PHYLOGENETIC ORIGINS OF ANTIBODY STRUCTURE

III. Antibodies in the Primary Immune Response of the Sea Lamprey, Petromyzon marinus*

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Recent studies of antibodies from lower vertebrates such as elasmobranchs (1–4) and amphibians (5) have established that these proteins resemble the immunoglobulins of mammals in chain structure and diversity. Although the sea lamprey, *Petromyzon marinus*, has been reported (6–8) to form antibodies in response to antigenic stimulation, relatively little is known of the chemical structure of the antibodies. The lamprey is particularly important to studies of the phylogeny of immunity because this cyclostome is the most primitive form known to have thymic and splenic tissue and a humoral antibody response (9).

In this paper, we describe the immunization of lampreys to bacteriophage f2 and the isolation and partial characterization of antibodies to the viral antigen.

Materials and Methods

Salts, Buffers, and Reagents.—All inorganic salts were reagent grade or better. Tris (hydroxymethyl) aminomethane[1] buffers were made by dissolving Trizma base (Sigma Chemical Co., St. Louis, Mo.) in water and adjusting the pH with HCl. 2-Mercaptoethanol (B.P. 153–157) was obtained from Matheson, Coleman and Bell (Norwood, Ohio). Iodoacetamide was obtained from K and K Laboratories (Plainview, N. Y.) and was recrystallized from water before use. The propionic acid was Eastman grade from Distillation Products Industries (Rochester, N. Y.). Urea was analytical reagent grade obtained from Mallinckrodt, St. Louis, Mo.

Immunization.—Three antigens, *Limulus* hemocyanin, sheep erythrocytes, and bacteriophage f2 were used. Spawning lampreys of both sexes were given a single subcutaneous injection of 5 mg hemocyanin, 10^9 red blood cells, or 0.1 mg f2 phage. In all cases the antigen was administered in 0.15 M saline, and the injection volume was 0.1–0.2 ml. The animals were maintained in outdoor tanks and spillways of running lake water at Hammond Bay Fisheries Laboratory, United States Department of the Interior, Hammond Bay, Mich. The water temperature varied from 13° to 27°C during the course of the immunization period.

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[1] Tris (hydroxymethyl) aminomethane = Tris.

Mortality was high among the spawning animals which were in a state of involution and did not feed. The experiments were begun with over 100 animals in each immunization group, but only about 25 of each group were alive at the end of the immunization period.

1 month after injection the surviving lampreys were sacrificed by cardiac exsanguination. The whole blood was allowed to clot at room temperature and the samples were refrigerated for 4 hr prior to centrifugation in a clinical centrifuge to remove the fibrin and cells. The sera were stored at 4°C during the course of the experiments.

Antigens and Assay of Antibody Activity.—Bacteriophage f2 was kindly supplied by Dr. N. Zinder. Sera and serum fractions were titrated for phage-neutralizing activity according to the procedure described by Adams (10). For purposes of comparison from species to species, the potency of the antisera is expressed in terms of the rate constant for phage inactivation:

$$K = -\frac{\ln (p/p_o)}{D\Delta T}$$

where p = number of plaques at time t; p_o = number of plaques at time $t = t_o$; D = serum dilution factor; and ΔT = time in minutes $(t - t_o)$.

When comparisons are made between lamprey antisera and antibody fractions, the dilution factor D is replaced by C, the total protein concentration expressed in milligrams per milliliter.

The neutralization constants were calculated for $\Delta T = 10$ min because inactivation followed apparent first-order kinetics during this interval. The potency of sera and serum fractions is also expressed here as per cent phage neutralized after 30 min of incubation. As shown by the kinetic studies, neutralization was essentially complete by this time. Studies on inactivation of antibodies were carried out by performing the usual titration procedure with solvents which contained 0.1 M 2-mercaptoethanol.

Sheep erythrocytes were purchased from Animal Blood Center, Syracuse, N. Y. They were washed three times with 0.15 M saline prior to use. Lamprey serum and serum fractions were tested for agglutinating activity using serial dilutions in test tubes (11) or microtitrator plates (Cooke Engineering Co., Alexandria, Va.). The standard diluent was 0.15 M saline buffered to pH 8.0 by the addition of Tris to a concentration of 0.05 M.[2] The activity in each tube or well was determined according to the settling patterns and resuspension behavior. Titers given in the results section represent the reciprocal of the dilution at which the final definite positive response was observed.

Limulus hemocyanin was prepared from hemolymph by zone electrophoresis on starch as previously reported (1). Qualitative analyses for precipitins were carried out using test tubes as well as microcapillary tubes according to the procedure described by Kabat and Mayer (11). Titrations were performed by means of a previously described modification (12) of the passive hemagglutination technique (13).

Isolation of Antibodies.—Lamprey antibodies were isolated from serum by the sequential application of zone electrophoresis on starch (14), column chromatography on diethylaminoethylcellulose[3] (15), and gel filtration on Sephadex G-200 (Pharmacia, Uppsala, Sweden). The electrophoretic fraction which contained antibody activity was further fractionated by chromatography on 30 × 1 cm columns of DEAE-cellulose (Schleicher and Schuell, Keene, N. H., 0.71 meq/g) equilibrated with 0.02 M sodium phosphate buffer, pH 7.2. The samples contained approximately 100 mg protein and were dialyzed against this buffer prior to loading on the column. Linear gradient elution was accomplished by running 0.2 M sodium phos-

[2] 0.15 M NaCl, 0.05 M Tris-HCl = Tris-NaCl.

[3] Diethylaminoethylcellulose = DEAE-cellulose.

phate, pH 7.2, which was 1.0 M in sodium chloride into a mixing chamber of 200 ml volume which initially contained 0.02 M sodium phosphate buffer. The conditions for gel filtration on Sephadex G-200 were: column dimensions, 1.5 × 90 cm; buffer, Tris-NaCl; flow rate, 12 ml/hr; temperature, 20°C.

Protein concentrations were determined by the modified Folin-Ciocalteu method (16). In addition, the absorbancy at 280 mμ was measured; for comparison $E^{1\%}_{1cm}$ was assumed to be 14.

Immunoelectrophoresis and Double Diffusion in Agar.—These procedures and the immunization schedule for rabbits have been previously described (1).

Reduction and Alkylation and Preparation of Polypeptide Chains.—The details of these procedures have been previously reported (17).

Ultracentrifugation.—Analytical ultracentrifugation was performed with a Spinco model E ultracentrifuge equipped with automatic temperature control and phase plate schlieren and interference optics. Molecular weights of intact immunoglobulins and polypeptide chains were determined under previously described solvent conditions (2, 5) according to the high speed equilibrium method of Yphantis (18). Partial specific volumes ($\bar{v}$) were calculated from amino acid compositions. Ultracentrifugation in sucrose density gradients was done in a Spinco model L preparative ultracentrifuge using the SW 39 swinging bucket rotor. This procedure has been described before (19, 20).

Starch Gel Electrophoresis in Urea.—The method has been described in a previous report (17).

Amino Acid Analysis.—Amino acid compositions were determined according to Spackman, Stein, and Moore (21) using the automatic amino acid analyzer (Beckman model 120C). Samples were hydrolyzed in 6 N HCl at 110°C for 24 hr. The results reported are the average of duplicate analyses; no corrections were made for losses during hydrolysis.

Digestion with Papain.—Hydrolysis with papain was performed according to the procedure of Porter (22). The digestion mixtures were incubated at 37°C for 18 hr.

Qualitative Determination of NH_2-Terminal Amino Acids.—This was carried out using the dansyl method of Gray (23). The dansyl derivatives were determined by polyamide layer chromatography according to Woods and Wang (24). The authors are grateful to Mr. Paul D. Gottlieb for carrying out the analyses.

RESULTS

Demonstration of Antibody Activity.—Table I compares bacteriophage neutralization by sera from individual uninjected lampreys with that of sera from animals injected with f2 phage. The lamprey sera were diluted 1:5 in these experiments. All of the normal sera showed less than 10% neutralization, whereas sera from three-fourths of the injected animals gave significant neutralization. Although the activity was weak in comparison with the responses of higher vertebrates to the same antigen (5), it was sufficiently great to follow through the course of subsequent chemical fractionations.

