Purification and Characterization of Two Classes of Immunoglobulins from the Marine Toad, *Bufo marinus*†

Ronald T. Acton,‡ E. Edward Evans,* Peter F. Weinheimer,§ William Niedermeier, and J. Claude Bennett¶

ABSTRACT: Following immunization with *Salmonella typhosa* H (STH) antigen, the marine toad was found to synthesize STH agglutinating antibody associated with the 17.9S fraction of sera. When the animals were challenged with T2 bacteriophage, T2 neutralizing antibody was found in both 17.9S and 7.3S fractions of sera at 21 days after primary immunization. At 14 days after a secondary injection of T2, no shift to the 7.3S fraction was observed. These immunoglobulins, designated immune macroglobulin and low molecular weight antibody, were purified and found to have molecular weights of ~880,000 and ~160,000, respectively. The immunoglobulins were also found to differ from each other in carbohydrate composition. Although the L chains from both molecules had a molecular weight of ~22,500, the H chain for the immune macroglobulin was ~67,000, while that of the low molecular weight antibody was ~53,000. In addition, the H chains of both classes of immunoglobulins were distinguishable from each other on the basis of amino acid compositions, peptide maps, and NH_2-terminal amino acid sequences. Thus, evidence is presented which clearly demonstrates the presence of at least two distinct classes of immunoglobulins in the marine toad.

To date, five classes of immunoglobulins have been described in man (reviewed in Adinolfi and Wood, 1969; Sterzl and Riha, 1970), while analogous forms of at least three of these classes, IgA,[1] IgG, and IgM, have been observed in several other species of mammals. These classes of immunoglobulins are distinguished on the basis of function, size, heavy (H) chain type, carbohydrate content, and antigenic characteristics. Phylogenetically (reviewed in Clem and Leslie, 1969; Grey, 1969) immunoglobulins resembling mammalian IgM have been reported for species representing the vertebrate classes Aves (Leslie and Clem, 1969; Grey, 1963), Reptilia (Marchalonis *et al.*, 1969; Acton *et al.*, 1972a), Amphibia (Marchalonis and Edelman, 1966; Diener and Marchalonis, 1970; Marchalonis *et al.*, 1970), and Chondrichthyes (Marchalonis and Edelman, 1965; Clem and Small, 1967; Johnston *et al.*, 1971). In addition, there have been molecules similar to the IgG class found in representatives of Aves (Clem and Leslie, 1969; Grey, 1969), Reptilia (Marchalonis *et al.*, 1969; Lykakis, 1968, Saluk *et al.*, 1970), and Amphibia (Marchalonis and Edelman, 1966; Diener and Marchalonis, 1970; Marchalonis *et al.*, 1970).

Studies of several species of Amphibia suggest that these animals are indeed capable of producing two distinct types of immunoglobulins (Marchalonis and Edelman, 1966; Diener and Marchalonis, 1970; Marchalonis *et al.*, 1970; Lykakis and Cox, 1968; Lykakis, 1969; Lin *et al.*, 1971). However, except for the work by Marchalonis and Edelman (1966) and Marchalonis *et al.* (1970) on the bullfrog and clawed toad there has been little information sufficient to permit evaluation of the relationship of amphibian immunoglobulins to those of other vertebrates. A report by Parkhouse *et al.* (1970) on the clawed toad, which described hexameric macroglobulins, implies more questions than answers.

In view of the limited structural data available, we have elected to study the immunoglobulins of a representative anuran Amphibian, the marine toad, *Bufo marinus*. This species has previously been shown to produce two molecular weight species of antibodies (Diener and Marchalonis, 1970; Lin *et al.*, 1971; Legler *et al.*, 1969). The purpose of this investigation was to elucidate the physical, chemical, and structural properties of the antibodies synthesized in response to different types of antigens. These studies were deemed necessary in light of the phylogenetically important position of the Amphibia. Animals in this class are transitional from a number of anatomical and physiological considerations and appear to have evolved from crassopterygian ancestors toward the latter part of the Devonian period (Romer, 1968).

Materials and Methods

Animals. Adult specimens of the marine toad, *Bufo marinus*, were obtained from the Lemberger Co., Oshkosh, Wis. Animals of both sexes were used and ranged in weight from 200 to 450 g. These were maintained at a constant temperature of 25 ± 2° as previously described (Evans *et al.*, 1966). The toads were force-fed twice weekly with ground horsemeat.

