PHYLOGENETIC ORIGINS OF ANTIBODY STRUCTURE*

I. Multichain Structure of Immunoglobulins in the Smooth Dogfish (Mustelus canis)

By J. MARCHALONIS and G. M. EDELMAN, M.D.

(From the Rockefeller Institute)

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Although the ability of lower vertebrates to produce circulating antibodies was established over 60 years ago (1, 2), little is known of the chemical structure of these antibodies. Recent progress in the analysis of the structure and relationships (3–5) of mammalian immunoglobulins has made feasible comparative chemical studies of the antibodies of lower forms. The results of such studies may be helpful in understanding the mechanism of the immune response, particularly since humoral antibody production appears to occur only among vertebrate species (6, 7).

Among the most primitive of the immunologically competent vertebrates are the elasmobranchs (8) which arose during the Devonian period. In the present paper, we provide evidence that the immunoglobulins of the elasmobranch, *Mustelus canis* (smooth dogfish), are multichain structures similar to those found in higher forms. The serum of *M. canis* was found to have a single predominant immunoglobulin class consisting of 17S and 7S components, each composed both of light and heavy (3) polypeptide chains. Antibody activity was found in the 17S component following immunization with hemocyanin.

These results bear upon the phylogenetic origins of the immunoglobulin classes and suggest that the multichain structure of antibodies evolved early in the development of humoral immunity.

Materials and Methods

1. Immunization and Bleeding.—Purified hemocyanin from the hemolymph of *Limulus polyphemus* was prepared by zone electrophoresis on starch (see section 4, below). Each of five mature dogfish was given a subcutaneous injection of 1 ml of an emulsion composed of 5 mg of hemocyanin in 0.5 ml of 3 per cent NaCl mixed with an equal volume of complete Freund's adjuvant. The injection site was on the dorsal surface lateral to the dorsal fin. Eight days later, each dogfish was given a booster injection of 1 ml of the same mixture at a symmetrically located site. Bleeding was accomplished by cardiac puncture at periods from 11 to 29 days following the initial immunization. The temperature of the running sea water in the tank which housed the animals averaged 20.5°C.

2. Assay of Antibody Activity.—Dogfish sera and serum fractions were titrated for anti-

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hemocyanin activity by means of a modification (9) of the passive hemagglutination technique (10). The antigen solution used to coat the tanned erythrocytes contained 1 mg of hemocyanin per ml. Sheep red cells were obtained from the Animal Blood Center, Syracuse, New York. Qualitative analysis for precipitins was carried out using test tubes as well as microcapillary tubes (11).

3. Preparation of Immunoglobulins.—Immunoglobulins were prepared either by zone electrophoresis on starch (12, 13) or chromatography on diethylaminoethyl-cellulose,[1] (14). The conditions for starch zone electrophoresis have been reported previously (12). The chromatographic fraction containing immunoglobulins was prepared by loading 1 ml of normal or immune serum on 30 x 1 cm columns of diethylaminoethyl-cellulose[1] (Schleicher and Schuell, Keene, New Hampshire) equilibrated with sodium phosphate buffer pH 7.2, 0.02 M. Serum components other than immunoglobulins, in particular the albumin-like material, were retained by the column. Larger volumes of normal dogfish sera (5 to 10 ml) were fractionated on larger columns measuring 40 x 2 cm. The fractions containing the immunoglobulins were concentrated to approximately 1.0 per cent by ultrafiltration (12).

Separation of the immunoglobulins of different molecular weight was achieved by means of gel filtration on sephadex G-200 (Pharmacia, Uppsala). The conditions for gel filtration were: column dimensions 2.0 x 90 cm; buffer, tris (hydroxymethyl) aminomethane, pH 8.0, 0.05 M, 0.15 M in sodium chloride;[2] flow rate 15 ml/hour.

The fractions obtained by gel filtration were further purified by starch zone electrophoresis.

4. Preparation of Limulus Hemocyanin.—Ten ml of hemolymph obtained from *Limulus polyphemus* were subjected to zone electrophoresis on starch (12, 13). Hemocyanin, the predominant component, was dialyzed against distilled water and lyophilized.

5. Determination of Protein Concentrations.—Protein concentration was determined by means of the modified Folin-Ciocalteu method (15). In addition, the absorbancy at 280 mμ was measured. For purposes of comparison, the absorbancy of a 1 per cent solution ($E_{1\,cm}^{1\,per\,cent}$) was assumed to be 13.5.

6. Starch Gel Electrophoresis in Urea.—This method has been previously described (16). Human γG-immunoglobulin (Cohn fraction II, Lederle Laboratories, Pearl River, New York) was used as a control in all gel experiments. In some cases, immunoglobulins were reduced in tris-NaCl but not alkylated prior to gel electrophoresis.

7. Immunoelectrophoresis and Double Diffusion in Agar.—These procedures have been previously described (12). Antisera to whole dogfish sera were obtained by immunizing rabbits with two intramuscular injections each consisting of 0.25 ml serum mixed with complete Freund's adjuvant to a final volume of 1.0 ml. The injections were spaced 3 weeks apart. The rabbits were bled 2 weeks after the second injection. Antisera to purified dogfish immunoglobulins were prepared similarly: the injection mixture consisted of 2 mg immunoglobulin in 0.5 ml of 0.15 M NaCl mixed with an equal volume of adjuvant.

8. Analytical Ultracentrifugation.—This was performed in a Spinco model E ultracentrifuge equipped with automatic temperature control and phase plate schlieren optics.

9. Ultracentrifugation in Sucrose Density Gradients.—The procedure followed represents a previously reported modification (17) of the method of Martin and Ames (18). The conditions of centrifugation were: linear sucrose gradient (5 to 20 per cent in tris-NaCl, pH 8.0); duration 16 hours; centrifugal field, 60,000 *g*; rotor, SW 39 swinging bucket.