Kinetic studies of phage inactivation by an active immune serum are given in Fig. 1. The fraction of surviving phage (p/p_o) decreased as the time of incubation with antiserum increased. The neutralization did not follow first-order kinetics throughout the time course, but showed pronounced curvature after 10-min of incubation (curve a). When the titration was carried out in 0.1 M 2-mercaptoethanol, neutralization was markedly inhibited (curve b). The

specificity of neutralization is illustrated by curve c which indicates that the serum had no effect on the unrelated bacteriophage f1. Curve d shows that serum from an uninjected animal did not neutralize bacteriophage f2.

Since the neutralization was approximately linear for the first 10 min, we calculated a neutralization constant (see Materials and Methods for definition) of 0.48 min^{-1} for this region. The normal serum, mercaptoethanol-treated antiserum and antiserum incubated with heterologous phage, had neutralization constants of less than 0.05 min^{-1}.

Sera from 25 hemocyanin-injected animals did not react with the antigen

TABLE I

Neutralization of Bacteriophage f2 by Sera from Lampreys Injected with f2

Neutralization class*	No. of animals in each neutralization class	
	Normals	In.ected with f2
%		
0–10	17	9
11–20		4
21–30		6
31–40		5
41–50		8
51–80		5
Total.....	17	37

* All sera were diluted 1:5 in the final reaction mixture. Data are expressed as fraction of phage neutralized × 100; i. e., $(1-p/p_o) \times 100$ where p/p_o is the fraction of infective phage at 30 min.

as tested by precipitin reactions in microcapillary tubes and by passive hemagglutination assays. Two of 23 sera from animals injected with sheep erythrocytes showed a weak agglutinating response (titer of four). This activity was too low to follow through the course of chemical fractionation.

All lamprey sera examined were capable of agglutinating horse red blood cells to a titer of approximately 10,000 when titrations were performed in a solvent consisting of 0.15 M NaCl, 0.1 M $CaCl_2$. This suggested that a natural hemagglutinin is present in lamprey serum. Because of the possibility that this natural hemagglutinin might resemble the natural hemagglutinins of higher vertebrates, which are immunoglobulins, the localization of hemagglutinating activity was attempted in the serum fractionation procedures.

Isolation and Characterization of Phage-Neutralizing Antibodies.—Because both total serum protein concentration (approximately 30 mg/ml) and neutralizing activity were low, 40 ml of pooled sera from 11 immunized animals

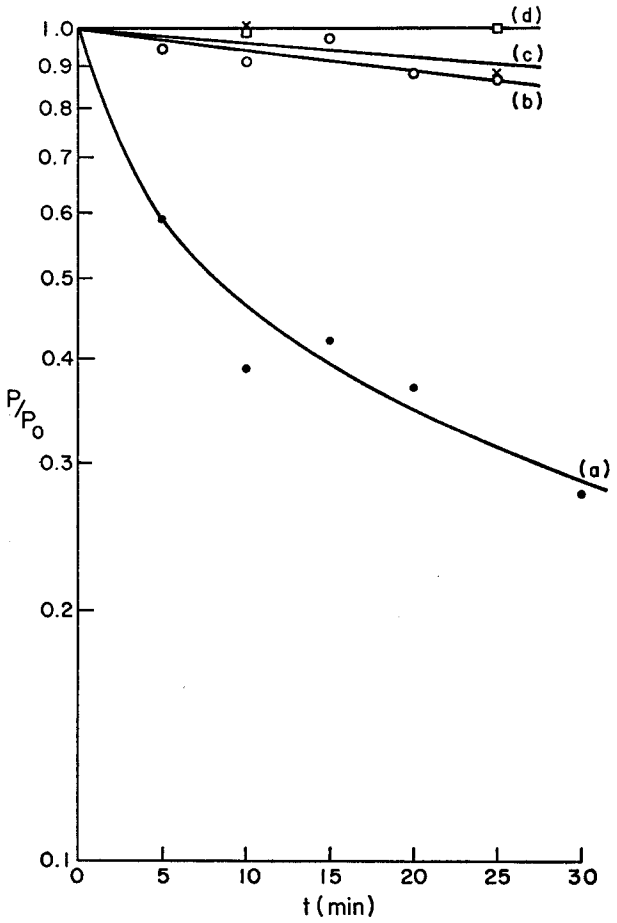

FIG. 1. Bacteriophage neutralization by lamprey serum. Ordinate: fraction of surviving phage, p/p_o; abscissa, time in minutes, t. The same lamprey antiserum to bacteriophage f2 was used in curves a, b, and c. Serum from an uninjected animal was used in curve d. Sera were diluted 1:5 with nutrient broth for these experiments. ●, curve a, lamprey antiserum, bacteriophage f2. ○, curve b, lamprey antiserum, bacteriophage f2; titration performed in the presence of 0.13 M mercaptoethanol. ×, curve c, lamprey antiserum tested with bacteriophage f1. □, curve d, normal serum, bacteriophage f2.

was concentrated to a protein concentration of 70 mg/ml. In control experiments, sera from normal animals gave protein fractionation patterns identical to those described below for immunized lampreys.

Fig. 2 shows the protein distribution pattern of pooled lamprey serum obtained after zone electrophoresis on starch. The electrophoretic pattern differs from those of higher vertebrates in that the major component migrates as an

α-globulin and constitutes over 85% of the serum protein. A small amount of pigmented material migrated towards the cathode in a zone corresponding in mobility to that of mammalian γG-immunoglobulins. This material possessed no antibody activity and has been identified as a transferrin by other workers (25). Bacteriophage-neutralizing activity was localized on the anodal side of the origin in the β-region. The natural hemagglutinating activity towards horse erythrocytes was also found in this region.

The protein fractions possessing antiphage activity were pooled, concen-

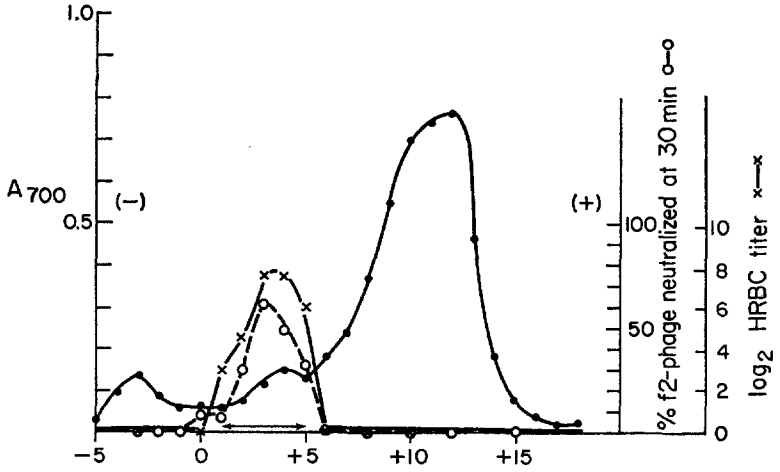

FIG. 2. Zone electrophoresis on starch of serum from lampreys immunized with bacteriophage f2. ●—●, absorbancy of the Folin reaction at 700 mμ; ○--○, per cent f2-phage neutralized at 30 min; ×—×, $\log_2$ hemagglutination titer to horse erythrocytes; (−), cathode; (+), anode; o, origin; and ↔, fractions pooled for density gradient centrifugation and chromatography.

trated to about 10 mg/ml, and subjected to ultracentrifugation on linear sucrose gradients (Fig. 3). Three components were observed when the fraction was centrifuged at 25,000 *g* for 16 hr (Fig. 3, *a*). Natural hemagglutinating activity was found to have a sedimentation coefficient of approximately 46S. Bacteriophage-neutralizing activity was found in a diffuse zone which included proteins ranging in sedimentation velocity from 7S to 14S. In Fig. 3, *b*, the conditions were modified (60,000 *g* for 16 hr) so that the 46S material was pelleted and a better resolution of the 14S and 7S components was achieved. Two distinct peaks of phage-inactivating activity were then observed.