Antigens and Immunization. Antigens used in this study were *Salmonella typhosa* "H" flagella antigen (STH)[2] and T2 bacteriophage. Detailed aspects concerning the preparation of these antigens as well as immunization procedures and antibody titrations have been described (Legler *et al.*, 1969; Evans *et al.*, 1966).

† From the Departments of Microbiology and Medicine, Division of Clinical Immunology and Rheumatology, University of Alabama in Birmingham, Birmingham, Alabama 35233. *Received November 1, 1971.* This investigation was supported by grants from the U. S. Public Health Service (AI-02693, AI-9153, and AM-03555), the National Science Foundation (Grant GB-5983), and the John A. Hartford Foundation. A preliminary report of this work was presented before the Federation of American Societies for Experimental Biology in April 1969, at Atlantic City, N. J.

‡ Present address: Division of Biology, California Institute of Technology, Pasadena, Calif. 91109.

* To whom reprint requests should be addressed.

§ Postdoctoral Research Fellow supported by the U. S. Health Service Research Fellowship 1 F02 AM42874-01A1.

¶ Recipient of a Research Career Development award (K3GM025,-386).

[1] This nomenclature is at outlined by the World Health Organization.

[2] 5th, *Salmonella typhosa* "H."

Immunoglobulin Purification. Immune sera that had been stored at −20° were allowed to thaw at room temperature and centrifuged at 2980*g* for 15 min at 5° to remove precipitated material. An aliquot of whole sera was subjected to gel filtration on Sephadex G-200 to determine the distribution of STH agglutinating and T2 neutralizing antibody. An additional aliquot was exhaustively dialyzed against 0.015 M Tris-HCl (pH 8.0), centrifuged at 2980*g* for 15 min at 5°, and applied to a DEAE-Sephadex (A-50) column (45 × 2.5 cm) equilibrated with 0.015 M Tris-HCl (pH 8.0). Flow rate was 40 ml/hr. Fractions of 10 ml were collected and monitored by absorption at 280 mμ. Elution was accomplished with a linear gradient (0.015 M Tris-HCl, pH 8.0, to 0.4 M NaCl, pH 8.0). The fractions collected from the DEAE column which were demonstrated to have antibody activity were then pooled and concentrated by ultrafiltration utilizing Dialflo XM-100 membranes (Amicon Corp.) and an aliquot applied to an upward-flowing Sephadex G-200 column (2.5 × 100 cm, bed volume 480 ml, flow rate 20 ml/hr) equilibrated with 0.14 M NaCl–0.01 M Tris, 0.01% sodium azide buffer adjusted to pH 7.4 with HCl (Tris-NaCl, pH 7.4 buffer). Fractions of 5 ml were collected and monitored by absorption at 280 mμ. The excluded fraction was concentrated by ultrafiltration and an aliquot applied to an upward-flowing Sepharose 6-B column (2.5 × 100 cm, bed volume 500 ml, flow rate 15 ml/hr) equilibrated with Tris-NaCl, pH 7.4 buffer. The fraction which appeared in the region where human IgG eluted was concentrated and recycled on Sephadex G-200. The final products, referred to as purified immune macroglobulin and low molecular weight antibody respectively, were concentrated by ultrafiltration and used immediately or stored at 5° until studies could be completed.

Immunoelectrophoresis. Rabbit antisera to whole marine toad serum utilized in the immunoelectrophoretic studies were prepared by intramuscular and footpad injections in Freund's complete adjuvant (Difco) at 0, 1, 3, and 6 weeks. The antisera obtained at 5 weeks after the last stimulation were used in the studies herein reported.

Immunoelectrophoresis was performed on film strips 12.7 × 3.5 cm utilizing 175 V and 5 mA/strip for 1.5 hr as described by Alford *et al.* (1967). The strips were coated with 6 ml of agar prepared in 0.06 M barbital buffer (pH 8.8).

Physical Measurements. Sedimentation velocity measurements were conducted at 20° in a Spinco Model E ultracentrifuge using double-sector 12-mm cells and schlieren optics. Sedimentation coefficients were calculated according to procedures described by Schachman (1959). Diffusion measurements were made in the same centrifuge at 20° and a speed of 3617 rpm, using a capillary-type synthetic boundary cell as described by Ehrenberg (1957). Diffusion coefficients were calculated by the maximun ordinate method (Ehrenberg, 1957). The sedimentation and diffusion photographic patterns were measured on a Nikon microcomparator. A detailed description of these determinations has previously been described (Acton *et al.*, 1971a,b).