10. Reduction and Alkylation and Preparation of Polypeptide Chains.—The 17S and 7S immunoglobulins were dissolved in tris-NaCl made 0.2 M and 0.1 M respectively, in 2-mercaptoethanol (16, 19). The protein concentration varied from 0.5 to 1.0 per cent. After 2 hours of reduction at room temperature, the chains were alkylated by dialysis (20) against 0.02 M

[1] DEAE-cellulose.

[2] Tris-NaCl.

iodoacetamide in tris-NaCl. The polypeptide chains were separated by gel filtration on sephadex G-100 using 1.0 M propionic acid as solvent (21, 22). Gel filtration in 0.5 M propionic acid resolved chains from the 7S protein but yielded poor resolution of the chains of the 17S protein.

Preparation of completely reduced chains for peptide mapping has been previously reported in detail (21). The macroglobulin was dissolved in 8 M urea, 0.2 M in 2-mercaptoethanol. Other conditions were exactly as described by Fougereau and Edelman (21).

11. Two-Dimensional High Voltage Electrophoresis of Tryptic Hydrolysates of Polypeptide Chains (Peptide Mapping).—The details of this procedure have been reported (21, 23).

TABLE I

Antibodies to Limulus Hemocyanin as Detected by Passive Hemagglutination Assay of Sera from M. canis

Serum	Days after initial immunization	Additional reagents or treatment*	Hemagglutination titer
N-1	0	None	10
H-5	29	None	2560
H-5	29	*Limulus* hemocyanin	40
H-5	29	Snail hemocyanin	640
H-4	21	None	640
H-4	21	*Limulus* hemocyanin	10
H-4	21	Bovine serum albumin	640
H-4	21	2-mercaptoethanol‡	<5

SerumN-1 was from an unimmunized animal. Sera H-4 and H-5 were from two immunized animals (see Materials and Methods).

* 0.1 mg of each protein was added to each tube in the passive hemagglutination system just before addition of the red cells.

‡ The serum was made 0.1 M in 2-mercaptoethanol and was allowed to stand for 2 hours at room temperature. It was then dialyzed against 0.15 N NaCl buffered to pH 8.0.

RESULTS

Antibody Activity in Sera of Immunized Animals.—The sera from normal and immunized dogfish were examined for antihemocyanin activity by means of passive hemagglutination and qualitative precipitin analysis. Two of the five sera from immunized animals (H-4 and H-5) showed very faint precipitin reactions in capillary tubes. Table I summarizes the data obtained by passive hemagglutination assay of these two sera and of serum from an unimmunized animal. The reactions of both immune sera were almost completely inhibited by addition of *Limulus* hemocyanin to the assay system. Passive hemagglutination by serum H-5 was slightly inhibited by snail hemocyanin. Treatment of serum H-4 with 2-mercaptoethanol followed by dialysis against buffered saline led to complete inactivation of specific passive hemagglutination.

Isolation and Characterization of Immunoglobulins.—Zone electrophoresis on starch of immune serum H-5 yielded the pattern shown in Fig. 1 *a*. The over-all

pattern differed from the patterns of mammalian sera in having a relatively low albumin peak. Passive hemagglutinating activity was localized in fractions around the origin. A small peak of material containing pink pigment was found to move towards the cathode; no antibody activity was associated with this material.

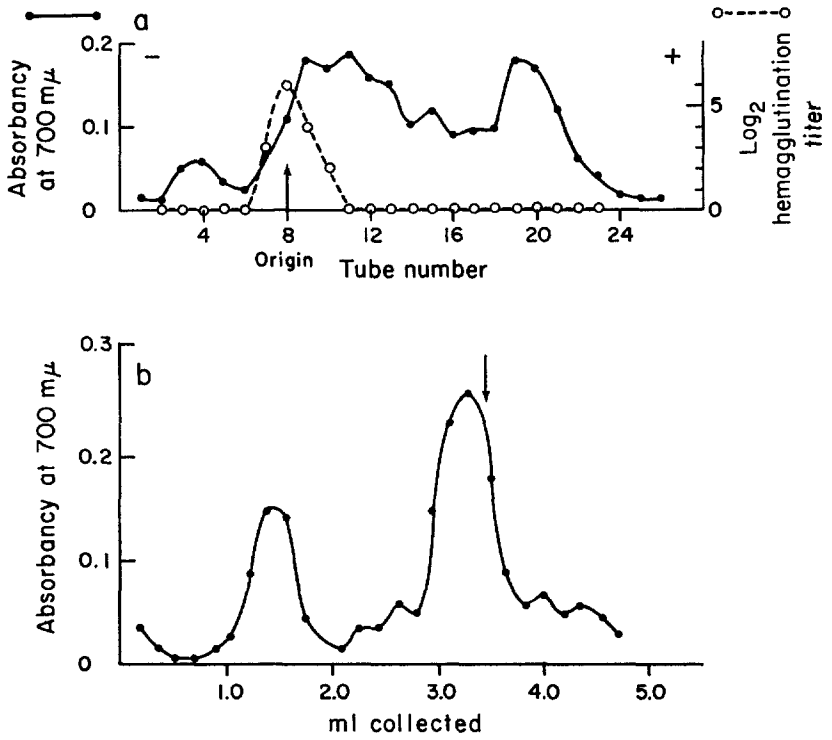

FIG. 1. Fractionation of dogfish serum.

(*a*) Zone electrophoresis on starch of serum from *M. canis* after immunization with *Limulus* hemocyanin. ●——● absorbancy of the Folin reaction at 700 mμ. O····O $\log_2$ of the passive hemagglutination titer. (+) anode, (−) cathode.

(*b*) Density gradient centrifugation of electrophoretic fractions 7 through 10. Sedimentation proceeded from right to left. ↓, position of alkaline phosphatase marker (~6S).

The fraction containing the specific hemagglutinating activity was concentrated by ultrafiltration and subjected to density gradient centrifugation (Fig. 1 *b*). Two components were resolved, one having a sedimentation coefficient of approximately 7S and the other having a sedimentation coefficient of about 17S. Together the two components were present in serum in a concentration of approximately 9.0 mg/ml. The ratio of 7S to 17S component was 1.6/1.0.