When subjected to antigenic analysis with rabbit antiserum to whole lamprey serum, the active fractions were found to contain a number of components and therefore an additional purification step was attempted. The active frac-

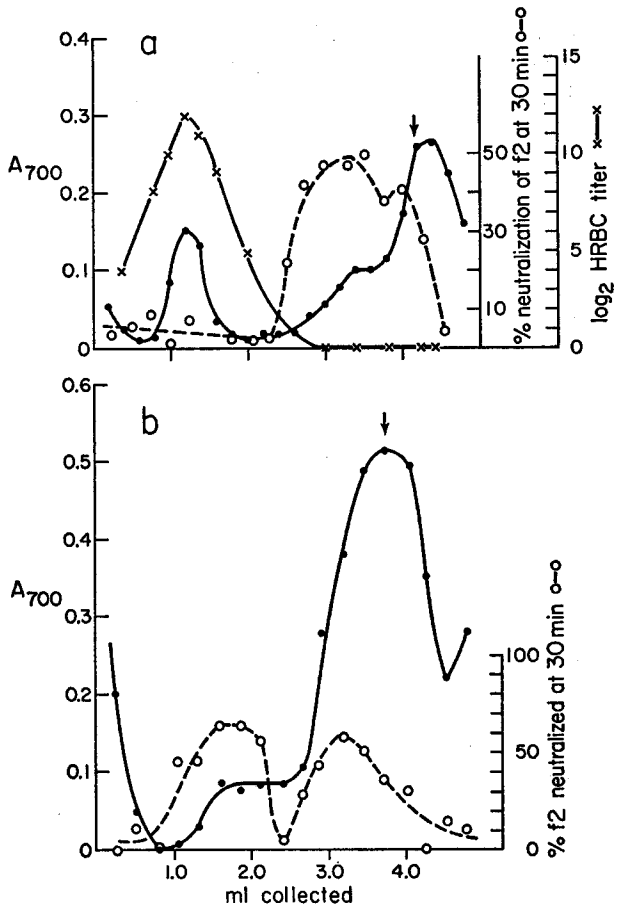

FIG. 3. Density gradient ultracentrifugation of electrophoretic fractions shown in Fig. 2. Sedimentation proceeded from right to left. ↓, position of alkaline phosphatase marker (6.3S); ●—●, absorbancy of the Folin reaction at 700 mμ; o—o, per cent neutralization of bacteriophage f2 at 30 min; and ×—×, $\log_2$ horse erythrocyte agglutination titer. (*a*) Centrifugation conditions: 25,000 *g*, 16 hr; 5–20% sucrose. (*b*) Centrifugation conditions: 60,000 *g*, 16 hr; 5–20% sucrose.

tion obtained from starch block electrophoresis was fractionated further by chromatography on DEAE-cellulose as shown in Fig. 4. Pigmented material was eluted when the column was washed with the starting buffer. This material showed no phage-inactivating or hemagglutinating activity. Bacteriophage-neutralizing activity was localized in the first fraction which was eluted after the gradient was applied. In the analytical ultracentrifuge, this active fraction was composed of approximately equal amounts of proteins with sedimentation coefficients of 46S and 6.6S.

The fraction obtained by chromatography on DEAE-cellulose was centrifuged for 5 hr at 100,000 *g* (SW 39 swinging bucket rotor) to pellet most of the 46S material. The supernatant was then subjected to gel filtration on Sephadex G-200 in Tris-NaCl as shown in Fig. 5. The major peak was eluted

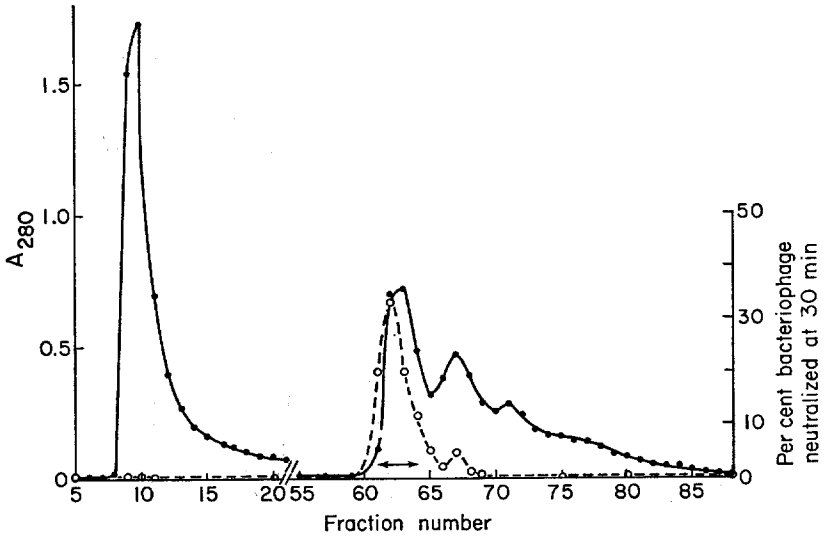

FIG. 4. Subfractionation of electrophoretic fractions with anti-f2 activity (see Fig. 2) by chromatography on DEAE-cellulose. The absorbancy at A280 mμ diminished smoothly to 0 by fraction 40. Gradient elution was begun at this point. ●—●, absorbancy at 280 mμ; ○–○, per cent neutralization of bateriophage f2 at 30 min; and ↔, fractions pooled for further analysis.

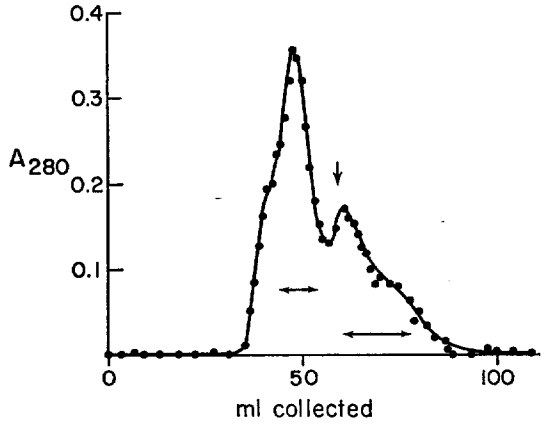

FIG. 5. Gel filtration on Sephadex G-200 of fraction obtained by chromatography on DEAE-cellulose (Fig. 4). Elution buffer, Tris-NaCl; ↓, position of alkaline phosphatase marker (6.3S); and ↔, fractions pooled for further study.

ahead of the alkaline phosphatase marker which is known to have a sedimentation coefficient of 6.3S and a molecular weight of about 90,000. The material in this fraction was pooled and tested for phage-neutralizing activity. The whole fraction had a neutralization constant of 0.17 mg^{-1} ml min^{-1} as

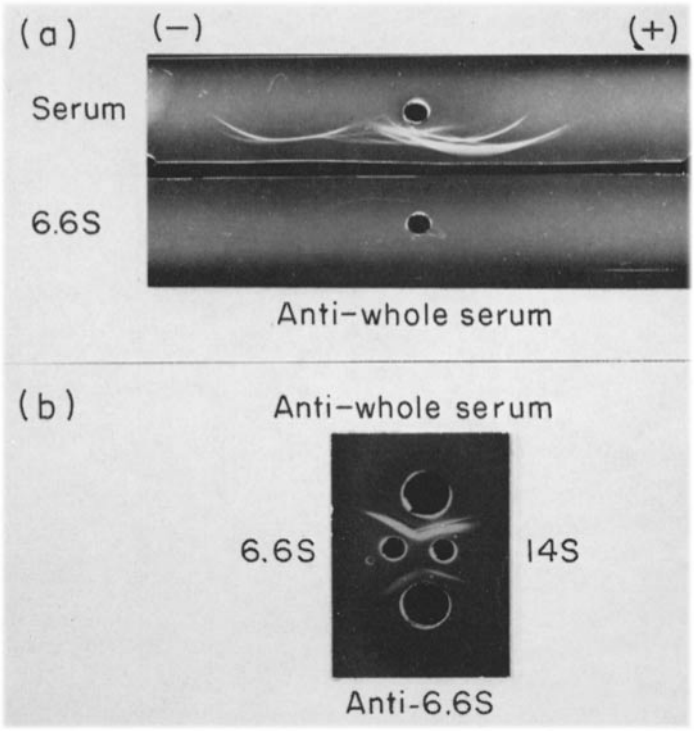

FIG. 6. Immunological analysis of lamprey serum and antibody fractions. (*a*) Comparison by immunoelectrophoresis of lamprey 6.6S immunoglobulin and whole serum. Rabbit antiserum to whole lamprey serum was employed. (*b*) Comparison by double diffusion in agar of lamprey 6.6S immunoglobulin and a fraction rich in 14S immunoglobulin obtained by density gradient ultracentrifugation. The upper well contains rabbit antiserum to whole lamprey serum. The lower well contains rabbit antiserum to the purified 6.6S immunoglobulin.

compared with a constant of 0.02 mg^{-1} ml min^{-1} for the original antiserum. Material in fractions emerging after the alkaline phosphatase marker had no phage-neutralizing activity.