The partial specific volumes of the marine toad immunoglobulins were calculated from the amino acid and carbohydrate compositions of the whole immunoglobulin molecules as described by Cohn and Edsall (1943). The extinction coefficients at 280 mμ were measured in 0.85% sodium chloride solution in a Beckman DB spectrophotometer. Protein concentration of the solutions were calculated from the nitrogen content determined with Nessler's reagent after Kjeldahl digestion. A nitrogen content of 16% was assumed. These analyses were performed in duplicate.

Carbohydrate Analysis. Prior to carbohydrate analysis of the purified immunoglobulin, it was necessary to remove possible carbohydrate contaminants that could arise from Sephadex gel. This was accomplished by twice precipitating the isolated immunoglobulins with 50% $(NH_4)_2SO_4$, solubilizing with distilled water, and then precipitating twice with 50% ethanol. The immunoglobulins were then exhaustively dialyzed against water and lyophilized.

The alditol acetates of the neutral and amino sugars were quantified by the gas chromatographic method of Niedermeier (1971). This procedure utilizes a Hewlett-Packard Model 402 gas chromatograph and an Infotronics Model CRS 104 electronic integrator. All analyses were performed in duplicate. Sialic acid was determined by the thiobarbituric acid method of Warren (1959).

Reduction, Alkylation, and Preparation of Polypeptide Chains. Titration of disulfide groups in the purified immunoglobulins was carried out with 0.5 M 2-mercaptoethanol in 0.1 M Tris-glycine buffer (pH 7.0), containing 0.5 mg of EDTA/ml, as described by Habeeb (1966). The concentration of guanidine hydrochloride varied from 0 to 5 M in the final reaction mixture. The number of liberated sulfhydryl groups was determined by the 5,5-dithio[bis(2-nitrobenzoic acid)] reagent of Ellman (1959) as described by Habeeb (1966).

The purified immunoglobulins were extensively reduced and alkylated in the presence of 8 M guanidine hydrochloride as previously described (Acton *et al.*, 1971a). Following reduction and alkylation, the H and L polypeptide chains were isolated by gel filtration on a Sephadex G-200 upward-flowing column (2.5 × 120 cm) equilibrated with 5 M guanidine hydrochloride as described by Small and Lamm (1966). Purity of the separated H and L chains was ascertained by electrophoresis in 5% polyacrylamide gel containing 5 M urea by a procedure adapted from Maizel (1966) as described by Acton *et al.* (1969). Molecular weights of the purified H and L chains were determined on the same Sephadex G-200 column in the presence of 5 M guanidine hydrochloride by the method described by Andrews (1964).

Amino Acid Analysis. Samples of the purified H and L chains were hydrolyzed in duplicate with 6 N HCl under nitrogen at 106° for 20 hr. Following hydrolysis, HCl was removed in a vacuum over NaOH. Analyses were performed on the Beckman Model 120C automatic amino acid analyzer utilizing a 55-cm column for separation of neutral and acidic amino acids and an 8-cm column for basic amino acids as described by Hubbard (1965). Corrections were not made for losses of serine and threonine. Tryptophan was determined spectrophotometrically by the *N*-bromosuccinimide method of Spande and Witkop (1967).

Peptide Maps. The purified H and L chains were subjected to two-dimensional peptide mapping analyses as described by Bennett (1967). Samples were digested with trypsin, 1:50 (w/w) ratio of enzyme to protein, and then subjected to chromatography followed by high-voltage electrophoresis in the second dimension. Peptides were detected by staining with ninhydrin–collidine color dip. The Pauly stain was utilized to detect tyrosine- and histidine-containing peptides.

