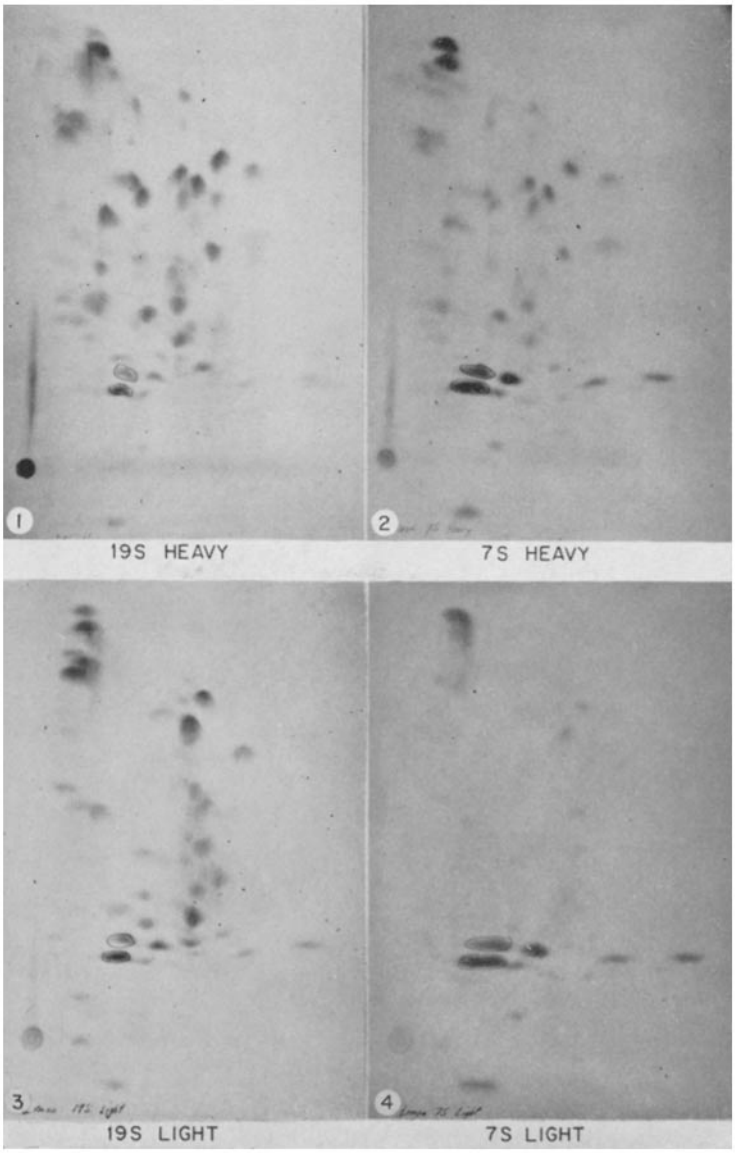

FIG. 10. Fingerprints of lemon shark immunoglobulin heavy and light chains. Sample was applied to the lower left-hand corner. Non-blue spots were circled. Descending chromatography was from left to right: the anode is to the bottom.

partial reduction and alkylation in the absence of denaturing agents of the lemon shark immunoglobulins was performed (16). The partially reduced materials were fractionated by gel filtration on a Sephadex G-100 column equilibrated with 1 M propionic acid and each gave three discernible fractions (Fig.

11). The largest of these (fraction I) was present in the column exclusion volume. Fraction III consisted of about 22–25% of the recovered optical density and was antigenically dissimilar to the material fraction I and II; these latter two were antigenically indistinguishable (discussed below). Thus to conform with the generally accepted terminology for mammalian immunoglobulins, fraction III will be referred to as L chains and fraction II as H chains. Fraction I most probably represents dimers of H chains and perhaps some H-L chain aggregates; L chains could be detected in some of the fraction I preparations examined (see below). After removal of the propionic acid by dialysis against successive changes of distilled water, saline, and buffered saline (pH 7.2) the