Another procedure used for isolating immunoglobulins from dogfish serum

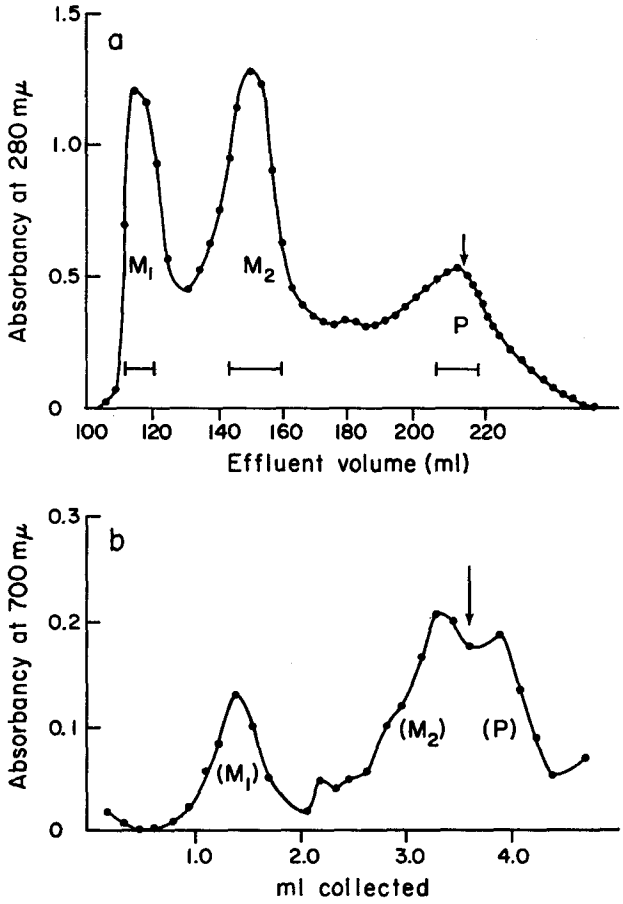

FIG. 2. Gel filtration and density gradient ultracentrifugation of fraction obtained by chromatography of dogfish serum on DEAE-cellulose.

(*a*) Gel filtration over sephadex G-200, ↓-position of alkaline phosphatase marker.

(*b*) Density gradient ultracentrifugation. Sedimentation proceeded from right to left. M_1, M_2, P refer respectively to the 17S, 7S, and 5S pigmented protein. Passive hemagglutinating activity was found only in fraction M_1.

was chromatography on DEAE-cellulose and single step elution with 0.0 M2 phosphate buffer, pH 7.2. This chromatographic fraction contained three components separable by gel filtration on sephadex G-200 and by density gradient ultracentrifugation (Fig. 2 *a* and *b*). In addition to the 7S and 17S peaks, a 5S shoulder was found in the ultracentrifugal pattern. This material was pink and appeared to correspond to the slowest moving peak found in electrophoresis of whole serum (Fig. 1 *a*).

The 17S fraction (M_1, Fig. 2 *b*) was found to contain all of the detectable hemagglutinating activity. In accord with these findings, only the first peak of material from the sephadex G-200 column (Fig. 2 *a*) showed hemagglutinating activity.

The 17S, 7S, and 5S components were compared by means of immunoelectrophoresis and double diffusion in agar, in which rabbit antiserum to dogfish serum (Fig. 3 *a*, *b*, and *c*) was employed. The 5S component moved farthest towards the cathode. The major precipitin lines of the 17S and 7S component fused completely with each other (Fig. 3 *c*). In addition to these major lines, the 17S component showed one fine line and the 7S component showed two lines. The materials corresponding to these lines could not be detected in the immunoelectrophoretic patterns and were not investigated further. It should be emphasized that in all cases the 5S component showed no cross-reaction with either the 17S or the 7S component (Fig. 3 *c*). Since antibody activity was located in the 17S component and the 7S and 17S components were antigenically related, both will be termed immunoglobulins.

An ultracentrifugal analysis of these immunoglobulins was performed (Fig. 4). Under the conditions indicated in the legend for Fig. 4, their sedimentation coefficients were found to be 6.7S and 17.1S (Fig. 4 *a* and *b*). In addition, the fraction containing 17.1S material showed a component with a sedimentation coefficient of 32.8S (Fig. 4 *b*). The pattern of dogfish serum (Fig. 4 *d*) also showed these components.

In view of reports that antibodies of lower vertebrates could be inactivated by reduction (24, 25), the 17.1S immunoglobulin was reduced and alkylated. After this treatment the sedimentation coefficient diminished to 7.2S (Fig. 4 *c*), suggesting dissociation into smaller units (20). This dissociation was confirmed by an additional experiment in which the crude DEAE fraction of antiserum H-5 was reduced and alkylated. All hemagglutinating activity was lost and comparison by density gradient ultracentrifugation showed a disappearance of the "17S" peak and concomitant increase in the "7S" peak (Fig. 5). Reduction of another sample of the DEAE fraction followed by reoxidation by dialysis against tris-NaCl showed only slight reconstitution of 17S material.

The Polypeptide Chain Structure of the Immunoglobulins of M. canis.—A number of methods were used to determine whether dogfish immunoglobulins consisted of light and heavy polypeptide chains. The principal approach was cleavage of disulfide bonds followed by exposure to dissociating solvents during fractionation (16, 19). Reduction and alkylation were performed both in the presence and absence of urea since it was found that complete dissociation could not be accomplished by reduction in the absence of urea.