Amino-Terminal Sequence Analysis. NH_2-terminal sequence analyses of the isolated H and L chains were performed by the Edman degradation method utilizing methyl isothiocyanate as the coupling agent. The methylthiohydantoin derivatives of the amino acids were resolved and identified by gas–liquid chromatography according to the methods of Waterfield and Haber (1970). Polypeptide chains which were found to be nonreactive in Edman procedure were digested

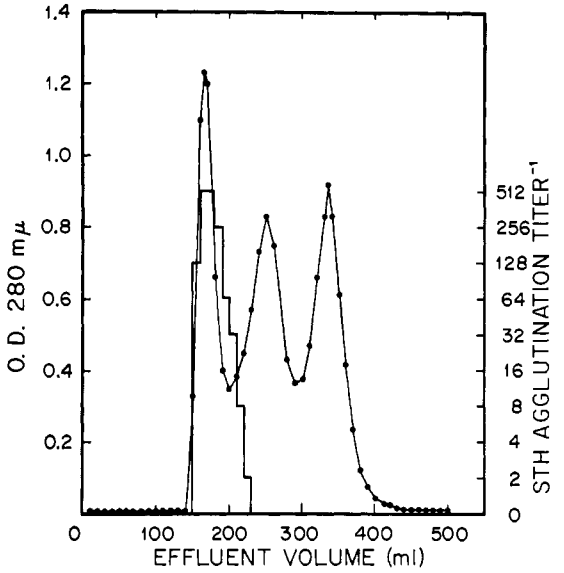

FIGURE 1: Elution pattern of marine toad whole immune serum from a Sephadex G-200 column (2.5 × 100 cm) equilibrated with Tris-NaCl buffer (pH 7.4). The vertical bar represents STH agglutinin titers; (●) absorbance at 280 mμ.

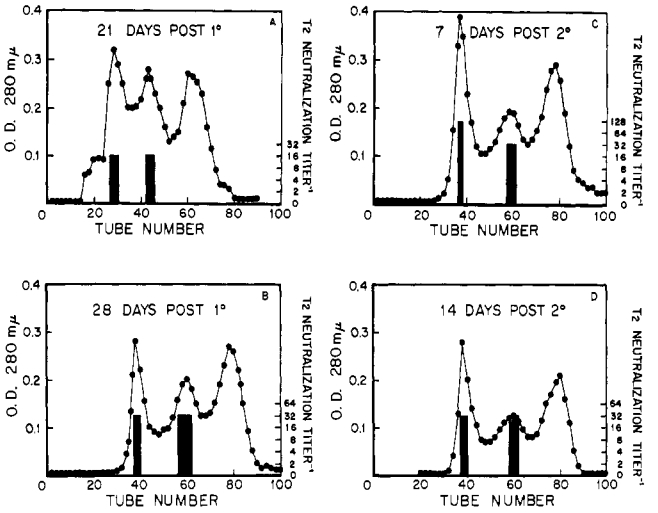

FIGURE 2: Elution patterns of marine toad whole immune serum from a Sephadex G-200 column (2.5 × 45 cm) equilibrated with Tris-NaCl buffer (pH 7.4). The vertical bar represents T2 bacteriophage neutralization titers in the indicated fractions; (●) absorbance at 280 mμ.

with Pronase and the blocked NH_2-terminal peptides from each of the Pronase digests were isolated from a Dowex 50-X2 column as described by Bennett (1968).

Results

Immunoglobulin Purification. Previous work by Evans and coworkers (1966) has shown that the marine toad, while capable of synthesizing antibodies in high titer to STH, lacks the ability to elicit an enhanced antibody response to repeated injections of this antigen. Utilizing sera obtained from marine toads immunized with STH, we attempted to determine the size distribution of STH agglutinating antibodies in whole serum. When immune anti-STH marine toad serum was subjected to gel filtration on Sephadex G-200, as shown in Figure 1, three components were resolved which were analogous to the excluded volume as well as the 7S and 4S regions respectively, as determined by standard molecular weight markers. The STH agglutinating activity was confined to the material eluting in the excluded volume. This same elution profile was observed, even when sera collected over a 241-day period following secondary and tertiary injections of STH were used. In no instance was there an indication of STH agglutinating antibodies in the material eluting in the 7S region. However, when the marine toad was challenged with T2 bacteriophage, as described by Evans *et al.* (1966), neutralizing antibody was found to be associated with the 7S region as well as material eluting in the void volume. As shown in Figure 2, gel filtration of marine toad immune T2 neutralizing sera on Sephadex G-200 revealed that at 21 and 28 days after the primary injection as well as 7 and 14 days after the secondary stimulation, the activity resided in a pool of both the excluded and the 7S fractions.