The active fraction obtained by gel filtration on Sephadex G-200 gave a single band after immunoelectrophoresis against rabbit antiserum to whole lamprey serum (Fig. 6, *a*). Although the pattern of lamprey serum was quite complex, the band corresponding in position to the purified (6.6S) fraction could readily be discerned. The active 6.6S fraction was also compared by

immune diffusion with material from the sucrose density gradient (Fig. 3, *b*) which had a sedimentation coefficient of 14S. Although the 14S preparation contained more than one component when analyzed with antiserum to whole lamprey serum, the major band fused completely with that of the 6.6S protein. When antiserum to the purified 6.6S protein was used, both fractions gave single components which fused completely. The capacity to neutralize bacteriophage f2 could be completely removed from active lamprey serum or electrophoretic fractions by absorption with this antiserum. Similar treatment with normal rabbit serum had no effect on the neutralizing activity of lamprey antisera.

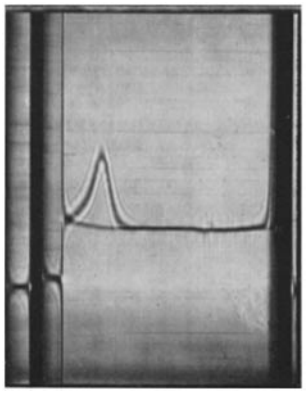

FIG. 7. Sedimentation velocity pattern of purified lamprey antibody fraction. Protein concentration, 4 mg/ml; speed, 52,000 rpm; time of photograph, 35 min; phase plate angle, 40°; and solvent, Tris-NaCl. Sedimentation proceeded from left to right.

In the analytical ultracentrifuge (Fig. 7), the active fraction showed a peak which was asymmetrical on the centripetal side, indicating the presence of lighter components. The $s^{\circ}_{20,w}$ of the main peak was 6.6S. In view of its association with antibody activity and its electrophoretic behavior and sedimentation properties, the 6.6S material will be designated provisionally as lamprey immunoglobulin. The 14S protein is also probably an immunoglobulin on the basis of its activity and antigenic relationship to the 6.6S protein.

Dissociation Properties of Lamprey Immunoglobulin.—The procedures used to examine the polypeptide chain structure of lamprey immunoglobulins were those used in studies with immunoglobulins from higher vertebrates, namely, treatment with agents which cleave disulfide bonds followed by separation under dissociating conditions (17, 26, 27). Lamprey 6.6S immunoglobulin and 46S hemagglutinin, both untreated and reduced with 2-mercaptoethanol, were compared with reduced human γG-immunoglobulin, reduced sting ray immunoglobulin, and heavy chains from dogfish immunoglobulin by starch gel electrophoresis in formate-urea buffer (Fig. 8). The patterns of the lamprey

hemagglutinin and immunoglobulin were quite different. Although the untreated hemagglutinin did not penetrate the gel, two faint bands which did migrate in the gel were observed after reduction. Both untreated and reduced 6.6S immunoglobulin showed diffuse bands in the light chain region. After

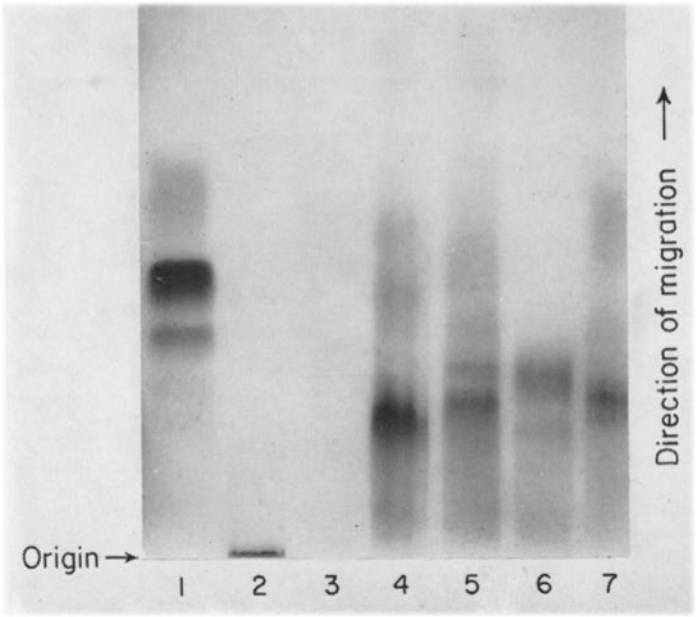

FIG. 8. Comparison by gel electrophoresis in 8 M urea formate of lamprey 6.6S antibody fraction and hemagglutinin with immunoglobulins from elasmobranchs and man. Reduction was performed at pH 8.0 in Tris-NaCl. Origins:

1. Reduced human γG-immunoglobulin.
2. Untreated lamprey hemagglutinin.
3. Reduced lamprey hemagglutinin.
4. Untreated lamprey 6.6S antibody fraction.
5. Reduced lamprey 6.6S antibody fraction.
6. Heavy chain from dogfish immunoglobulin.
7. Reduced sting ray immunoglobulin.

reduction, the penetration in the gel of the slower band of the 6.6S immunoglobulin was similar to that of the dogfish heavy chain and sting ray immunoglobulin heavy chain. As shown previously (1, 2), heavy chains from elasmobranch immunoglobulins resemble mammalian μ-chains in their behavior on starch urea gels.

Similar patterns were obtained for the untreated and the reduced lamprey immunoglobulin. This suggested that dissociation of the 6.6S protein in the gel buffer which was 8 M in urea did not require cleavage of interchain disulfide bonds. In order to confirm this dissociation behavior and to prepare subunits

for chemical analysis, untreated and reduced-alkylated 7S immunoglobulins were subjected to gel filtration on Sephadex G-100 both in neutral aqueous buffer and under dissociating conditions. The behavior of the immunoglobulin in 0.15 M NaCl at pH 8.0 (0.05 M in Tris-HCl) is shown in Fig. 9, *a*. All of the

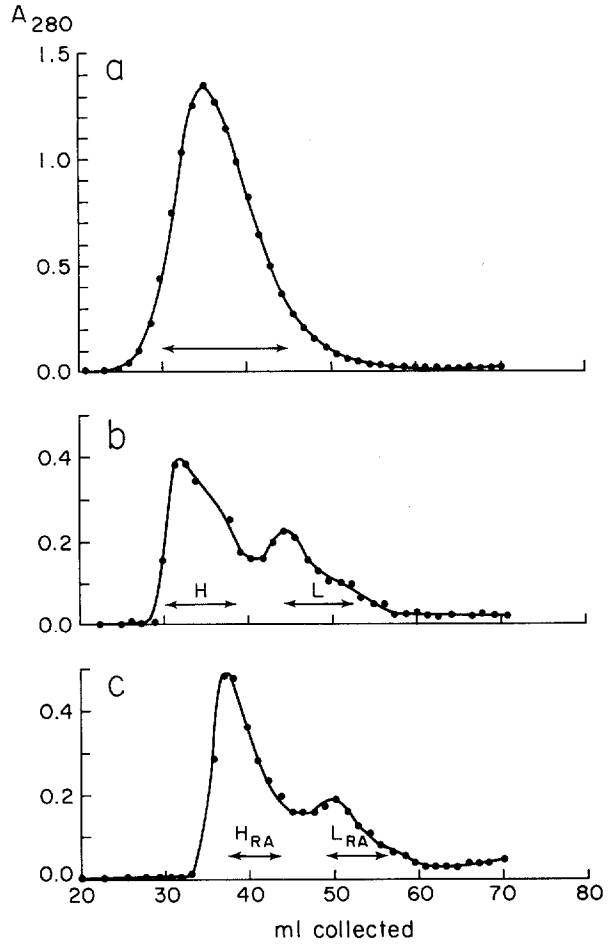

FIG. 9. Gel filtration of lamprey 6.6S immunoglobulin in Tris-NaCl and in propionic acid. Sephadex G-100, Column dimensions 100 × 1 cm. (*a*) Untreated immunoglobulin. Column equilibrated with Tris-NaCl. (*b*) Untreated immunoglobulin. Column equilibrated with 1 M propionic acid. (*c*) Immunoglobulin reduced and alkylated in Tris-NaCl prior to loading on column equilibrated with 1 M propionic acid. L, light component from untreated immunoglobulin. H, heavy component from untreated immunoglobulin. L_{RA}, light component from reduced-alkylated immunoglobulin. H_{RA}, heavy component from reduced-alkylated immunoglobulin.

