PHYLOGENY OF IMMUNOGLOBULIN STRUCTURE AND FUNCTION

V. Valences and Association Constants of Teleost Antibodies to a Haptenic Determinant*

By L. W. CLEM, Ph.D., and P. A. SMALL, Jr., M.D.

(From the Department of Microbiology, College of Medicine, University of Florida, Gainesville, Florida 32601 and Marineland Research Laboratories, Marineland, Florida 32074)

(Received for publication 31 March 1970)

Numerous reports have now described the structural relationships between the immunoglobulins of various lower and higher vertebrates (see reference 1). However, an important void in our knowledge of lower vertebrate antibodies is that of valences and association constants. Since quantitative data of this type can only be readily obtained by using haptens and specifically purified antibodies, studies on the antibody responses of various species of lower vertebrates have been initiated. Elasmobranchii (sharks), fish (both holosteans and teleosteans), amphibians (marine toads), reptiles (alligators), and fowl (chickens) can make antibody to the 2,4-dinitrophenyl (DNP)[1] determinant (2). Teleosts in general appeared to synthesize higher levels of antibody to this determinant than did the other taxonomic groups. Thus we have chosen to focus initially on teleost antibodies to DNP. For these studies the giant grouper, *Epinephelus itaira*, was chosen; the use of this species instead of many of the smaller teleosts circumvents the need for using pooled sera.

This report describes the isolation of grouper antibodies to DNP and the determinations of their valences and average intrinsic association constants. The physicochemical properties of grouper antibodies and immunoglobulins are considered in another paper.[2]

Materials and Methods

Preparation of Antigens.—Dinitrophenylated bovine gamma globulin (DNP_{50}-BGG) and dinitrophenylated bovine serum albumin (DNP_{40}-BSA) were prepared and the degrees of substitution characterized spectrophotometrically as described by Eisen (3).

Immunization of Groupers.—Two giant groupers, weighing about 250 pounds each, designated fishes 3 and 4, were used. These animals are two of the numerous marine animals on

* Supported by National Institutes of Health Research Grant AI 07713-03 and National Science Foundation Grant GB 8632.

[1] *Abbreviations used in this paper:* BBOT, 2,5-bis[2-(5-tert-butyl-benzoxazolyl)]-thiophene; BGG, bovine gamma globulin; BSA, bovine serum albumin; DNP, dinitrophenyl; EACA, ϵ-amino caproic acid.

[2] Clem, L. W. 1970. Phylogeny of immunoglobulin structure and function. IV. Immunoglobulins of the giant grouper. Submitted for publication.

exhibit in one of the large show-tanks at Marineland of Florida. This tank (400,000 gal) is constantly replensihed with fresh sea water and the temperature is that of the adjacent surf (21°–26°C). The animals are fed daily on a diet of raw fish and have lived in this tank for over 10 yr.

The fish were injected subcutaneously on the dorsal surface in four sites with a total of 10 mg of alum-precipitated (4) DNP–BGG on day 0. Subsequent antigen injections, also given in multiple sites, were as follows: 16 mg at 1 wk, 20 mg at 3 wk, and 20 mg at 1, 2, 4, 10, and 12 months. The animals, under MS-222 anesthesia (Sandoz Pharmaceuticals, Switzerland), were bled from the caudal vessels of the hemal arch by using 8 inch, 15-gauge needles and 300 ml vacuum bottles; 600–900 ml of blood could be obtained from each fish at any bleeding. The blood was allowed to clot at room temperature for 3–5 hr and overnight in the cold; serum yields usually approximated 40% of the blood volume. Sera were stored at −20°C.

Precipitin curves were carried out as follows: 0.3 ml samples of grouper serum were mixed with equal volumes of varying concentrations of DNP–BSA in NaCl—[tris(hydroxymethyl)-aminomethane](Tris)buffer,[3] at 37°C for 1 hr followed by 2.5°C for 48 hr. The washed immune precipitates were then assayed for protein content employing the Folin-Ciocalteu reagent (5) using rabbit IgG as a standard. Equivalence points were estimated by supernatant analysis using immunodiffusion in agar gel. Serum antibody concentrations were estimated by subtracting the antigen precipitated at equivalence from the total protein precipitated.

Purification of Specific Antibody—Grouper antibodies specific for the DNP determinant were purified according to the method of Farah et al. (6) with one exception, as noted below. Washed specific precipitates formed at equivalence with DNP–BSA were extracted with 0.1 M 2,4-dinitrophenol (DNP–OH), (Fisher Scientific Co., Fairlawn, N.J.) in NaCl–Tris buffer, pH 8.0 at 37°C for 1 hr; streptomycin (35 mg/ml) was employed to precipitate the DNP–BSA. Initial experiments employed Dowex 1 × 8 (200–400 mesh) anion exchange columns to remove the DNP–OH. However, considerable loss of antibody (especially macroglobulin) was noted and this method was discontinued. An effective way of removing the DNP–OH, and simultaneously separating gouper antibodies of differing molecular weights, was found to be Sepharose 4B (Pharmacia Fine Chemicals, Inc., Upsala, Sweden) gel filtration. An upward flow 2.5 × 100 cm column equilibrated with NaCl–Tris buffer, pH 7.4, at 2.5°C (flow rate = 2 ml/cm^2 per hr) was employed for this purpose.

Antibody preparations were concentrated by positive pressure dialysis to 2–5 mg/ml and stored at 2.5°C until used. These preparations were determined to be free of measurable DNP–BSA (absorbancies at 360 mμ were less than 1% of that at 280 mμ) and specific antibody accounted for greater than 70% of the protein present; this is discussed in detail in the Results section.