The immunoglobulins of the marine toad could be separated from other serum proteins by passage through an anion-exchange column. In the particular elution profile shown in Figure 3A, immune anti STH serum was applied to the column, and as can be seen, the STH agglutinating activity was confined to the first fraction eluted. When the other serum components were eluted with a linear salt gradient and assayed, no additional activity could be found. The active fraction (FI) was pooled as indicated, concentrated and subjected to gel filtration on Sephadex G-200. As shown in Figure 3B, two components were resolved in regions correspond-

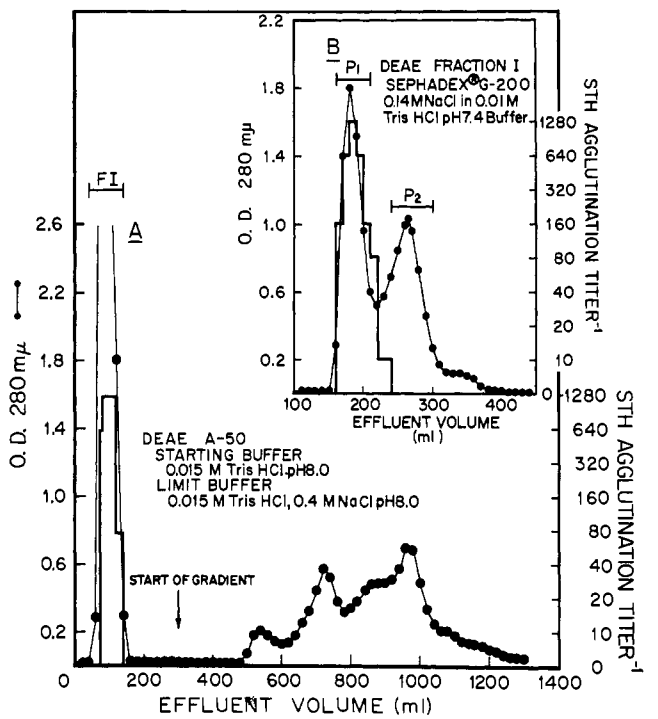

FIGURE 3: (A) Fractionation of marine toad whole immune serum from a DEAE A-50 anion-exchange column (2.5 × 45 cm). A gradient (---) with 0.015 M Tris-HCl (pH 8.0) as the starting buffer and 0.015 M Tris-HCl + 0.4 M NaCl (pH 8.0) as the limit buffer was used. FI indicates the fraction pooled for gel filtration Sephadex G-200. (B) Gel filtration of FI on a Sephadex G-200 column (2.5 × 100 cm) equilibrated with Tris-NaCl buffer (pH 7.4). The horizontal bars indicate the fractions pooled (P1 and P2). The vertical bar represents STH agglutin titers; (●) absorbance at 280 mμ.

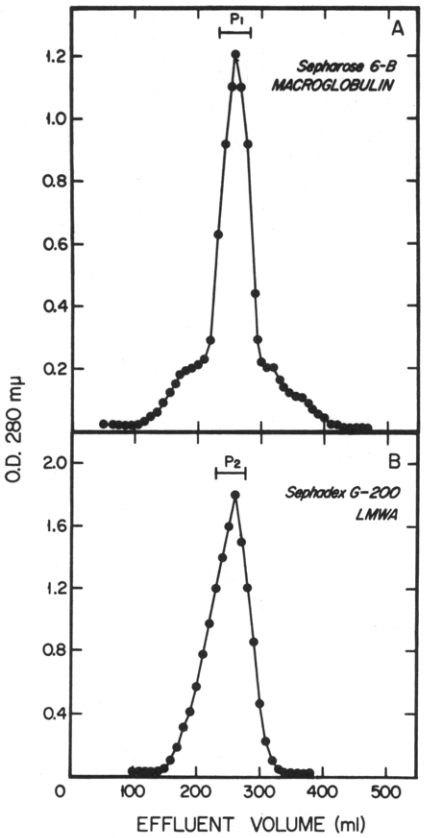

FIGURE 4: (A) Gel filtration of P1 from Sephadex G-200 (Figure 3B) on a Sepharose 6-B column (2.5 × 100 cm). (B) Gel filtration of P2 from Sephadex G-200 (Figure 3B) on a Sephadex G-200 column (2.5 × 100 cm). Both columns were equilibrated with Tris-NaCl buffer (pH 7.4). The horizontal bars (P1 and P2) represent the fractions pooled for further analysis and will be referred to as purified immune macroglobulin and purified low molecular weight antibody (LMWA), respectively; (●) absorbance at 280 mμ.