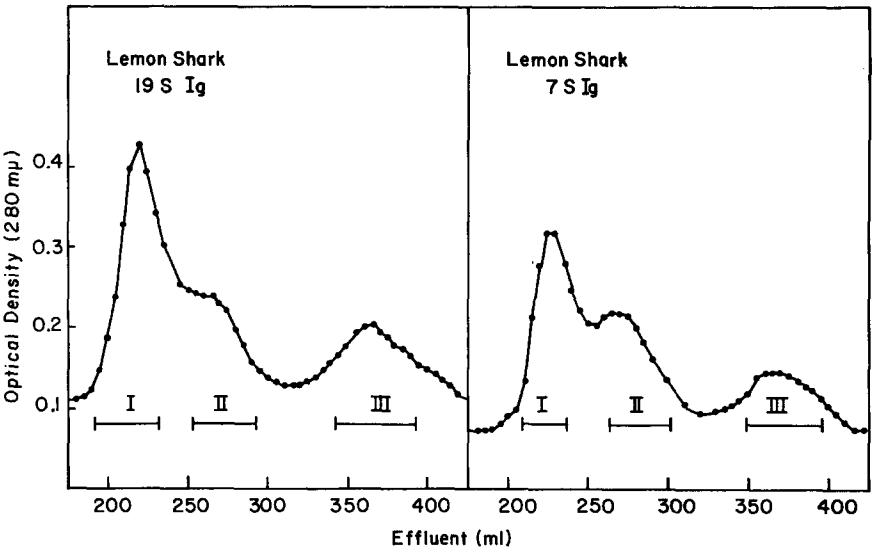

FIG. 11. Sephadex G-100 gel filtration in 1 M propionic acid of partially reduced and alkylated lemon shark immunoglobulins.

fraction I and II material remained essentially totally soluble whereas fraction III exhibited considerable but not complete precipitation.

Rabbit antisera were prepared against the 7S immunoglobulin H (pool of fractions I and II) and L (fraction III) chains obtained by partial reduction. The results of antigenic analysis with these antisera are shown in Fig. 12. Rabbit antiserum to the 7S H chains showed reactions of identity with the 7S and 19S H chains as well as with the intact molecules but did not react with either of the L chains. Rabbit antiserum to the 7S L chains showed reactions of identity between the 19S and 7S L chains and the intact 19S immunoglobulin to be similar and did not react with 19S or 7S H chains. In experiments not shown here this anti-L chain serum indicated that fraction I from both the 19S and 7S proteins contained traces of L chains. The amount of L chains in

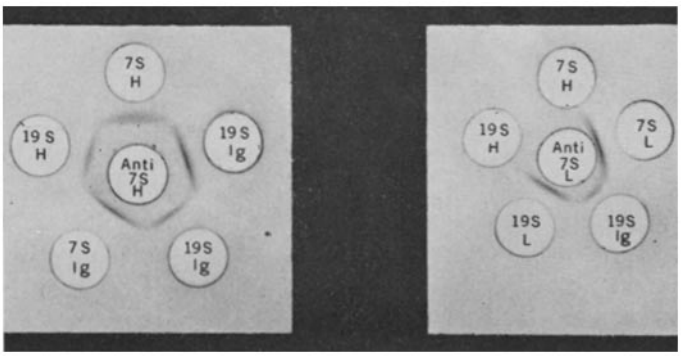

FIG. 12. Ouchterlony analysis of lemon shark heavy and light polypeptide chains and intact immunoglobulins with rabbit antisera. Protein concentrations were between 1.5 and 2 mg/ml. Antisera were used undiluted.

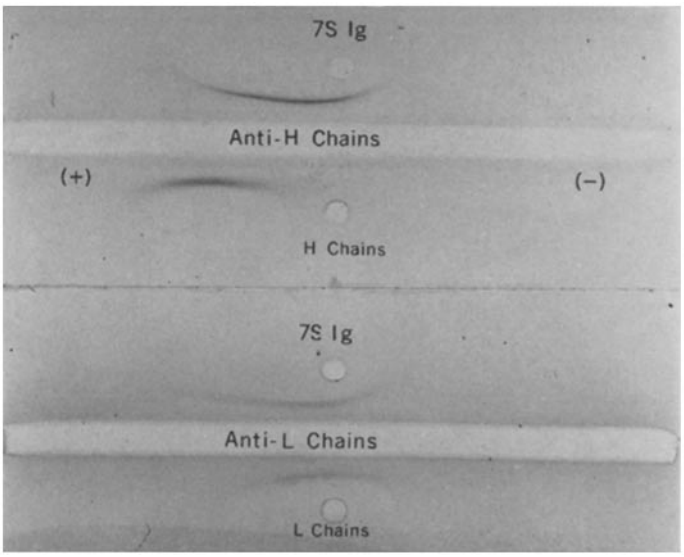

FIG. 13. Immunoelectrophoretic analysis of lemon shark heavy and light polypeptide chains. Protein concentrations were approximately 2 mg/ml and antisera were used undiluted.

the H chain preparation used to immunize the rabbits apparently was not sufficient to be immunogenic. However, when obtained after prolonged immunization, such H chain anti-sera reacted with L chains. Immunoelectrophoresis (Fig. 13) showed the 7S L chains to remain at the origin whereas the 7S H chains are relatively fast moving and as such can be readily distinguished from the intact immunoglobulin which has a slight anodic mobility. Similar results were obtained with the 19S H and L chains (not shown).