Equilibrium Dialysis.—Equilibrium dialysis was performed essentially as described by previous workers (3) using tritiated 2,4-dinitrophenyl-ε-amino caproic acid. Lucite cells in which the two compartments were separated by a dialysis membrane (cellulose tubing, size 36, Union Carbide Corp., Chicago, Ill.) were employed; the antibody side usually contained 0.3–0.4 ml of 0.4–0.6 mg/ml of antibody solution and the hapten side contained a similar volume of hapten-containing solution (between 1.59×10^{-6} M and 84.4×10^{-6} M). The solvent for these experiments was the previously mentioned NaCl–Tris buffer, pH 7.4. The cells were rotated (radius ~ 30 cm, 3–4 rpm) at 2.5°C for 48 hr to equilibrium; in experiments conducted at 18.5° and 36.5°C rotation for 24–26 hr was used. Duplicate 100 μl samples were then counted for tritium using 10 ml BBOT scintillation fluid (Packard Instrument Co., Inc., Downers Grove, Ill.) in a Packard Tri-Carb liquid scintillation spectrometer. Each experimen-

[3] The NaCl-Tris buffer used here (and throughout this paper) consisted of 0.14 M NaCl, 0.01 M Tris-HCl, pH 7.4, and 0.01 M EDTA.

tal point depicted in the Scatchard plots reported here consists of the average of duplicate 10 min counts of duplicate samples from duplicate cells i.e., 8 individual determinations. Control experiments indicated that corrections for quenching by the protein were unnecessary. Similarly, control experiments with ^{125}I-labeled grouper antibodies showed protein binding to the cells or dialysis membrane to be undetectable.

^{3}H-DNP–EACA was prepared by reacting ^{3}H-dinitrochlorobenzene (87 mCi/mM, New England Nuclear Corp., Boston, Mass.) with excess ϵ-amino caproic acid (K&K Laboratories, Plainview, N.J.) overnight under alkaline conditions. The radiolabeled DNP–EACA was purified by repeated acid precipitation and quantified spectrophotometrically at 360 mμ assuming a molar extinction coefficient of 17,400 at pH 7.4 (3).

The concentration of grouper antibodies was determined by assuming similar extinction coefficients to those determined for the immunoglobulins from this species.[2] The 16S immunoglobulin $\epsilon_{280\ m\mu}\ {}^{2\%}_{2cm} = 13.8$ in saline, the 6.4S immunoglobulin $\epsilon_{280\ m\mu}\ {}^{1\%}_{1cm} = 16.6$ in saline. The molecular weight of the 16S antibody was determined to be $\sim$ 700,000 and that of the 6.4S antibody to be $\sim$ 120,000.[2]

Physicochemical Methods.—Analytical ultracentrifugation, immunodiffusion, and immunoelectrophoresis tests were conducted as previously described (7).

Radioiodination of antibody and immunoglobulin preparations was accomplished with ^{125}I (New England Nuclear Corp.) using the Chloramine T (Matheson, Coleman and Bell, Cincinnati, Ohio) method (8).

RESULTS

Antibody Response of Groupers to DNP–BGG.—Sera obtained from each of the two immunized groupers at 3 wk and at 1, 2, 4, 10, 12, and 22 months were examined qualitatively for precipitating antibody by immunodiffusion in agar gels. Each serum, with the exception of the 22 month serum from fish 3, showed precipitating antibodies in agar gels to DNP–BGG; normal serum did not precipitate with DNP–BSA or DNP–BGG. Since none of these sera formed visible precipitates with BGG or BSA and since 0.1 M DNP–OH inhibited precipitate formation, it was assumed that the antibody present was specific for the DNP determinant.

Quantitative precipitin curves using DNP–BSA as the antigen were employed to measure the concentration of antibody present. A representative grouper anti-DNP–BGG precipitin curve with DNP–BSA (Fish 3, 1 month) is depicted in Fig. 1. For comparative purposes this figure also contains results obtained with a rabbit antiserum containing antibodies (predominantly IgG) to DNP. The rabbit antibody curve (including the formation of soluble complexes in antigen excess) is very similar to that obtained by others with rabbit antiserum. In contrast the teleost curve showed little or no inhibition of precipitation in antigen excess and is thus more similar to the curves obtained with mammalian macroglobulin antibodies (9, 10). Attempts at forming soluble complexes with grouper antiserum in extreme antigen excess (> 10 times equivalence) have been severely hampered by the formation of nonspecific (even in normal serum) precipitates in the presence of such high antigen concentrations.

The quantitative data from each of the grouper sera studied are presented in Table I. The anti-DNP levels range from a high level of 300 μg/ml to a low of 75 μg/ml. It should also be noted that these levels appear to persist for long periods of time. For example fish 3, when "boosted" at 4 months, contained

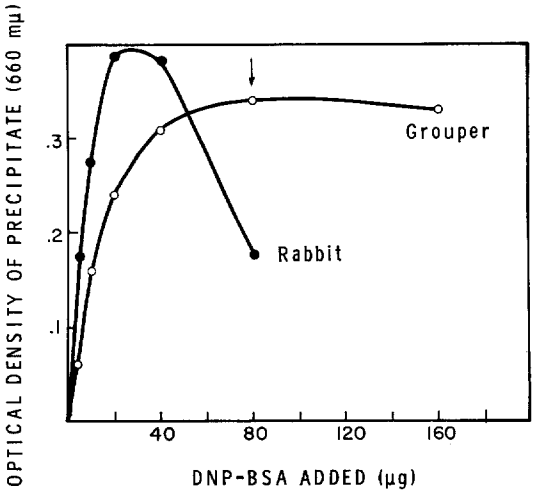

FIG. 1. Representative quantitative precipitin curves of grouper (○——○) and rabbit (●——●) anti-DNP–BGG with DNP–BSA. The arrow indicates the equivalence point for the grouper antiserum.