protein emerged as a single peak in the void volume of the column. The fractions indicated were pooled, concentrated, and separated into two aliquots. The first was loaded on a column of Sephadex G-100 of the same dimensions which had been equilibrated with 1 M propionic acid (Fig. 9, *b*). The second

TABLE II

Molecular Weights of 6.6S Immunoglobulin and Dissociated Components

Sample	Solvent	Molecular weight*
6.6S Immunoglobulin Preparation 1	Tris-NaCl pH 8.0	115,000 ± 8,000
6.6S Immunoglobulin Preparation 1	20% HAc‡ ½% sucrose	88,900 ± 11,000
6.6S Immunoglobulin Preparation 2	Tris-NaCl pH 8.0	100,400 ± 17,600
6.6S Immunoglobulin Preparation 2	20% HAc ½% sucrose	74,300 ± 8,500
H	20% HAc ½% sucrose	71,100 ± 4,000
H_{RA}	20% HAc ½% sucrose	69,600 ± 2,000
L	20% HAc ½% sucrose	29,000 ± 4,900
L_{RA}	20% HAc ½% sucrose	24,600 ± 2,000

* Weight average molecular weights obtained by the Yphantis long column equilibrium centrifugation method (18). Partial specific volume ($\bar{v}$) used in all calculations was 0.733, as determined from the amino acid composition.

‡ HAc, acetic acid.

aliquot was reduced and alkylated prior to loading on the column equilibrated with propionic acid (Fig. 9, *c*). The elution patterns of reduced and unreduced 6.6S immunoglobulin were generally similar; approximately 70% of the absorbancy emerged close to the void volume whereas the remaining 30% eluted as a second peak which was significantly retarded by the gel matrix.

The molecular weights of materials from each of these peaks as well as of the original preparations are given in Table II. The original preparations had molecular weights which were significantly lower in 20% acetic acid, ½%

sucrose, than in Tris-NaCl. It should be emphasized that these values represent bulk averages (18) of preparations which were polydisperse with respect to molecular weight. For example, the point-by-point molecular weight of sample 2 in Tris-NaCl, determined by a sliding five-point least squares fit (see Yphantis, reference 18), varies almost continuously from 70,000 to 140,000 as the concentration of the protein increases with distance from the center of rotation. This heterogeneity is illustrated by curve a in Fig. 10. A relatively homogeneous preparation, the reduced light component (curve b), is shown for comparison.

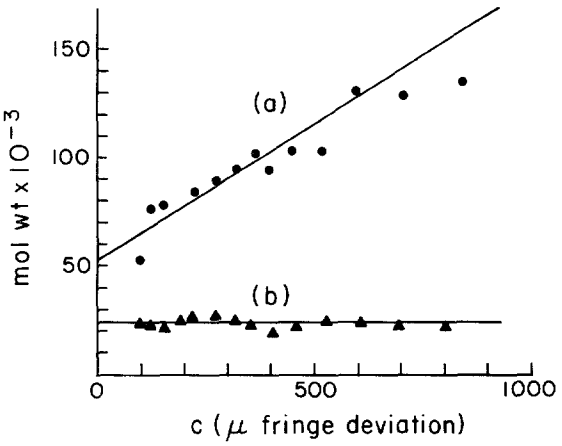

FIG. 10. Weight average molecular weights of lamprey antibody preparations plotted as a function of protein concentration, c. The molecular weights at each point were calculated according to a sliding five-point least squares analysis of the data from a single ultracentrifuge experiment (18). The lines are drawn according to the method of least squares. (*a*) 6.6S immunoglobulin preparation 2. (*b*) Light component from reduced-alkylated 6.6S mmunoglobulin (see Fig. 9, *c*).

The molecular weights of the heavy components determined in the acetic acid solvent were identical within experimental error and comparable with that of μ-chains of immunoglobulins of higher vertebrates (28). The molecular weights of the light components in the same solvent are in fair agreement with the value of 23,000 obtained for immunoglobulin light chains (29). It was observed that the light fraction from unreduced immunoglobulin contained two components: one with a molecular weight of approximately 23,000, the other with a molecular weight of about 40,000. When examined in 1 M propionic acid, the light component had a molecular weight of 40,000.

The light and heavy components from untreated and reduced immunoglobulins were dialyzed against Tris-NaCl. Neither the light nor heavy components alone showed phage-neutralizing activity. Preliminary observations suggest, however, that mixing the light and heavy components in propionic acid prior to dialysis can bring about complete recovery of activity.

The separated light and heavy components were compared antigenically to the 6.6S protein using rabbit antiserum to the 6.6S protein (Fig. 11). As illustrated in the diffusion experiment (Fig. 11, *a*), the light component was antigenically unrelated to the intact protein. The light components from reduced and untreated immunoglobulins were antigenically identical. The antiserum showed no precipitin line with the heavy component. In Figure 11, *b* is shown a comparison by immunoelectrophoresis of the light component and the intact protein. Instead of the single precipitin arc given by the intact protein, the light component shows three separate arcs under these conditions. Material

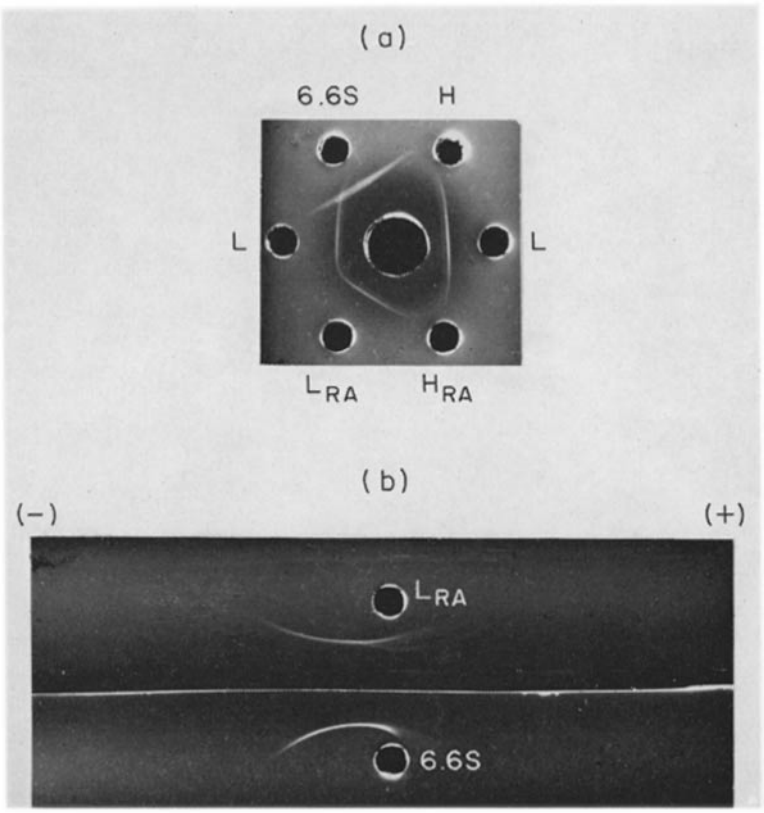

FIG. 11. Immunological comparison of lamprey 6.6S immunoglobulin and the polypeptide components isolated by gel filtration in propionic acid (see Fig. 9). Rabbit antiserum to the isolated 6.6S protein was used in both cases. (*a*) Immune diffusion. L, light component from untreated immunoglobulin. H, heavy component from untreated immunoglobulin. L_{RA}, light component from reduced-alkylated immunoglobulin. H_{RA}, heavy component from reduced-alkylated immunoglobulin. (*b*) Immunoelectrophoretic comparison of the light component from reduced immunoglobulin with the intact protein. The light component obtained from untreated immunoglobulin gave an identical pattern.

antigenically identical to the light component was present in the fraction which elutes after the alkaline phosphatase marker upon gel filtration on Sephadex G-200 in Tris-NaCl (Fig. 5). This finding suggests either that free light chains are normally present in the serum or that the immunoglobulin dissociates under the relatively gentle conditions used in the isolation procedure.