In Fig. 6 are shown comparisons by starch gel electrophoresis in urea of human γG-immunoglobulins and dogfish immunoglobulins before and after reduction and alkylation. Although the failure to penetrate the gel reflects the size

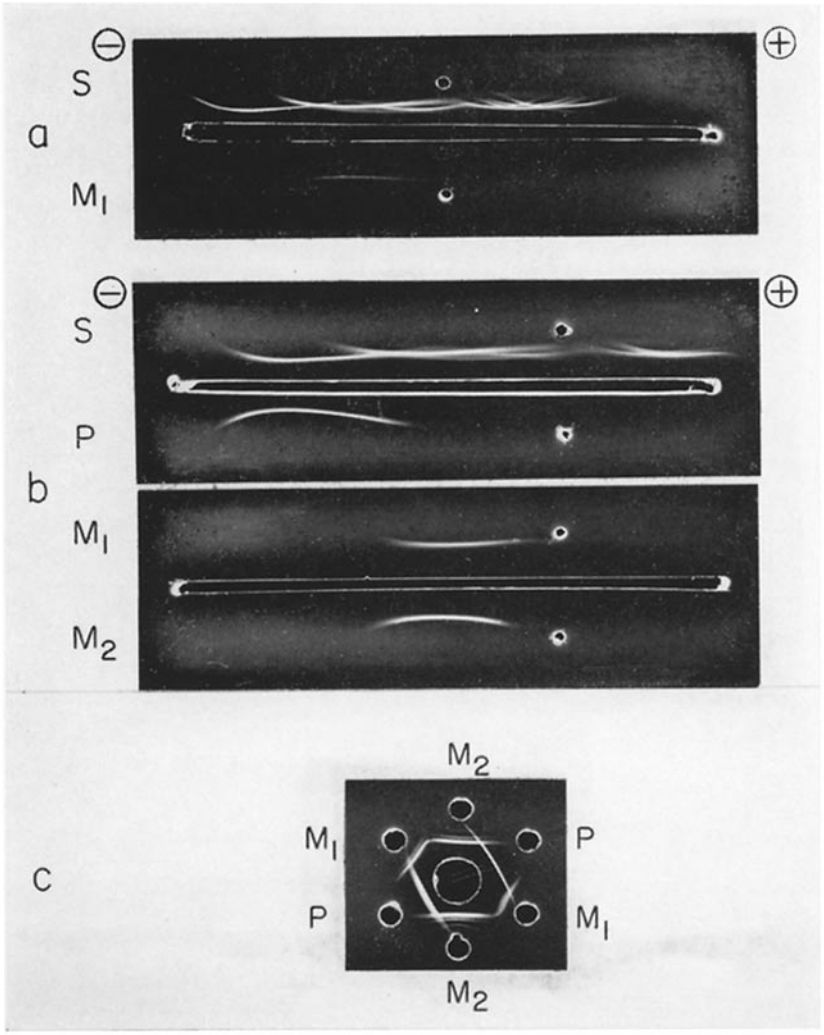

FIG. 3. Immunological comparisons of dogfish serum, dogfish immunoglobulins, and the 5S pigmented protein.

(*a*) Immunoelectrophoresis of whole serum (S) and fraction M_1 (17S immunoglobulin). Antiserum–rabbit antiserum 1 to dogfish serum. Electrophoresis proceeded for 2 hours.

(*b*) Immunoelectrophoresis of whole serum (S) and fractions M_1, M_2 and P (see Fig. 2). Antiserum–rabbit antiserum 2 to dogfish serum. Electrophoresis proceeded for 3 hours.

(*c*) Immune diffusion of immunoglobulins and 5S pigmented protein. Antiserum–rabbit antiserum 2 to dogfish serum. M_1—17S immunoglobulins; M_2—7S immunoglobulins; P—5S pigmented protein.

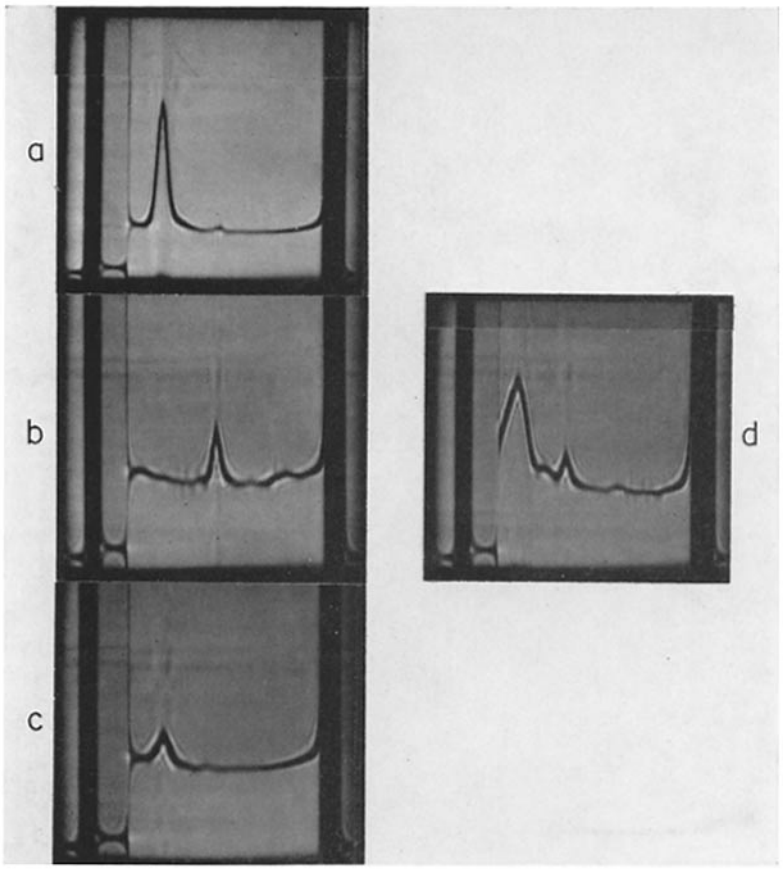

FIG. 4. Sedimentation velocity experiments on dogfish immunoglobulins and serum.
(*a*) 7S immunoglobulin (6.0 mg/ml)
(*b*) 17S immunoglobulin (2.3 mg/ml)
(*c*) reduced alkylated 17S immunoglobulin (1.2 mg/ml)
(*d*) whole serum (diluted to 8.6 mg/ml).
Solvent—tris-HCl buffer 0.05 M; pH 8, 0.15 NaCl; Speed—52,640 RPM. Time of photographs (*a*), (*b*), (*c*)—28 minutes; (*d*) 22 minutes. Phase plate angles—(*a*) 60°, (*b*) 45°, (*c*) 45°, and (*d*) 50°.