TABLE I

Antibodies from Groupers Immunized with DNP-BGG

Fish No.	Bleeding time	Serum antibody	Yields of antibody		Antibody purity		Average intrinsic association constant	
			16S	6.4S	16S	6.4S	16S	6.4S
	months	*μg/ml*	*μg/ml serum*	*μg/ml serum*	%	%	$K_0 \times 10^6\ M^{-1}$	$K_0 \times 10^6\ M^{-1}$
3	1	230	50	40	81 (78)§	75	2.1*	3.0‡
	2	300	130	40	93	83	2.2	2.5
	4	250	120	70	90	81	0.1	0.4
	10	200	90	70	70	60	~0.2	~0.4
	12	200	90	60	87	83	~0.06	~0.4
4	1	350	120	60	82	80	2.3	8.8
	4	175	40	40	79	66	<0.01	~0.1
	12	70	20	20	64	71	<0.01	~0.02
	24	180	110	50	86	72	<0.01	~0.01

* Assuming $n = 4$.

‡ Assuming $n = 1$.

§ Calculated from quantitative precipitin data.

250 μg/ml and 6 months later, with no additional immunization, still contained 200 μg/ml.

Purification of Grouper Antibodies to DNP.—Sepharose gel filtration of the supernatants from washed specific precipitates extracted with DNP–OH routinely yielded three separate fractions. One such Sepharose gel filtration profile is depicted in Fig. 2; much of the DNP–OH from this dissolved precipitate (fish 3, 1 month) was removed by dialysis prior to its application to the Sepharose column. The first two peaks, labeled 16 and 6.4S (discussed below), contained grouper antibodies to the DNP determinant. The third peak contained

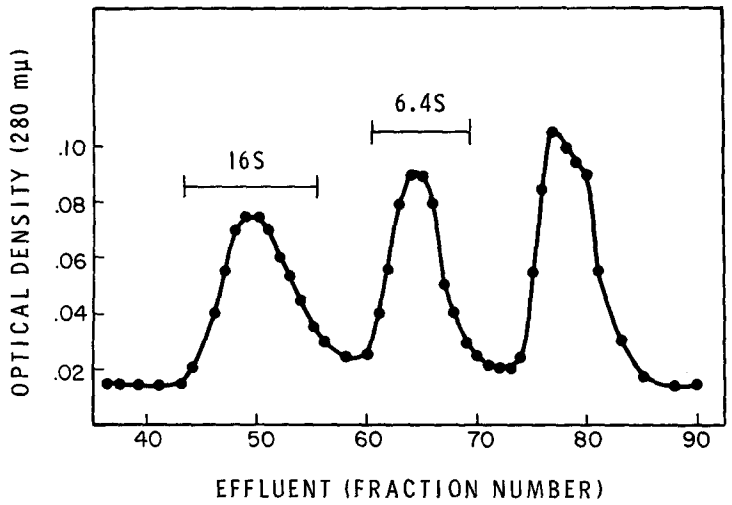

FIG. 2. The optical density elution profile obtained by Sepharose gel filtration of grouper antibodies extracted from immune precipitates with 0.1 M DNP-OH.

free DNP–OH. The serum depicted here yielded about 50 μg of 16S protein and 40 μg of 6.4S protein per ml serum. Thus, by quantitative precipitin analysis this serum contained 230 μg/ml of anti-DNP and represents ~40% recovery of antibody. The ratios of 16:6.4S protein and the per cent recoveries from other bleedings are summarized in Table I. Most of the recoveries were about 50%, although some were as high at 75–89%; these latter cases yielded only antibody of low affinity. One additional item of note here is that, while the weight ratio of 16:6.4S protein isolated ranged from 3.3 (fish 3, 2 months) to 1.0 (fish 4, 4 and 12 months), there was no discernible shift from high molecular weight to low molecular weight antibodies between 1 month and 2 yr of immunization.

Assessment of Antibody Purity.—The purity of each antibody preparation was determined by two different approaches, (*a*) physico- and immunochemical study and (*b*) antigen binding activity. Each purified antibody preparation was

studied for mass homogeneity in the analytical ultracentrifuge. The schlieren patterns of two such preparations (fish 3, 1 month) are presented in Fig. 3. The $S_{20,w}$ of each macroglobulin antibody preparation was 15–16S at concentrations of 3–5 mg/ml and all preparations were homogeneous by this criteria. The low molecular weight grouper antibody was also homogeneous in the ultracentrifuge and had an $S_{20,w}$ of $\sim$ 6.4S (at 3–5 mg/ml).

Antigenic analysis for purity using rabbit antisera prepared against whole grouper serum was performed by immunoelectrophoresis and immunodiffusion. As can be seen in Fig. 4 (fish 3, 1 month) both 16 and 6.4S antibody preparations appeared pure within the limits of the method and to have similar fast gamma mobilities. Other preparations were similar, i.e., there were no noticeable changes with prolonged immunization. Similarly Ouchterlony analysis showed

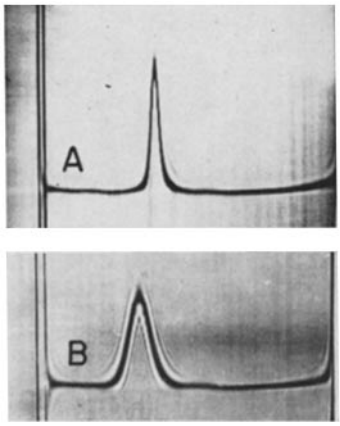

FIG. 3

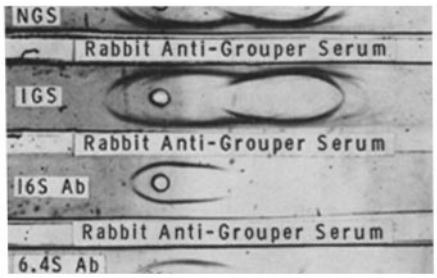

FIG. 4

FIG. 3. Schlieren patterns of grouper anti-DNP 16S (A) and 6.4S (B) antibodies after extraction from immune precipitates and Sepharose gel filtration. The concentration of each protein was $\sim$ 5 mg/ml.