Amino acid compositions of the intact lamprey 6.6S immunoglobulin, the components obtained by gel filtration in propionic acid, and the lamprey

TABLE III

Amino Acid Compositions of Lamprey Hemagglutinin (HA), the 6.6S Antibody Fraction and its Polypeptide Chains

Amino acid*	Lamprey HA	Lamprey 6.6S	Lamprey light	Lamprey heavy
Lys	6.5	7.0	7.2	7.5
His	2.6	2.7	3.1	2.6
Arg	4.8	7.2	7.4	6.6
Asp	14.3	10.3	10.3	10.2
Thr	6.7	7.0	6.3	6.7
Ser	6.3	5.4	5.3	5.6
Glu	12.0	12.4	13.4	12.5
Pro	4.3	5.6	5.6	5.2
Gly	5.7	4.1	4.1	4.4
Ala	5.5	5.0	6.0	5.0
Val	4.4	6.6	7.0	7.6
Met	0.5	2.2	2.2	2.2
Ile	4.6	4.8	4.1	4.8
Leu	8.0	8.8	10.4	8.5
Tyr	6.0	5.1	3.9	5.1
Phe	7.8	6.0	5.1	5.3

* Amino acid composition is expressed as g/100 g of carbohydrate-free protein. These values are the average of duplicate analyses. Cysteine and tryptophan are not included. Values are not corrected for destruction during hydrolysis.

natural hemagglutinin, are given in Table III. The compositions of the lamprey immunoglobulin and hemagglutinin are distinctly different from each other. The immunoglobulin chains show close similarity, but are significantly different in a number of amino acids; e.g., leucine and isoleucine.

Qualitative determinations by the dansyl method disclosed that the NH_2-terminal amino acid of both the light and the heavy components is aspartic acid or asparagine. In addition, a number of other amino acids were observed to be present in trace amounts in both preparations.

Behavior of Lamprey Immunoglobulin after Exposure to Papain.—Mammalian immunoglobulins are degraded into antigenically unrelated fractions by proteolysis with papain (30). We wished to determine if similar results were

obtained with lamprey immunoglobulins. Lamprey 6.6S immunoglobulin, the smooth dogfish 7S immunoglobulin which resembles 7S subunit from γM of higher vertebrates (1, 2), and human γG-immunoglobulin were incubated at 37°C for 18 hr with cysteine-activated papain (22, 30). The untreated and

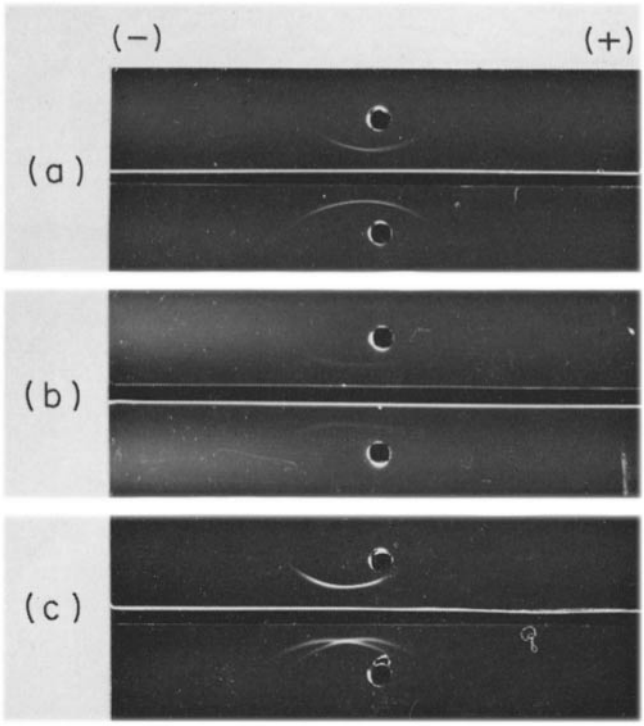

FIG. 12. Comparison by immunoelectrophoresis of untreated and papain-digested immunoglobulins from lamprey, *Mustelus canis*, and man. (*a*) Lamprey 6.6S immunoglobulin: upper well, untreated; lower well, incubated with papain; and antiserum, rabbit antiserum to the 6.6S protein. (*b*) *M. canis* 7S immunoglobulin: upper well, untreated; lower well, incubated with papain; and antiserum, rabbit antiserum to the 7S immunoglobulin. (*c*) Human γG-immunoglobulin: upper well, untreated; lower well, incubated with papain; antiserum, rabbit antiserum to the γG-protein.

treated proteins were compared by immunoelectrophoresis against rabbit antisera with the respective purified proteins (Fig. 12). Under these conditions, the human γG-immunoglobulin was converted almost completely into Fab and Fc fragments. As tested by immunoelectrophoresis and zone electrophoresis on cellulose acetate strips, neither the dogfish nor the lamprey immunoglobulin was affected by this treatment.

DISCUSSION

The sea lamprey is the most primitive vertebrate reported to be capable of producing circulating antibodies (6–8). We have found that this animal can produce antibodies to bacteriophage f2 following a single injection of the viral antigen. The evidence supporting this conclusion can be summarized as follows: (*a*) only sera from immunized animals neutralized phage, (*b*) the neutralization was specific, and (*c*) neutralizing activity was consistently localized in definite serum protein fractions.

Neutralization of bacteriophage by immune lamprey serum was extremely weak in comparison with the serum activities observed in amphibians (5, 31, 32), birds (31), and mammals (33) that have received a single injection of phage antigen. The neutralization constants for lamprey sera, calculated in the initial region of approximate linearity, were less than 1 min^{-1} whereas the higher vertebrates cited above produced antisera with neutralization constants as great as 100 min^{-1}. Phage inactivation by lamprey sera was, however, comparable with that produced by antibodies from certain elasmobranchs (6, 8) and chondrostean fishes (8). Despite the weak response, the sensitivity of the kinetic assay allowed localization of activity throughout all chemical fractionation steps.

We injected lampreys with two other antigens, *Limulus* hemocyanin and sheep red blood cells, but were either unable to detect antibodies to them or found extremely low titers of activity. Good and his coworkers (6–8) detected antibodies only to the bacterium *Brucella abortus*, although they tested a variety of protein, cellular, and viral antigens. The responses of the lamprey to *B. abortus* antigens and to human erythrocyte antigens (25) were quite feeble.

Antibodies to bacteriophage f2 were found to sediment as 14S and 6.6S proteins which migrated electrophoretically in the fast γ- or β-region. Our results are consistent with those of other workers (25, 34) who found that lamprey antibodies possess fast γ- or β-electrophoretic mobility. Boffa et al. (25) reported that antibodies to human erythrocyte antigen H were localized in the two heaviest components prepared by gel filtration of lamprey antiserum on Sephadex G-200. They tentatively identified these fractions with the 10.9S and 6.6S proteins observed in analytical ultracentrifugation of whole serum. Pollara et al. (34) found that anti-*Brucella* activity is associated with a protein having a sedimentation coefficient intermediate between 7S and 19S.