of the 17S immunoglobulin of the dogfish, the chain patterns of this protein and the 7S immunoglobulin from the same species were indistinguishable. After reduction and alkylation, both immunoglobulins showed bands corresponding to heavy chains and light chains. The heavy chains did not penetrate the gel as deeply as the heavy chains of γG-immunoglobulin (γ-chain) (4, 26). Their penetration corresponded to that obtained with heavy chains of human macroglobulins (μ-chains) (4). The bands of reduced alkylated dogfish immunoglob-

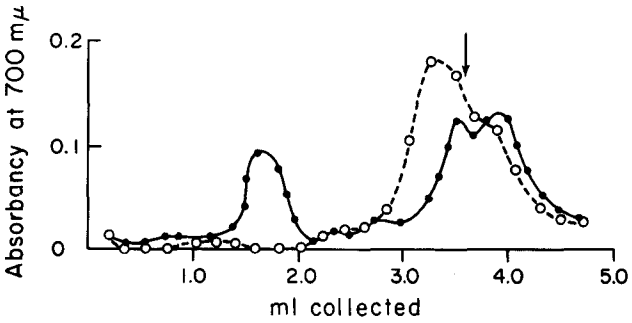

FIG. 5. Density gradient ultracentrifugation of chromatographic fraction of dogfish serum before and after reduction and alkylation. (Fraction was obtained by chromatography on DEAE-cellulose; compare with Fig. 2.) ●——● chromatographic fraction of immune serum H-5. At the concentration used, the entire fraction had a passive hemagglutination titer of 64. ○····○ chromatographic fraction after reduction and alkylation. No passive hemagglutinating activity was detected. Sedimentation proceeded from right to left. ↓ position of alkaline phosphatase marker.

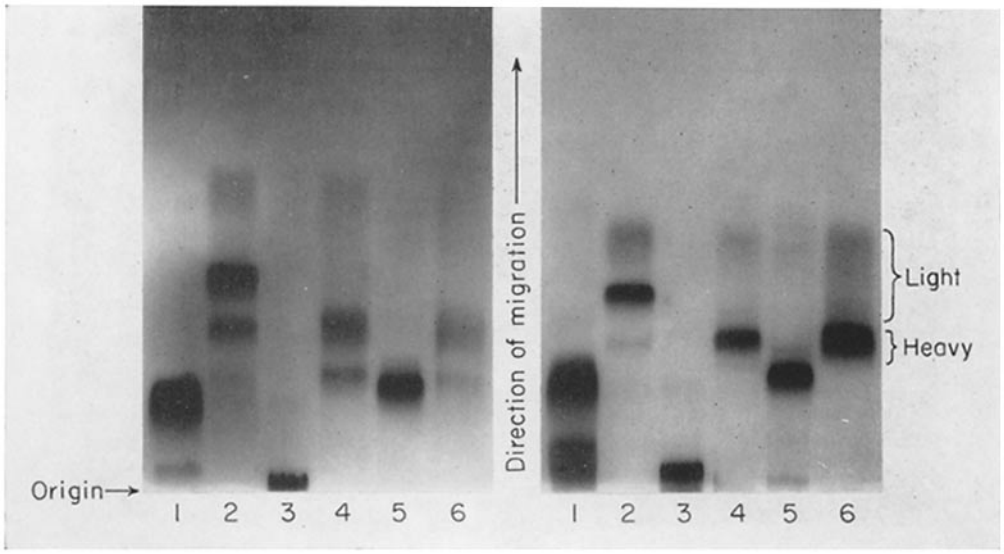

FIG. 6. Starch gel electrophoretic comparisons of untreated and reduced alkylated immunoglobulins of dogfish and man.

(*a*) Samples reduced in the absence of urea.

(*b*) Samples reduced in the presence of 8 M urea.

Origins: (1) Human γG-immunoglobulin, (2) reduced alkylated human γG-immunoglobulin, (3) dogfish 17S immunoglobulin, (4) reduced alkylated dogfish 17S immunoglobulin, (5) dogfish 7S immunoglobulin, (6) reduced alkylated dogfish 7S immunoglubulin. "Light" and "heavy" refer only to the light chain and heavy chain regions for sample in origin 6. (Electrophoresis was performed in 8 M urea formate buffer.)

ulins which corresponded to light chains were spread in a diffuse zone comparable to that of their counterparts in human immunoglobulins.

More complete reduction in 8 M urea yielded similar results (Fig. 6 *b*). In the case of the elasmobranch immunoglobulins, however, there was little or no evidence of residual bands corresponding to 7S material (compare Fig. 6 *a* and *b*, origins 4 and 6).

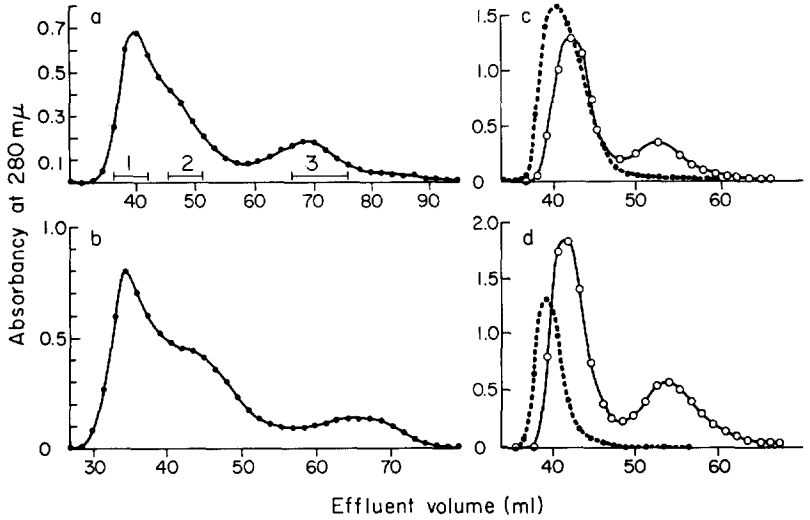

FIG. 7. Separation of polypeptide chains of dogfish immunoglobulins reduced in the pres ence and absence of 8 M urea (gel filtration over sephadex G-100).