FIG. 4. Immunoelectrophoretic examination of grouper serum and specificially purified antibodies. Abbreviations used: NGS, normal grouper serum; IGS, immune grouper serum. Concentrations of reagents: sera were used undiluted; 16S and 6.4S antibodies were used at $\sim$ 5 mg/ml.

each preparation to be pure by this criteria; Fig. 5 presents typical results of such studies (fish 3, 1 month). The gel diffusion studies also show that the 6.4S protein is antigenically deficient relative to the 16S protein and that the 16S antibody readily precipitates with DNP–BSA whereas the 6.4S antibody does not. The possible reasons for these findings are discussed below.

Purity of the grouper anti-DNP preparations was assessed by determining the extent of binding of ^{125}I-labeled antibody to small ($\sim$ 0.3 ml) columns of ϵ-DNP-lysine coupled to Sephadex G25 (Pharmacia Fine Chemicals, Inc.,

Upsala, Sweden) (11). ^{125}I-labeled normal grouper 16 and 6.4S immunoglobulins adsorbed to such a column only about 1–2% (of 20 μg of labeled protein applied) in the presence of 5% normal grouper serum or 0.5% gelatin; the normal serum or gelatin was necessary to prevent nonspecific adsorbtion. Under similar conditions radiolabeled specific antibodies adsorbed to the immunoadsorbant columns as indicated in Table I. These values ranged from a high of 93% to a low of 65%. These estimates of purity are probably only minimal in that denaturation during the radiolabeling procedure would result in a low value. The 6.4S antibody, which has a measurable valence of about one, may not adsorb as well as the multivalent 16S antibody. The denaturation argument would not, however, appear to be a serious objection as shown in the

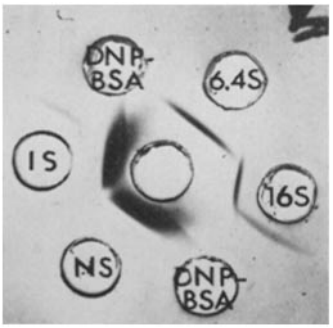

FIG. 5. Ouchterlony examination of specifically purified grouper antibodies. The center well contained rabbit antiserum to whole grouper serum. Abbreviations used: IS, immune serum (to DNP–BGG); NS, normal serum. Concentration of reagents: sera were used undiluted; 16 and 6.4S antibodies were used at $\sim$ 3 mg/ml; DNP–BSA was used at $\sim$ 1 mg/ml.

results of the following experiment. The unlabeled 16S antibody preparation from fish 3, 1 month was precipitated as suggested by Farah et al. (6) at 1 mg/ml with varying concentrations of radiolabeled DNP–BSA. Analysis of the precipitates indicated that 78% of the antibody in this preparation was precipitable with DNP–BSA, thus comparing favorably with the 81% binding by the immunoadsorbant method.

One additional experiment was performed to ensure that the purified antibody did not contain significant amounts of DNP–OH. 1 mg of purified 16S antibody (fish 3, 1 month) was dialyzed for 72 hr at 2.5°C in an equilibrium dialysis cell against 10^{-3} M ^{3}H–DNP–EACA. The antibody-side contents were then subjected to Sepharose gel filtration and the 16S peak concentrated to 0.1 ml. This entire concentrate was then counted for ^{3}H in duplicate vials and none was detected. If one-half mole of ^{3}H–DNP–EACA were found per mole of protein, about 2000 counts/10 min per vial would have been anticipated instead of the observed 75 (background level). Since counts about twice back-

ground level would have been easily detectable there was less than 0.02 moles of bound hapten/mole of antibody.

Valences and Association Constants of Grouper Antibodies to DNP.—Each of the grouper anti-DNP preparations was studied by equilibrium dialysis employing ^{3}H-ε-DNP–amino caproic acid as hapten. Scatchard plots of the data (uncorrected for antibody purity) from these experiments are presented in Fig. 6. The 16S antibody preparation from fish 3, 2 months, showed what may