Demonstration of the Lack of Chemical or Immunological Relationship between the Pink Mixture and the Immunoglobulins of the Lemon Shark.—Although the pink 5S mixture contained no detectable antibody activity it did contain proteins with immunoelectrophoretic mobilities comparable to gamma G immunoglobulin and could conceivably contain a "half molecule" of immunoglobulin. The major component of this mixture was the pink protein which after extensive reduction and alkylation still eluted from the 5 M guanidine Sephadex

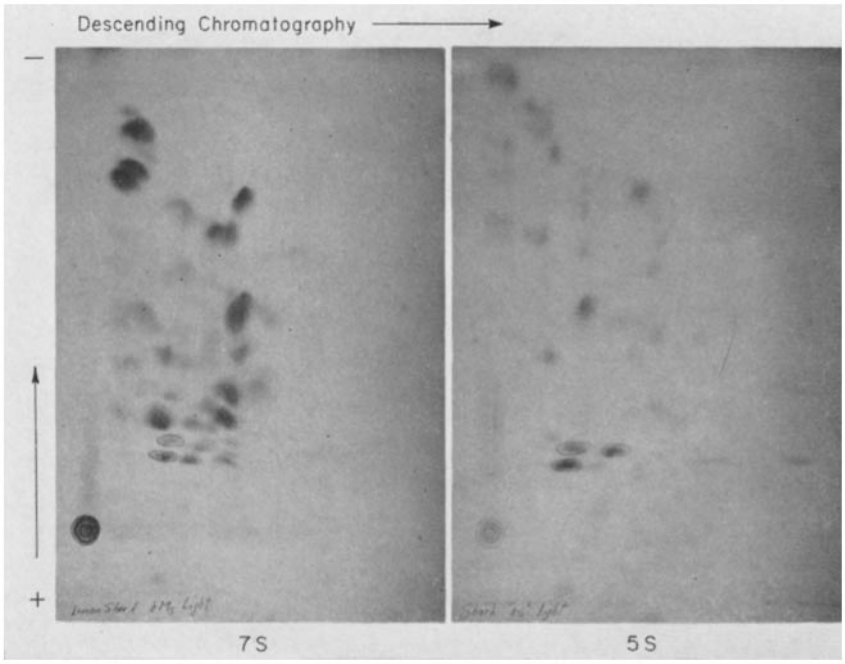

FIG. 14. A comparison of the fingerprints of lemon shark 7S immunoglobulin L chains and the small chains derived from the pink mixture.

column as a single component in a volume indicating a molecular weight of 70,000–75,000. The pink protein therefore could not have contained light chains. When the pink mixture was subject to similar treatment 86% of the recovered ultraviolet absorption eluted as a single peak in a volume similar to that of the pink protein, while the remaining 14% eluted as a discrete peak in a volume expected to contain immunoglobulin light chains. Experiments were therefore initiated in order to investigate the relationship of these later chains to immunoglobulin L chains. Fig. 14 shows a comparison of the fingerprints of these chains. These fingerprints reveal major differences between the 7S L chains and the chains derived from the pink mixture; the three or four lower spots that are common to both fingerprints are seen on essentially all our fingerprints and

probably represent free amino acids. Disc electrophoretic patterns, shown in Fig. 15, demonstrate heterogeneity in both preparations but there is little if any correspondence of the bands in terms of either position or intensity of staining. The pattern of the material obtained from the pink mixture lacks the regularity seen with immunoglobulin L chains.