(*a*) 17S immunoglobulin reduced and alkylated in tris-NaCl, pH 8.0. Gel filtration solvent—1.0 M propionic acid.

(*b*) 7S immunoglobulin reduced and alkylated in tris-NaCl, pH 8.0. Gel filtration solvent—1.0 M propionic acid.

(*c*) O——O 17S immunoglobulin reduced and alkylated in 8 M urea -●-●- untreated 17S immunoglobulin. Solvent—6 M urea, 1 M propionic acid.

(*d*) O——O 7S immunoglobulin reduced and alkylated in 8 M urea ●——● untreated 7S immunoglobulin. Solvent—6 M urea, 1 M propionic acid.

Additional evidence that reduction in the absence of urea did not result in complete dissociation into polypeptide chains was obtained by gel filtration in propionic acid (Fig. 7 *a*, *b*). After reduction in urea and fractionation on sephadex G-100 in 6 M urea, 1 M propionic acid, similar patterns were obtained for 7S and 17S immunoglobulins (Fig. 7 *c* and *d*). The position of peaks corresponding to heavy chains and light chains was the same for reduced alkylated 7S and 17S dogfish immunoglobulins. Under all conditions used, the untreated 7S and 17S immunoglobulins emerged within the void volumes of the columns. The ratio of yields on a weight basis of heavy chains and light chains was 2.7/1 for

the 17S immunoglobulin and 2.1/1 for the 7S protein. These ratios were calculated on the assumption that the extinction coefficients of the two types of chains were equal.

The chains obtained from partially reduced 17S immunoglobulin were compared by immunoelectrophoresis and double diffusion (Fig. 8 *a* and *b*). Light chains moved more towards the cathode than the heavy chains (Fig. 8 *a*). Both chains were antigenically unrelated to each other and showed antigenic deficiency with respect to the 17S immunoglobulins (Fig. 8 *b*). It should be noted

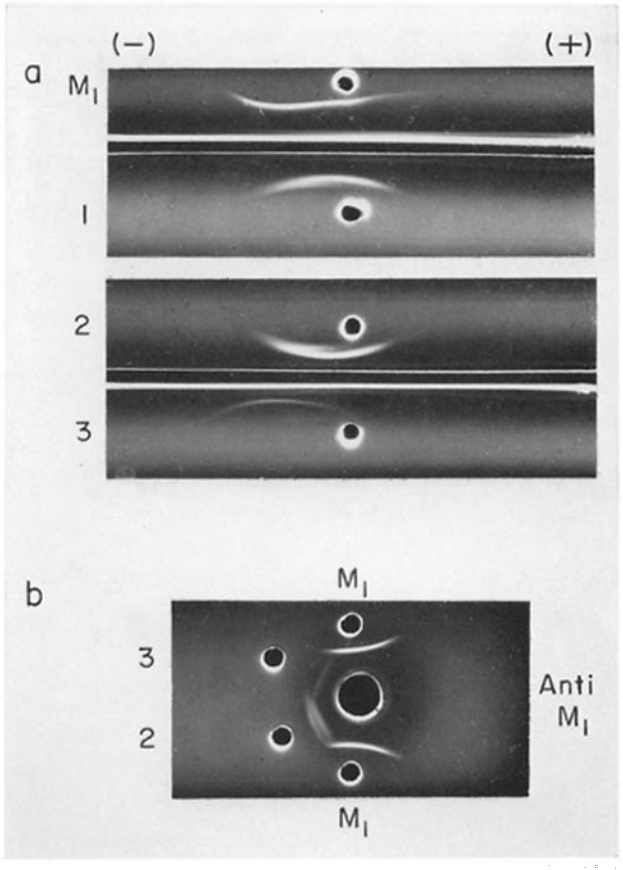

FIG. 8. Immunological comparisons of polypeptide chains of dogfish 17S immunoglobulin (chains were obtained from partially reduced samples; see Fig. 7 *a*).

(*a*) Immunoelectrophoresis of 17S immunoglobulin (M_1) and chain fractions obtained by gel filtration. 1, first fraction; 2, heavy chain fraction; 3, light chain fraction (see Fig. 7 *a*).

(*b*) Immune diffusion of 17S immunoglobulin (M_1), heavy chain fraction (2) and light chain fraction (3). Antiserum—rabbit antiserum to dogfish 17S immunoglobulin.

that in both experiments, there was evidence of contamination of heavy chain fractions with material having the antigenic determinants of light chains. In Fig. 8 *b* the heavy chain fraction shows an outer line which appears to fuse with the light chain line in the adjoining well.

Relation between the 7S and 17S Immunoglobulins.—The antigenic relationships between the 7S and 17S immunoglobulins, the similarity in the patterns of separation of their chains, and the dissociation of the 17S immunoglobulin to 7S units all suggested that the 7S immunoglobulin in dogfish serum is similar to or identical with the units of the 17S immunoglobulin. This relationship between 7S and 17S serum immunoglobulins is different in higher vertebrates in which the immunoglobulins belong to different classes. Each class contains dis-

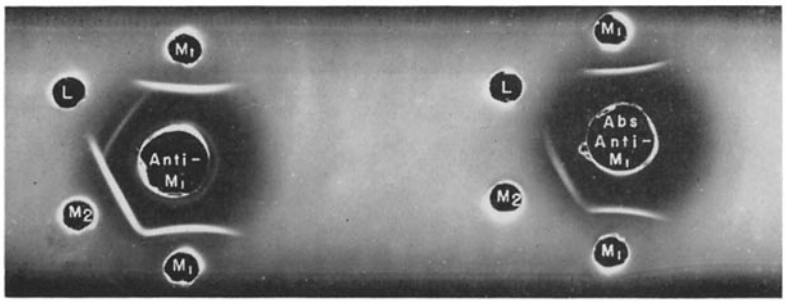

FIG. 9. Cross-reaction of 17S and 7S dogfish immunoglobulins.