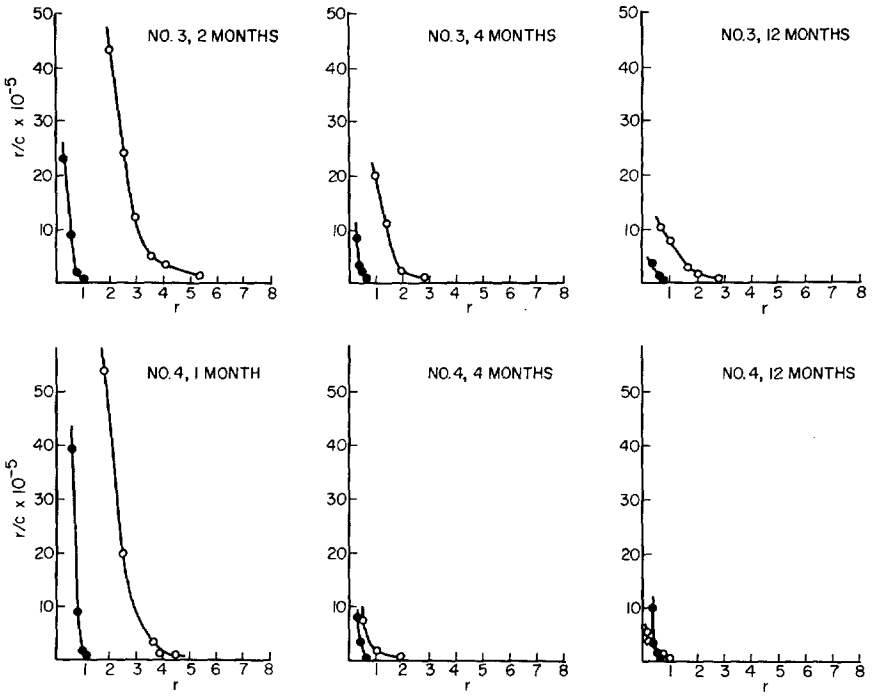

FIG. 6. Scatchard plots of equilibrium dialysis data obtained with grouper antibodies to DNP and ^{3}H-DNP-ε-amino caproic acid at 2.5°C.

be interpreted as representing at least two distinct populations of combining sites of differing association constants (K_a). The higher K_a population extrapolated to a valence of about four and thus the average intrinsic association constant (K_o) of this population of sites would be 2.2×10^6 M^{-1} (obtained from the relation $K_o = 1/c$ when one-half the antibody sites are occupied, i.e., 2.0 for this population). The low K_a population of sites, of which there also appeared to be four, had a K_o of $\sim 8 \times 10^4$ M^{-1} or about 4% that of the high K_a sites. The data presented here are based upon the assumption that all the protein used was antibody and if corrections are made to minimal estimates of

purity, the observed values are increased by only 7%. Similarly, the high K_o (based upon $n = 4$) would only change from 2.2×10^6 to 2.7×10^6 M^{-1}.

The Scatchard plot (Fig. 6) of the data obtained with the 16S antibody preparation from the 1 month bleeding of Fish 4 (and also that from the 1 month

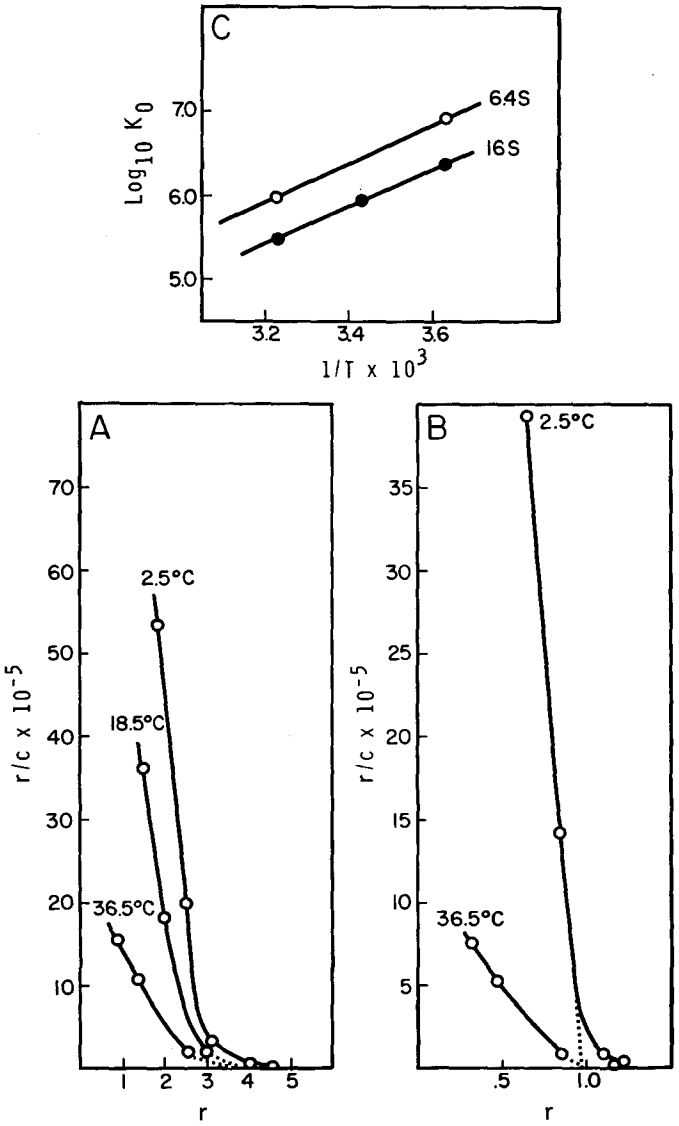

FIG. 7. Results obtained by equilibrium dialysis of grouper antibodies to DNP and ^{3}H-DNP-ε-amino caproic acid at different temperatures. A. Scatchard plot of 16S antibody. B. Scatchard plot of 6.4S antibody. C. Arrhenius plot of both antibodies.

bleeding of fish 3, not shown here) showed a similarly shaped curve to that described above. Assuming four for the valence of the high K_a sites, K_o was calculated for this preparation as done for similar sites with the 16S preparation from fish 3, 1 month. These data are summarized in Table I and the K_o constants are $\sim 2 \times 10^6$ M^{-1}. Although low K_a sites obviously exist for this population of antibody molecules, it was impossible to quantify them; however, the K_o is probably $\sim 10^4$ M^{-1}. The total valence for these antibody populations appears to be between six and eight.