Perhaps the most convincing evidence for the lack of any relationship between the proteins of the pink mixture and the lemon shark immunoglobulins was obtained by immunologic analysis. Antisera to 19S or 7S intact immunoglobulins or to the 7S H or L chains gave no reactions with the pink protein or the concentrated fractions of the pink mixture eluted from the CMC column represented in Fig. 4. Antisera to whole lemon shark sera (see Fig. 7) also showed

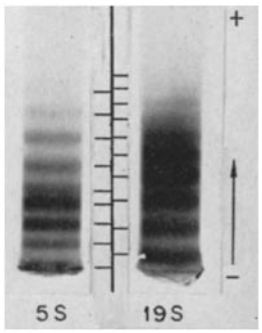

FIG. 15. Disc electrophoretic patterns run simultaneously of lemon shark 19S immunoglobulin L chains and the small chains derived from the pink mixture. The solid bars represent the position of bands readily visible on the original stained gels.

no evidence of any relationship between the pink mixture and the immunoglobulins.

DISCUSSION

The main ideas to be developed in this discussion are (*a*) that the lemon shark has but one class of immunoglobulin, (*b*) that this shark immunoglobulin class is comparable to γM of mammals in terms of molecular architecture and electrophoretic heterogeneity, and (*c*) that studies on the lemon shark immunoglobulins raise questions regarding functional aspects of mammalian γM and the evolution of immunoglobulins in general.

The lemon shark appears to have fewer classes of immunoglobulins than mammals. Mammalian immunoglobulins are principally differentiated by the heavy chain present in the molecule (2). Since the heavy chains of the lemon shark 19S and 7S immunoglobulins showed similar peptide maps, disc patterns, molecular weights, and antigenic properties, these two proteins were felt to be

of the same class. These findings are consistant with those obtained for the dogfish shark (29) and more recently have been extended to include the nurse shark.[1] Since different immunoglobulin classes of a given mammalian species have similar light chains, the failure to detect other proteins in lemon shark sera reacting with anti-light chain sera indicates the probable absence of γG and γA-like immunoglobulins in this species of shark. The lemon shark protein with γG-like electrophoretic mobility referred to as the pink protein and also present in dogfish sera (29), contained no detectable antibody activity and structurally was a single polypeptide chain which was unrelated antigenically to the shark immunoglobulins. The comparable protein in nurse shark sera has been shown to bind iron and thus behaves like transferrin.[1] Therefore the pink protein from other elasmobranchs is probably also transferrin.

The lemon shark immunoglobulins appear to belong to a class comparable to γM as defined for mammals. The lemon shark 19S and 7S immunoglobulins each have a relatively high hexose content as does γM. The 19S protein has a molecular weight (800,000–900,000) and a molecular structure similar to mammalian γM. Specifically the lemon shark 7S and 19S immunoglobulins contain equimolar heavy and light chains based on column yields from extensively reduced material. The heavy chain molecular weight was $\sim$70,000 and the light chain molecular weight was $\sim$22,000 and therefore molecules composed of two heavy and two light chains would have a molecular weight of $\sim$180,000 which is in fair agreement with the observed molecular weight $\sim$160,000 for the naturally occurring 7S immunoglobulin. The lemon shark 19S immunoglobulin is composed of 7S subunits and since these subunits seem to be similar in mass to the naturally occurring 7S molecules, the value of 160,000–180,000 for the molecular weight of the latter suggests the 19S molecule to be a pentamer of 7S subunits. This proposed structure of lemon shark γM is thus identical to that of rabbit (27) and man (30).

One of the most unique aspects of immunoglobulins in general is the electrophoretic heterogeneity of their chains. In this regard it is interesting to note that the lemon shark light chains are apparently as heterogeneous as their mammalian counterparts. This finding suggests the presence of common and distinctive regions as in mammalian light chains (31–33).

Perhaps the most perplexing aspect of this investigation was the apparent polyvalency of the lemon shark 7S γM antibody contrasted with the behavior of the 7S subunits obtained by reduction of the 19S molecule. That is to say that the naturally synthesized 7S antibody-agglutinated antigen coated erythrocytes and that this activity was resistant to reduction, whereas the 19S antibody lost this reactivity upon reduction. This phenomena may be explained

[1] DeBoutaud, F., L. W. Clem, and M. M. Sigel. Phylogeny of immunoglobulin structure and function. II. The transferrin-like properties of shark "gamma globulin." In preparation.

by a relative inefficiency of 7S molecules in agglutination reactions as is the case for mammalian γG (34–36), or by the presence of only one binding site on the reductive subunit as suggested for rabbit γM 7S subunits (37). If this latter possibility is correct the structural difference between the naturally synthesized and the reductive subunits remains to be elucidated. The existance of polyvalent (hemagglutinating) 7S γM antibody has recently been demonstrated for the nurse shark[1] but was not reported in the dogfish (29). This negative observation on the dogfish shark may have been due to the short immunization period employed. However, it is also possible that this represents a species difference since limited observations with the margate, a marine teleost, indicate the presence of 7S γM antibody that is apparently incapable of agglutination reactions.[2] It should also be mentioned that 7S antibodies of probable polyvalent nature have been reported in other lower vertebrates (38, 39), but no immunoglobulin class analyses have as yet been reported for these species.