M_1—17S immunoglobulin, M_2—7S immunoglobulin, L—light chains from 17S immunoglobulin.

Anti M_1—rabbit antiserum to dogfish 17S immunoglobulin, abs anti M_1—an aliquot of the same antiserum after complete absorption with light chains of 7S immunoglobulin.

tinctive heavy chains whereas the light chains are the common structural units among the different classes (3, 4).

To rule out the possibility that the observed antigenic identity of the 7S and 17S dogfish serum immunoglobulins resulted from the presence in the antiserum used of antibodies directed only against light chains, the following experiment was performed. The rabbit antiserum to 17S immunoglobulin was absorbed with light chains of the 7S immunoglobulin and the absorbed antiserum was used to compare the 7S and 17S immunoglobulins by double diffusion (Fig. 9). A complete fusion of the precipitin lines of 7S and 17S immunoglobulins was obtained with this absorbed antiserum.

A more specific comparison of the two major serum immunoglobulin components was made by two-dimensional high voltage electrophoresis of tryptic hydrolysates of their polypeptide chains (Fig. 10). In each case, the peptide maps of light chains were grossly different from those of heavy chains. On the other hand, a comparison of hydrolysates of corresponding chains from the 7S

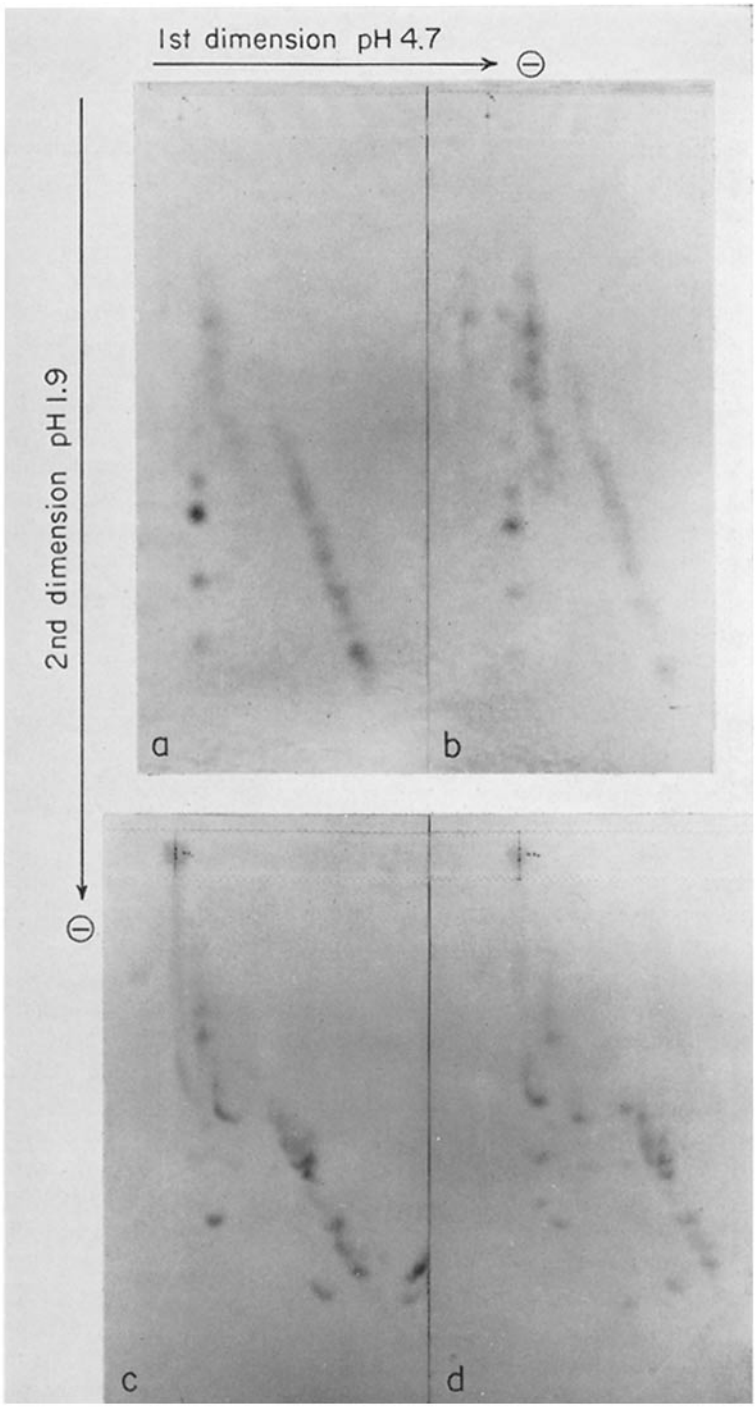

FIG. 10. Peptide maps obtained by two-dimensional high voltage electrophoresis of tryptic hydrolysates of polypeptide chains from dogfish 17S and 7S immunoglobulins.

(*a*) Light chains of 17S immunoglobulin

(*b*) Light chains of 7S immunoglobulin

(*c*) Heavy chains of 17S immunoglobulin

(*d*) Heavy chains of 7S immunoglobulin.

Origins are located at the upper left-hand corner of each pattern.

and 17S immunoglobulins showed similar patterns. Only slight differences were observed, notably in one or two ninhydrin positive spots in the peptide maps of hydrolysates of heavy chains.

DISCUSSION

Elasmobranchs arose from the placoderms in the Devonian period. They are among the most primitive of vertebrates (27, 28), and have diverged less than the bony fishes during the 300 million years since the Devonian. A number of preliminary reports (8, 25, 29, 30) have indicated that elasmobranchs respond to immunization by producing specific antibodies of high molecular weight, the activity of which is destroyed by treatment with mercaptoethanol. In accord with these observations, we have found that, within one month after injection of *Limulus* hemocyanin, *M. canis* produces specific 17S antibodies having electrophoretic mobilities of so-called "fast γ-globulins." Reduction of these 17S immunoglobulins resulted in a decrease of the sedimentation coefficient to approximately 7S with concomitant loss of antibody activity.