16S antibody preparations obtained at later times from these two animals have grossly lower K_o values (see Fig. 6). These data are also summarized in

TABLE II

*Thermodynamic Results for the Binding of ε-DNP-Amino Caproic Acid by Grouper 6.4S and 16S Antibodies**

Antibody preparation	Temp.	ΔF°	ΔH°	ΔS°
	°C	(*K cal/mole*)	(*K cal/mole*)	(EU/*mole*)‡
6.4S	2.5	10.0	15.6	22.8
	36.5	9.3		
16S	2.5	9.5	15.7	22.8
	18.5	9.4		
	36.5	9.2		

* Obtained from fish 4, 1 month.

‡ Entropy unit/mole.

Table I. One problem with these late preparations is deciding upon the antibody valence. For example, the highest valence measured for the 12 month bleeding from fish 4 was 1.2 and for the 24 month bleeding it was only 0.9. Thus the indicated K_o constants in Table I were calculated for high K_0 sites assuming the valence to be four (see Discussion section). Control experiments with normal grouper 16S immunoglobulin (prepared as described in footnote 2) indicated nonspecific binding of hapten to be much less. No binding was observed with the lower initial hapten concentrations; higher hapten concentrations showed "r's" (from Scatchard plot) less than 0.5.

The Scatchard plots of the data obtained for the various 6.4S antibody preparations are also depicted in Fig. 6 and summarized in Table I. The curvature of these plots indicate heterogeneity of binding and is suggestive of high and low K_a sites. The valences of the high K_a sites for the early bleedings of each animal appear to extrapolate close to one. This is also consistent with the lack of precipitation seen in Fig. 5. Calculations of K_o (assuming $n = 1$) indicate a value of from 1.2×10^6 to 8×10^6 M^{-1}. 6.4S antibody preparations obtained at later times showed perceptible lowering of the K_o constants (still assuming n

= 1). It is also important to note that the K_o for each 6.4S antibody preparation was only slightly higher than that of the corresponding 16S antibodies.

Thermodynamic properties of grouper antibodies to DNP.—Association constants for the binding of DNP-ε-amino caproic acid by grouper 16 and 6.4S antibodies, isolated from fish 4, 1 month bleeding, were determined by equilibrium dialysis at differing temperatures. Scatchard plots and the temperature dependence of the K_o constants (assuming $n = 4$ for the 16S preparation and $n = 1$ for the 6.4S preparation) are shown in Fig. 7. Thermodynamic values, calculated from these data in the usual fashion, are given in Table II.

DISCUSSION

The data presented here indicate that the grouper can synthesize at least two different sizes of antibody molecules to the DNP determinant. The synthesis of high and low molecular weight antibodies to a variety of complex antigens has previously been reported for sharks (12–16), teleosts (16, 17) and amphibians (18, 19). The one notable difference between the antibody response of the grouper and the other species mentioned was the apparent lack of a temporal synthesis in the grouper of high and low molecular weight antibodies associated with prolonged immunization. However, based upon certain physicochemical properties of grouper antibodies, this difference may not represent a contradiction. The shark immunoglobulins appear to belong to the same immunoglobulin class, i.e. 19 and 7S IgM, whereas the amphibian high molecular weight immunoglobulin resembles IgM and the low molecular weight immunoglobulin resembles IgG. Thus a priori it appears that the class Osteichthyes (the intermediate phylogenetic group) may represent the transition point between one and two immunoglobulin classes. Therefore, considerable variations may exist between different species of teleosts.

In order to faciliate this discussion certain properties of grouper immunoglobulins presented in a previous paper[2] will be summarized here. The grouper 16S immunoglobulin has a molecular weight of $\sim$ 700,000 and a relatively high carbohydrate content. The molecular weight of the H and L chains are 70,000 and 22,000 respectively, and there appear to be equimolar amounts of each type of chain. Therefore, while the molecular weight of the grouper 16S molecule is relatively low, based upon polypeptide chain characteristics, it appears to resemble IgM. For a more general discussion of the assignment of class designations to immunoglobulins from different phylogenetic levels, a recent review dealing with this subject is suggested (1). The grouper 6.4S immunoglobulin appears to be different from established immunoglobulin classes of other animals, i.e. molecular weight of 120,000, low carbohydrate content, H chain molecular weight of 40,000 and L chain molecular weight of 22,000. However, antigenetic and peptide map studies have shown the 6.4S molecule to be related to the 16S molecule; the apparent difference being that the 6.4S

molecule is missing $\sim$ 30,000 daltons (and the associated antigenic determinants) from the H chain. It is thus tempting to postulate that the 6.4S molecule is a fragment of the 16S molecule. If indeed the 6.4S molecule is a fragment of the 16S molecule it will be necessary to determine whether it is a synthetic intermediate or a catabolic product. Regardless of the above considerations, the grouper 16S molecule will be considered as a 700,000 mol wt form of IgM (presumably tetrameric).

The equilibrium dialysis studies, reported herein, demonstrate several important factors relative to the phylogeny of antibody activity. First, early grouper 16S antibodies to DNP gave binding curves similar to those reported by Onoue et al., (20) for rabbit IgM antibodies. These curves included an extrapolated valence of one for each H-L chain pair and an indication of two populations of combining sites. The extrapolated valence of eight for grouper 16S antibody is also consistent with that reported for human IgM anti-*Salmonella* O antigen (21) and for a Waldenstrom IgM which possesses anti-DNP activity (22). It should also be pointed out here that Voss et al., (23) have recently demonstrated that the valence of nurse shark 18S IgM antibodies to DNP appeared to be about five, i.e., one for each subunit. Since the K_o of the measurable sites on these shark antibodies was only about 2×10^5 M^{-1}, the possibility remains that a population of 5 low K_o sites (low K_o < than 5% of the high K_o) were present but undetected. The apparently erroneous findings of pentavalent macroglobulin antibodies previously reported in groupers (24) and rabbits (25) attests to the difficulty of measuring the low K_o sites which may be present within an IgM antibody preparation.