In the course of our studies on antibody activity, we observed a natural hemagglutinin in the lamprey serum. This protein has also been detected by Gewurz et al. (35) who found that it migrated in the same electrophoretic region as did anti-*Brucella* activity. The hemagglutinin was located by these workers in the heaviest fraction eluted from Sephadex G-200, whereas anti-*Brucella* activity was retarded by passage through the gel matrix. Our data indicate that

the hemagglutinin is a 46S protein which differs from lamprey immunoglobulins in antigenic properties, subunit structure, and amino acid composition. The starch gel electrophoretic studies suggest that it consists of subunits which may be linked by disulfide bonds. Because of the large size of the intact molecule, we considered the possibility that it might be a hemagglutinating virus infecting the lamprey. This possibility was excluded by the fact that no nucleic acid absorption maximum at 260 mμ was observed. The lamprey hemagglutinin showed no obvious resemblance to serum proteins from higher vertebrates and also differed from invertebrate hemagglutinins such as that of *Limulus* (36, 36 *a*).

Studies of various lamprey proteins such as hemoglobin (37) and thyroglobulin (38) have indicated that they resemble corresponding proteins from higher vertebrates in many respects, but also differ markedly in certain properties. In particular, the lamprey proteins show a greater tendency to dissociate into subunits. In the present experiments, we have observed that the 6.6S lamprey antibodies dissociate more readily than antibodies of other vertebrates (1–5, 39, 40). Although the concentration of immunoglobulin in lamprey serum is extremely low (approximately 0.2 mg/ml), we were able to prepare a sufficient amount of purified 6.6S antibody fraction for dissociation studies. The 14S protein could not be isolated in quantity, but immunological data suggest the two proteins are antigenically similar or identical. The 6.6S antibody could be dissociated in the absence of reducing agents by denaturing solvents such as acetic acid, propionic acid, and urea. In this respect, the lamprey antibody differs from the immunoglobulins of other vertebrates ranging from elasmobranchs (1–5) to mammals (17, 40) which require cleavage of disulfide bonds prior to dissociation. The dispersity of molecular weight averages obtained for the immunoglobulin (Table II, Fig. 10) and the sedimentation velocity patterns (Fig. 7) suggest that concentration-dependent dissociation may occur even in neutral aqueous buffers.

When the active 6.6S protein was subjected to gel filtration in propionic acid, two smaller polypeptide fractions were isolated which differed in amino acid composition and did not possess antibody activity. These polypeptide components resembled light chains and heavy chains of the μ-type from immunoglobulins of other vertebrates in (*a*) molecular weights, 24,000 and 70,000, respectively, (*b*) electrophoretic mobility on starch urea gels and diffuse heterogeneity of the pattern in the light chain region, (*c*) relative yields of the light (30%) and heavy (70%) components as determined by gel filtration in propionic acid, (*d*) presence of equimolar amounts of each component in the intact protein as indicated by the molecular weights and relative yields, and (*e*) presence of aspartic acid or asparagine as an NH_2-terminal amino acid in the light component.

The immunological relationship of the polypeptide components to the intact

molecule differs markedly from that observed with immunoglobulins from other species in which light chains and heavy chains are antigenically distinct, but show partial identity with the whole molecule (39). The heavy component did not give a precipitin line with rabbit antiserum to the intact 6.6S molecule. This may be the result of aggregation which was observed when the preparation was dialyzed against Tris-NaCl. Light components from reduced-alkylated and untreated 6.6S immunoglobulin are antigenically identical. These polypeptides represent a system of antigenically unrelated components which do not cross-react with the intact molecule. These results indicate that the antigenic properties depend strongly upon conformational differences of the polypeptide chains in the dissociated state and in the intact molecule.

Proteolysis with papain cleaves mammalian γG-immunoglobulins into two antigenically unrelated fragments, Fab and Fc (30). Similar treatment of mammalian γM- and γA-proteins produces an analogous degradation (41, 42). Digestion of lamprey 6.6S immunoglobulin and *Mustelus canis* 7S immunoglobulin with papain under conditions sufficient for complete conversion of human γG into Fab and Fc pieces did not bring about the formation of fragments. This raises the possibility that under similar conditions, immunoglobulins from lower vertebrates may be less susceptible to proteolytic enzymes than those of birds (43) and mammals (40).

A consistent interpretation of the data obtained in the present study is that lamprey antibodies are structurally similar to immunoglobulins of the elasmobranchs but lack interchain disulfide bonds or have extremely labile interchain bonds. The 6.6S protein would consist of two light and two heavy chains held together by weak interactions. The fact that the observed average molecular weight of the intact protein is lower than that predicted on the basis of two light and two heavy chains probably reflects the tendency of the protein to dissociate even in neutral aqueous buffers (Fig. 10). The 14S protein, which could not be isolated in quantity, is most likely a higher aggregate of the 6.6S protein inasmuch as the two fractions appeared to be antigenically identical.

Because of the striking singularities in dissociation behavior and antigenic behavior of lamprey immunoglobulin, the above interpretations must be considered provisional. If confirmed, they would indicate that the interchain disulfide bonds of immunoglobulins emerged at some time after the emergence of light and heavy chains. One might expect that proteins composed of disulfide bonded subunits evolved from precursors which were linked by noncovalent interactions only. In any case, the data are compatible with the proposal that γM-immunoglobulins are the most primitive antibodies (1). In accord with previous hypotheses (44, 45), we may assume that the earliest immunoglobulin gene coded for a polypeptide resembling the light chain. The gene for μ-chains may have arisen by end-to-end duplication of this precursor gene approximately 400 million years ago, corresponding with the emergence of vertebrates. Sub-

sequently, the gene specifying γ-chains may have arisen from the μ-chain gene or by a second duplication of the light chain gene at some time prior to the emergence of higher amphibians (about 200 million years ago). Preliminary starch gel electrophoretic experiments with immunoglobulins of elasmobranchs (sting ray), holostean fishes (bowfin), and teleosts (carp, blue marlin, and lake trout), tend to substantiate this scheme.[4] The only immunoglobulin heavy chains present in these forms resemble the human μ-chain; γ-type chains were not observed below the phylogenetic level of higher amphibians. A more precise determination of the evolutionary interrelationships among immunoglobulin chains of the various vertebrate species should emerge from detailed studies of amino acid sequences (4).

We have provided unequivocal evidence that the sea lamprey produces specific circulating antibodies in response to immunization. These antibodies resemble the immunoglobulins of other vertebrates in properties such as multichain structure and diversity, but differ with respect to dissociation behavior. The similarities substantiate the conclusion that the multichain structure is found in antibodies from all vertebrate species. The differences probably reflect the early evolutionary divergence between the lamprey and other vertebrates. Analysis of these differences should yield new information bearing on the problems of chain interaction in antibody molecules.

SUMMARY

The sea lamprey, *Petromyzon marinus*, has been found to produce specific antibodies after immunization with bacteriophage f2. Antibody activity is localized in 6.6S and 14S fractions of lamprey serum. The 6.6S antibodies were purified by a combination of zone electrophoresis, ion exchange chromatography, and gel filtration. Antigenic analysis of the 6.6S antibodies showed them to be free of other serum proteins and antigenically similar or identical to the 14S fraction. Evidence has been obtained which suggests that the 6.6S immunoglobulins consist of light components (molecular weight 25,000) and heavy components (molecular weight 70,000). In the immunoglobulin, these polypeptides appear to be linked via weak interactions but not by interchain disulfide bonds. Molecular weight analyses support the view that the chains can undergo concentration-dependent dissociation in aqueous solutions. Amino acid analyses showed that the compositions of the light and heavy components were similar and that aspartic acid or asparagine was the predominant amino terminal residue. Starch gel electrophoresis indicated that the subunits of lamprey antibodies are diffusely heterogeneous. The heavy chain mobility corresponded to that of μ-chains and resembled that of heavy chains of shark and sting ray immunoglobulins.

[4] Marchalonis, J., nd G. M. Edelman. Unpublished observations.

In the course of the fractionation a 46S natural hemagglutinin composed of lower molecular weight subunits was isolated. This hemagglutinin did not resemble the lamprey immunoglobulin although it had a similar zone electrophoretic mobility in the β-region.

These studies are consistent with the hypothesis that μ-chains were the earliest of the heavy chain classes to emerge and further support the view that the multichain structure of immunoglobulins is a fundamental feature of antibody molecules.