Phylogenetically, the studies on the lemon shark are consistent with the hypothesis of Marchalonis and Edelman (29), that the γM-class preceded γA and γG and that therefore μ-chains appeared before α- or- γchains. It is not known however whether the 7S γM- or the 19S γM-class came first. Further studies on the presence of a 9S immunoglobulin in the more primitive lamprey (40) may help to clarify this point. The finding of 7S γM protein in sera from patients with systemic lupus erythematosus (41) and ataxia telangiectasia (42) suggests that this form of γM may be more prevalent than one might expect.

In light of the suggestion that γG and not γM is involved in the anamnestic response of mammals (43–45), the finding of only γM immunoglobulin in sharks raises questions regarding the presence or absence of immunologic memory in such ancient vertebrates. Thus, the failure to find evidence of immunoglobulin memory in the studies reported here for the lemon shark may perhaps be attributable to an absence of γG. This generalization would not appear, however, to apply to all lower vertebrates, since certain teleosteans and holosteans lacking in γG immunoglobulin do show anamnestic responses of 19S antibody under certain conditions of antigenic stimulation (46).[3] Similarly the horned shark (also probably lacking in γG) has been demonstrated to show secondary responses to certain antigens (5). Further studies are thus required prior to making meaningful correlations between the presence or absence of specialized functions, such as memory, and the presence or absence of different immunoglobulin classes in lower vertebrates.

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[2] Clem, L. W. Phylogeny of immunoglobulin structure and function. III. Immunoglobulins of marine teleosts. In preparation.

[3] Bradshaw, C., and L. W. Clem. Immune responses in the Florida garfish. Unpublished observations.

SUMMARY

Lemon sharks immunized with bovine serum albumin produced two molecular forms of antibodies detectable by passive hemagglutination of antigen-coated, tanned sheep erythrocytes. Throughout the course of immunization 2-ME–sensitive antibody was associated with a 19S immunoglobulin fraction (4–5 mg/ml serum) while late in the course of immunization antibody was found also associated with a 7S immunoglobulin fraction (7–8 mg/ml serum). No evidence for any anamnestic response was found in these animals. Naturally occurring hemagglutinins for sheep erythrocytes were found to be 2-ME–sensitive and present in the 19S immunoglobulin fraction.

These immunoglobulin fractions were readily purified by DEAE-cellulose chromatography and Sephadex G-200 gel filtration. Both immunoglobulin molecules yielded equimolar amounts of H and L polypeptide chains when subjected to extensive reduction and alkylation followed by gel filtration in 5 M guanidine–HCl. Antigenically reactive H and L chains were obtained by partial reduction and alkylation followed by gel filtration in 1 M propionic acid. The 7S and 19S immunoglobulin H chains were indistinguishable by fingerprints of tryptic digests, disc electrophoretic patterns, antigenic properties, and mass (molecular weight ~70,000), thus suggesting these two molecules to belong to the same immunoglobulin class. The shark 19S and 7S immunoglobulin L chains were indistinguishable from each other by similar criteria and were different from the H chains. These L chains exhibited the electrophoretic heterogeneity of their mammalian counterparts.

The 7S (shark immunoglobulin) molecule was shown to have a molecular weight of ~160,000 and to consist of 2H and 2L polypeptide chains (total mass ≅180,000). The 19S molecule was shown to have a molecular weight of 800,000–900,000; therefore, there were probably five 7S subunits per 19S molecule, comparable to mammalian γM. Other reasons for considering the 7S and the 19S lemon shark molecules to belong to a class of immunoglobulins comparable to the γM class of mammals are that they both have high carbohydrate contents, and H chains of mass similar to μ chains.

The lemon shark serum proteins with electrophoretic mobilities comparable to gamma G of mammals were not related to the immunoglobulins of this species. These proteins had no antibody activity and had no antigenic or chemical similarity to either the H chains, the L chains, or the intact immunoglobulin molecules from the lemon shark.

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