One important unresolved question regarding the grouper 16S antibodies is whether the two populations (high and low K_o) of combining sites are on the same or different molecules. Two preliminary approaches have been employed in attempting to answer this question. The first was a study of subunit binding as done by Onoue et al (20). This has thus far been unsuccessful because mild reduction does not yield subunits with appreciable binding activity; this lack of binding may be related to the difficulties in obtaining any subunits subsequent to partial reduction (see footnote 2). A second approach was an attempt to elute the low K_o population from immune precipitates with lower amounts of DNP-OH (0.01 M); this failed to fractionate the two populations since the lower DNP-OH concentration eluted as much antibody as could be eluted with 0.1 M DNP-OH. Thus our tentative (but as yet unproven) hypothesis is that the two major populations of combining sites are on one population of antibody molecules. Regardless of these considerations it appears that the valence of teleost IgM antibody is eight, or one for each H-L chain pair, and that the low K_o sites may not function in many antibody assays.

Possibly the most exciting data obtained from the binding studies with grouper 16S antibodies to DNP were the apparent decline of K_o with the

increase of time after immunization. This observation would appear to contradict that of Eisen and Siskind (26) wherein K_o increased with time after initial immunization of rabbits with DNP proteins. However, it must be emphasized that the grouper antibody resembles IgM whereas the rabbit antibodies were of the IgG class. In addition to species and immunoglobulin class differences, the schedule of immunization is also a variable between the two experiments. It therefore becomes important for future studies to examine changes in K_o of mammalian IgM antibodies associated with time and different schedules of immunization. For example, is the well documented decrease in titer of mammalian 19S antibody to a variety of antigens really due to a decrease in antibody concentration, or alternatively, is there a decrease in affinity such that the 19S antibodies are no longer readily detected?

The grouper 6.4S antibodies would appear to represent an enigma in that they have a measurable valence of about one and do not precipitate with DNP proteins; apparently they are initially purified by virtue of coprecipitating with grouper 16S antibody–DNP—BSA complexes. However, as stated above, these 6.4S molecules appear to resemble a fragment or subunit of the 16S molecule and therefore may have both high and low K_o sites; the latter may be unmeasurable by equilibrium dialysis. The drop in K_o of the 6.4S antibodies with an increase in the time of immunization parallels that of the 16S molecules. This would be expected if the 6.4S is a subunit or fragment of the 16S molecule. Voss et al (23) have also recently described a univalent 7S IgM antibody to DNP from the nurse shark. The K_o of this univalent shark antibody was similar to that observed with the 18S antibody from the same bleeding, i.e., $2 \times 10^5 M^{-1}$. This observation led them to suggest that the shark 7S antibody is a subunit or fragment of the 18S molecule, as suggested here also for the grouper. Unfortunately, no data are available regarding the interconvertability of the grouper proteins, but data from our laboratory indicate that the nurse shark 7S immunoglobulin is not an extracellular precursor or degradation product of the 18S molecule (27). Thus, if the lower vertebrate low molecular weight immunoglobulins are not extracellular catabolic products of the high molecular weight proteins, or synthetic subunits of them, the metabolic interrelationships of these two proteins can only be examined at the intracellular level. In all likelihood, further studies along these lines may help delineate the mechanisms of macroglobulin assembly.

The thermodynamic properties of the grouper 16 and 6.4S antibodies studied were very similar to each other. These properties were also similar to those reported by Eisen and Siskind (26) for rabbit IgG antibodies to the DNP determinant. Therefore, from the phylogenetic viewpoint, it appears that although there were considerable changes between fish and mammals in terms of the evolution of multiple immunoglobulin classes, there may have been little in the way of gross evolutionary changes in the actual antigen-combining site of the

antibody. This hypothetical constancy of the combining site may account for the shared idiotypic specificities of IgM and IgG antibodies from certain higher animals (28).

SUMMARY

The giant grouper, *Epinephelus itaira*, was shown to synthesize 16 and 6.4S antibodies specific for the dinitrophenyl determinant (DNP). Sera obtained at various intervals between 1 month and 2 yr after initial immunization contained both species of antibody; no temporal synthesis was evident.

Equilibrium dialysis studies employing ϵ-dinitrophenyl-amino caproic acid were conducted with purified grouper antibodies specific for DNP. The 16S antibody preparations obtained at 1 and 2 months of immunization showed heterogeneity of hapten binding indicative of two populations of combining sites. One-half of these sites (an average of four sites per 16S molecule) exhibited an average intrinsic association constant (K_o) of $\sim 10^6 M^{-1}$; the K_o of the remaining four-sites was $\sim 10^4 M^{-1}$. Thus, the valence of the grouper 16S antibody molecule appears to be eight although the distribution of the high and low K_o sites is unknown, i.e., are they each on the same or on different molecules? The 16S antibody preparations obtained after more prolonged immunization exhibited increasingly lower K_o values; the so-called low K_o sites were no longer detectable. These findings are in contrast to reports of rabbit IgG antibodies showing an increase in K_o with increased time.

The 6.4S antibody preparations obtained from the 1 and 2 month antisera had K_o values of $\sim 10^6 M^{-1}$ and a valence of one. These antibodies would not precipitate with antigen. The 6.4S antibody preparations obtained at later times showed decreasing K_o values comparable to those of the 16S antibodies from the same bleedings.