The main purpose of the experiments described in the present paper was to establish whether the immunoglobulins of *M. canis* were structurally similar to those of higher vertebrate species (3, 4, 5, 31). This required isolation and characterization of immunoglobulins from normal and immune dogfish. Dogfish serum was found to contain both 7S and 17S immunoglobulins. The total concentration of immunoglobulins in the sera was about 9 mg per ml, and the ratio of concentrations of 7S to 17S immunoglobulins was about 1.5 to 1.0. Both of the serum components were antigenically indistinguishable when examined by immunoelectrophoresis and double diffusion in agar using rabbit antisera to whole dogfish serum and to the 17S immunoglobulin.

Reduction and alkylation of the 17S and 7S immunoglobulins followed by exposure to dissociating solvents resulted in separation of heavy and light polypeptide chains. Starch gel electrophoresis and gel filtration in dissociating solvents of the reduced alkylated immunoglobulins showed patterns of fractionation which closely resembled those of similarly treated immunoglobulins of higher vertebrates (4, 5, 12, 16, 17, 31). In both methods of fractionation, the 7S and 17S dogfish immunoglobulins gave very similar patterns; the relative yields of light chains varied from 27 to 32 per cent. From the yields of the chains and a comparison of their behavior after starch gel electrophoresis and gel filtration, it seems likely that the 7S component consists of two light and two heavy chains. The 17S component would most probably consist of five or six 7S units. Studies are in progress to verify these assignments.

Two additional observations are significant in comparing dogfish immunoglobulins with those of higher vertebrates. The starch gel electrophoretic mobility of the heavy chains of the 17S and 7S dogfish immunoglobulins was slower than that of the γ-chains of human γG-immunoglobulin. In this respect, the heavy chains of dogfish immunoglobulins resemble μ-chains of human and rab-

bit γM-immunoglobulin (4, 26). Preliminary starch gel electrophoretic experiments have indicated that μ-chains from rabbit immunoglobulin and heavy chains from the dogfish have the same mobilities. The second comparative observation of interest is that the starch gel electrophoretic bands of light chains of dogfish immunoglobulins were diffuse rather than sharp. This suggests that they are chemically heterogeneous, like their counterparts in higher forms (3, 4, 16, 31).

As shown by the peptide maps, the light and heavy chains of *M. canis* immunoglobulins differ grossly in primary structure. The peptide maps of corresponding light and heavy chains of the 7S and 17S immunoglobulins were similar, however. This similarity is in accord with the antigenic identity of the 7S and 17S serum components. Taken together with the fact that the 17S protein may be dissociated into 7S units, these findings suggest that the two serum components belong to the same immunoglobulin class (*i.e.*, have similar kinds of heavy chains) (4, 26, 31, 32). On the basis of properties so far established, this class most closely resembles the class of γM globulins found in higher vertebrates. Although the presence of trace amounts of other immunoglobulins cannot be excluded (see Fig. 3), *M. canis* is unlike the higher vertebrates in having one major class of immunoglobulins rather than three.

An interesting possibility emerges from these findings: the classes of molecules corresponding to γA- and γG-immunoglobulins may have emerged at later stages of vertebrate evolution than that represented by the elasmobranchs. This emergence would correspond to a selection for structural genes coding for heavy chains (α- and γ-chains) belonging to these classes (26, 31, 32). Unlike the dogfish, higher vertebrates do not show large amounts of a 7S serum component of the same class as γM-immunoglobulin. After immunization of individuals in these higher species, serum antibodies first appear in the 19S γM-immunoglobulins and subsequently in the 7S γG-immunoglobulins (33). The early appearance of γM-antibodies in response to immunization may be a reflection of the fact that γM-immunoglobulin appears to be the most primitive of the antibody classes. Thus, the sequence (*a*) γM-antibodies (*b*) γG-antibodies seen among immunoglobulins of higher forms may be related to the order of their evolutionary appearance.

A precisely similar sequence of events apparently is not possible in the dogfish. It still may be, however, that the 7S serum immunoglobulin of the dogfish would show antibody activity after prolonged immunization. Poikilothermic vertebrates such as the goldfish and the frog (24) show initial production of rapidly sedimenting antibodies followed by production of more slowly sedimenting antibodies. The high molecular weight antibodies persist for 3 to 5 months, however, and it has not been established whether the more slowly sedimenting antibodies of the goldfish and frog belong to a different immunoglobulin class than the more rapidly sedimenting antibodies.

The evolutionary emergence of separate classes of immunoglobulins among

the vertebrates obviously deserves further exploration. The present studies emphasize the connection of this problem to that of the relationship of the multichain structure of antibodies to their function. The fact that the multichain structure is an ancient evolutionary development is consistent with the notion (3, 32) that such a structure may be necessary for the emergence of antibody specificity.

SUMMARY

The elasmobranch *Mustelus canis* has been shown to produce antibodies to *Limulus* hemocyanin. The serum of both normal and immunized *M. canis* contains immunoglobulins having sedimentation coefficients of approximately 7S and 17S. Antibody activity was found in the 17S immunoglobulin which may be dissociated to 7S components with concomitant loss of activity. Both 17S and 7S serum immunoglobulins were antigenically identical. They consisted of light and heavy chains present in amounts comparable to those of higher vertebrates. Peptide maps indicated that the light chains had an entirely different primary structure than the heavy chains, but that the corresponding chains of 7S and 17S dogfish serum immunoglobulins were similar in primary structure. The heavy chains appeared to resemble the μ chains of immunoglobulins of higher vertebrates in their starch gel electrophoretic behavior. It is suggested that the elasmobranch *M. canis* may have only one major class of immunoglobulins resembling that of macroglobulins (γM-immunoglobulins) seen in higher vertebrates.

The results indicate that the multichain structure of antibodies is an ancient evolutionary development.

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