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BIBLIOGRAPHY

1. Marchalonis, J., and G. M. Edelman. 1965. Phylogenetic origins of antibody structure. I. Multichain structure of immunoglobulins in the smooth dogfish (*Mustelus canis*). *J. Exptl. Med.* **122**:601.
2. Marchalonis, J., and G. M. Edelman. 1966. Polypeptide chains of immunoglobulins from the smooth dogfish (*Mustelus canis*). *Science.* **154**:1567.
3. Clem, L. W., and P. A. Small, Jr. 1967. Phylogeny of immunoglobulin structure and function. I. Immunoglobulins of the lemon shark. *J. Exptl. Med.* **125**:893.
4. Suran, A. A., and B. W. Papermaster. 1967. N-Terminal sequences of heavy and light chains of leopard shark immunoglobulins: evolutionary implications. *Proc. Natl. Acad. Sci. U.S.* **58**:1619.
5. Marchalonis, J., and G. M. Edelman. 1966. Phylogenetic origins of antibody structure. II. Immunoglobulins in the primary immune response of the bullfrog, *Rana catesbiana*. *J. Exptl. Med.* **124**:901.
6. Papermaster, B. W., R. M. Condie, J. Finstad, and R. A. Good. 1964. Evolution of the immune response. I. The phylogenetic development of adaptive immunologic responsiveness in vertebrates. *J. Exptl. Med.* **119**:105.
7. Finstad, J., and R. A. Good. 1964. The evolution of the immune response. III. Immunologic responses in the lamprey. *J. Exptl. Med.* **120**:1151.
8. Finstad, J., and R. A. Good. 1966. Phylogenetic studies of adaptive immune responses in the lower vertebrates. *In* Phylogeny of Immunity. R. T. Smith, P. A. Miescher, and R. A. Good, editors. University of Florida Press, Gainesville. 173.
9. Good, R. A., J. Finstad, B. Pollara, and A. E. Gabrielsen. 1966. Morphologic studies on the evolution of the lymphoid tissues among the lower vertebrates. *In* Phylogeny of Immunity. R. T. Smith, P. A. Miescher, and R. A. Good, editors. University of Florida Press, Gainesville. 149.
10. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
11. Kabat, E. A., and M. M. Mayer. 1961. Experimental Immunochemistry. Charles C Thomas, Springfield, Ill. 2nd edition.
12. Stavitsky, A. B. 1954. Micromethods for the study of proteins and antibodies. I. Procedure and general applications of hemagglutination and hemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells. *J. Immunol.* **72**:360.

13. Boyden, S. V. 1951. The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. *J. Exptl. Med.* **93**:107.
14. Kunkel, H. G. 1954. Zone electrophoresis. *Methods Biochem. Analy.* **1**:141.
15. Peterson, E. A., and H. A. Sober. 1956. Chromatography of proteins. I. Cellulose ion-exchange adsorbents. *J. Am. Chem. Soc.* **78**:751.
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
17. Edelman, G. M., and M. D. Poulik. 1961. Studies on structural units of the γ-globulins. *J. Exptl. Med.* **113**:861.
18. Yphantis, D. A. 1964. Equilibrium ultracentrifugation of dilute solutions. *Biochemistry.* **3**:297.
19. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. *J. Biol. Chem.* **236**:1372.
20. Olins, D. E., and G. M. Edelman. 1964. Reconstitution of 7S molecules from L and H polypeptide chains of antibodies and γ-globulins. *J. Exptl. Med.* **119**:789.
21. Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Chem.* **30**:1190.
22. Porter, R. R. 1959. The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. *Biochem. J.* **73**:119.
23. Gray, W. R. 1967. Dansyl chloride procedure. *Methods Enzymol.* **11**:139.
24. Woods, K. R., and K.-T. Wang. 1967. Separation of dansyl-amino acids by polyamide layer chromatography. *Biochim. Biophys. Acta* **133**:369.
25. Boffa, G. A., J. M. Fine, A. Drilhon, and P. Amouch. 1967. Immunoglobulins and transferrin in marine lamprey sera. *Nature.* **214**:700.
26. Edelman, G. M. 1959. Dissociation of γ-globulin. *J. Am. Chem. Soc.* **81**:3155.
27. Fleischman, J. B., R. H. Pain, and R. R. Porter. 1962. Reduction of γ-globulins. *Arch. Biochem. Biophys.* (Suppl. 1):174.
28. Lamm, M. E., and P. A. Small, Jr. 1966. Polypeptide chain structure of rabbit immunoglobulins. II. γM-immunoglobulin. *Biochemistry.* **5**:267.
29. Small, P. A., Jr., J. E. Kehn, and M. E. Lamm. 1963. Polypeptide chains of rabbit gamma globulin. *Science.* **142**:393.
30. Edelman, G. M., J. F. Heremans, M.-Th. Heremans, and H. G. Kunkel. 1960. Immunological studies of human γ-globulin. Relation of the precipitin lines of whole γ-globulin to those of fragments produced by papain. *J. Exptl. Med.* **112**:203.
31. Uhr, J. W., M. S. Finkelstein, and E. C. Franklin. 1962. Antibody response to bacteriophage φX 174 in non-mammalian vertebrates. *Proc. Soc. Exptl. Biol. Med.* **111**:13.
32. Ching, Y., and R. J. Wedgwood. 1967. Immunologic responses in the axolotl, *Siredon mexicanum. J. Immunol.* **99**:191.
33. Uhr, J. W. 1964. The heterogeneity of the immune response. *Science.* **145**:457.
34. Pollara, B., J. Finstad, and R. A. Good. 1966. The phylogenetic development of immunoglobulins. *In* Phylogeny of Immunity. R. T. Smith, P. A. Miescher, and R. A. Good, editors. University of Florida Press, Gainesville. 88.
35. Gewurz, H., J. Finstad, L. H. Muschel, and R. A. Good. 1966. Phylogenetic inquiry

into the origins of the complement system. *In* Phylogeny of Immunity. R. T. Smith, P. A. Miescher, and R. A. Good, editors. University of Florida Press, Gainesville. 105.

36. Marchalonis, J., and G. M. Edelman. 1967. Isolation and characterization of a hemagglutinin from the horseshoe crab, *Limulus polyphemus*. *J. Mol. Biol.* In press.

36 *a*. Fernández-Morán H., J. Marchalonis, and G. M. Edelman. 1968. Electron microscopy of a hemagglutinin from *Limulas polyphemus*. *J. Mol. Biol.* In press.

37. Braunitzer, G. 1966. Phylogenetic variation in the primary structure of hemoglobins. *J. Cell. Physiol.* **67**(Suppl. 1):1.

38. Aloj, S., G. Salvatore, and J. Roche. 1967. Isolation and properties of a native subunit of lamprey thyroglobulin. *J. Biol. Chem.* **242**:3810.

39. Edelman, G. M., and B. Benacerraf. 1962. On structural and functional relations between antibodies and proteins of the gamma-system. *Proc. Natl. Acad. Sci. U.S.* **48**:1035.

40. Cohen, S., and R. R. Porter. 1964. Structure and biological activity of immunoglobulins. *Advan. Immunol.* **4**:287.

41. Rowe, D. S. 1962. The separation and antigenic characteristics of some fractions of papain digests of human serum gamma globulins and the antigenic relationships of human gamma globulins with the gamma-globulins of some other mammalian sera. *Immunology*. **5**:533.

42. Fahey, J. L. 1963. Studies of γ- and β_{2A}-globulins. Comparison of immunological properties of S and F (papain) fragments of myeloma proteins from inbred mice. *J. Immunol.* **90**:576.

43. Dreesman, G., and A. A. Benedict. 1965. Properties of papain-digested chicken 7S γ-globulin. *J. Immunol.* **95**:855.

44. Singer, S. J., and R. F. Doolittle. 1966. Antibody active sites and immunoglobulin molecules. *Science*. **153**:13.

45. Hill, R. L., R. Delaney, R. E. Fellows, Jr., and H. E. Lebovitz. 1966. The evolutionary origins of the immunoglobulins. *Proc. Natl. Acad. Sci. U.S.* **56**:1762.