Studies on the thermodynamic parameters of the hapten-antibody interaction showed the grouper 16 and 6.4S antibodies to be similar to each other. These data also showed that the enthalpy and entropy changes of grouper antibody-hapten reactions resemble those reported for rabbit IgG antibodies to this hapten. It is thus suggested that, although considerable evolution of immunoglobulin classes has occurred between fish and rabbits, the antibody combining site may have remained relatively unchanged during a large part of evolutionary time.

This study would have been impossible without the cooperation and assistance of Mr. Cecil Walker at Marineland. The authors also wish to acknowledge the excellent technical assistance of Mr. Edsel McLean and to thank numberous battered and drenched colleagues who assisted in immobilizing and bleeding the animals.

REFERENCES

1. Clem, L. W., and G. A. Leslie. 1969. Phylogeny of immunoglobulin structure and function. *In* Immunology and Development. M. Adinolfi, ed. Spastics International Medical Publications, London, England. 62.

2. Leslie, G. A., and L. W. Clem. 1969. Production of anti-hapten antibodies by several classes of lower vertebrates. *J. Immunol.* **103**:613.
3. Eisen, H. N. 1964. Equilibrium dialysis for measurement of antibody-hapten affinities. *Methods Med. Res.* **10**:106.
4. Kabat, E. A., and M. M. Mayer. 1961. Experimental Immunochemistry. Charles C Thomas, Publisher, Springfield, Ill. 2nd edition. 871.
5. Lowry, O. H., N. J. Roseborough, A. L. Farr, and R. H. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
6. Farah, F. S., M. Kern, and H. Eisen. 1960. The preparation and some properties of purified antibody specific for the 2,4-dinitrophenyl group. *J. Exp. Med.* **112**:1195.
7. Clem, L. W., and P. A. Small. 1967. Phylogeny of immunoglobulin structure and function. I. Immunoglobulins of the lemon shark. *J. Exp. Med.* **125**:893.
8. McConahey, P., and F. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* **29**:185.
9. Hill, W. C., and J. C. Cebra. 1965. Horse anti-SI immunoglobulins. I. Properties of γM-antibody. *Biochemistry* **4**:2575.
10. Lindquist, K., and D. C. Bauer. 1966. Precipitin activity of rabbit macroglobulin antibody. *Immunochemistry* **3**:373.
11. Porath, J., R. Axén, and S. Ernback. 1967. Chemical coupling of proteins to agarose. *Nature (London)* **215**:1491.
12. Clem, L. W., and F. DeBoutaud. 1967. Elasmobranch immunoglobulins. *In* Sharks, Skates, and Rays. P. W. Gilbert, R. F. Mathewson, and D. P. Rall, eds. Johns Hopkins Press, Baltimore, Md. 581.
13. Clem, L. W., F. DeBoutaud, and M. M. Sigel. 1967. Phylogeny of immunoglobulin structure and function. II. Immunoglobulins of the nurse shark. *J. Immunol.* **99**:1226.
14. Suran, A. A., M. H. Tarail, and B. W. Papermaster. 1967. Immunoglobulins of the leopard shark. I. Isolation and characterization of 16S and 7S immunoglobulins with precipitating activity. *J. Immunol.* **99**:679.
15. Sigel, M. M., and L. W. Clem. 1965. Antibody responses of fish to viral antigens. *Ann. N. Y. Acad. Sci.* **126**(1):662.
16. Uhr, J. W., M. S. Finkelstein, and E. C. Franklin. 1962. Antibody response to bacteriophage φX174 in non-mammalian vertebrates. *Proc. Soc. Exp. Biol. Med.* **111**:13.
17. Hodgins, H. O., R. Weiser, and G. Ridgeway. 1967. The nature of antibodies and the immune response in rainbow trout, (*Salmo gairdneri*). *J. Immunol.* **99**:534.
18. 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. Exp. Med.* **124**:901.
19. Lykakis, J. J., and F. E. G. Cox. 1968. Immunological responses of the toad, *Xenopus laevis*, to the antigens of the ciliate, *Tetrahymena pyriformis*. *Immunology*. **15**:429.
20. Onoue, K., A. L. Grossberg, Y. Yagi, and D. Pressman. 1968. Immunoglobulin M antibodies with ten combining sites. *Science (Washington)*. **162**:574.
21. Merler, E., L. Kartin, and S. Matsumato. 1968. The valency of human γM antibody. *J. Biol. Chem.* **243**:386.

22. Ashman, R., and H. Metzger. 1969. A Waldenström macroglobulin which binds nitrophenyl ligands. *J. Biol. Chem.* **244:**3405.
23. Voss, E. W., W. J. Russell, and M. M. Sigel, 1969. Purification and binding properties of nurse shark antibodies. *Biochemistry* **12:**4866.
24. Clem, L. W., and P. A. Small. 1968. Valence of teleost IgM antibody to DNP. *Fed. Proc.* **27:**2633.
25. Onoue, K., Y. Yagi, A. Grossberg, and D. Pressman. 1965. Number of binding sites of rabbit macroglobulin antibody and its subunits. *Immunochemistry.* **2:**401.
26. Eisen, H., and G. Siskind. 1964. Variations in affinities of antibodies during the immune response. *Biochemistry.* **3:**996.
27. Small, P. A., Jr., D. G. Klapper, and L. W. Clem. 1970. Half-lives, body distribution, and lack of interconversion of serum 19S and 7S IgM of sharks. *J. Immunol.* In press.
28. Oudin, J., and M. Michel. 1969. Idiotype of rabbit antibodies. II. Comparison of idiotype of various kinds of antibodies formed in the same rabbits against *Salmonella typhi*. *J. Exp. Med.* **130:**619.