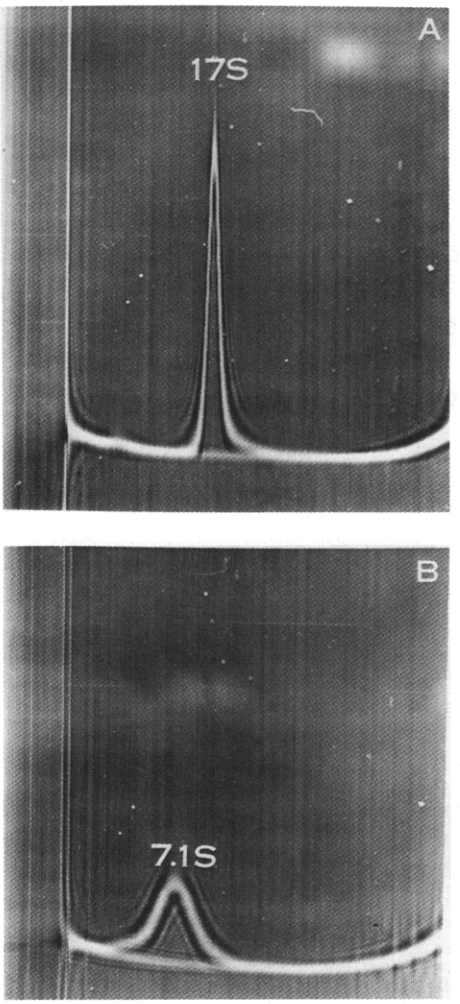

FIGURE 5: Ultracentrifugation patterns of purified marine toad immunoglobulins (P1 and P2, Figure 4A, B). (A) Purified immune macroglobulin at a protein concentration of 2.8 mg/ml. The photograph was taken 16 min after reaching a speed of 56,000 rpm. (B) Purified LMWA at a protein concentration of 3.0 mg/ml. The photograph was taken 24 min after reaching a speed of 56,000 rpm. The buffer employed was 0.076 M sodium phosphate (pH 7.5). The centrifugations were conducted at a phase-plate angle of 60° and a temperature of 20°.

ing to the excluded volume and 7S markers. STH agglutinating activity was associated only with the material in the excluded volume.

T2 neutralizing antibody could also be found in the first fraction eluted from an anion-exchange column analogous to the profile seen in Figure 3A when marine toad immune anti-T2 sera was applied. When this fraction was chromatographed on Sephadex G-200, activity was found in both the excluded and 7S fractions.

To obtain homogeneous preparations of immunoglobulins, the fractions from several Sephadex G-200 runs were pooled as indicated in Figure 3B, (P1 and P2), concentrated, and rechromatographed (Figure 4A,B). These rechromatographed materials will be referred to as purified immune macroglobulin and purified low molecular weight antibody, respectively. Ultracentrifuge analyses of these preparations are shown in Figure 5. Both fractions contained predominantly homogeneous material with only trace amounts of low or high molecular weight contaminants. The immune macroglobulin and low molecular weight antibody had $s_{20,w}$ values of 17 and 7 S, respectively. The purified immunoglobulins were also found to be free of contaminating proteins as determined by immunoelectrophoresis (Figure 6).

Ouchterlony analysis of the purified immunoglobulins utilizing rabbit antiserum to whole marine toad serum revealed precipitin arcs with a reaction of partial identity. The low molecular weight antibody was shown to be antigenically deficient with respect to the immune macroglobulin.

Physical Studies. The extinction coefficients at 280 mμ for the purified immunoglobulins measured in 0.85% sodium chloride were found to be 14 and 16 for the immune macroglobulin and low molecular weight antibody, respectively.

The sedimentation of the immune macroglobulin was found to be concentration dependent and a sedimentation coefficient of 17.9 S was obtained by extrapolation to infinite dilution. Sedimentation of the low molecular weight antibody did not appear to be concentration dependent and an $s^0_{20,w}$ value of 7.3 S was obtained. The diffusion coefficients were obtained by extrapolation of the $D_{20,w}$ values plotted as a function of protein concentration. This plot revealed $D^0_{20,w}$ values of 1.78×10^{-7} and 3.89×10^{-7} cm²/sec for the immune macroglobulin and low molecular weight antibody, respectively. The immune macroglobulin exhibited concentration dependence of diffusion which is to be expected in view of the large frictional ratio of this protein. Molecular weights for the purified immunoglobulins were calculated from the $D^0_{20,w}$ and $s^0_{20,w}$ constants according to the equation of

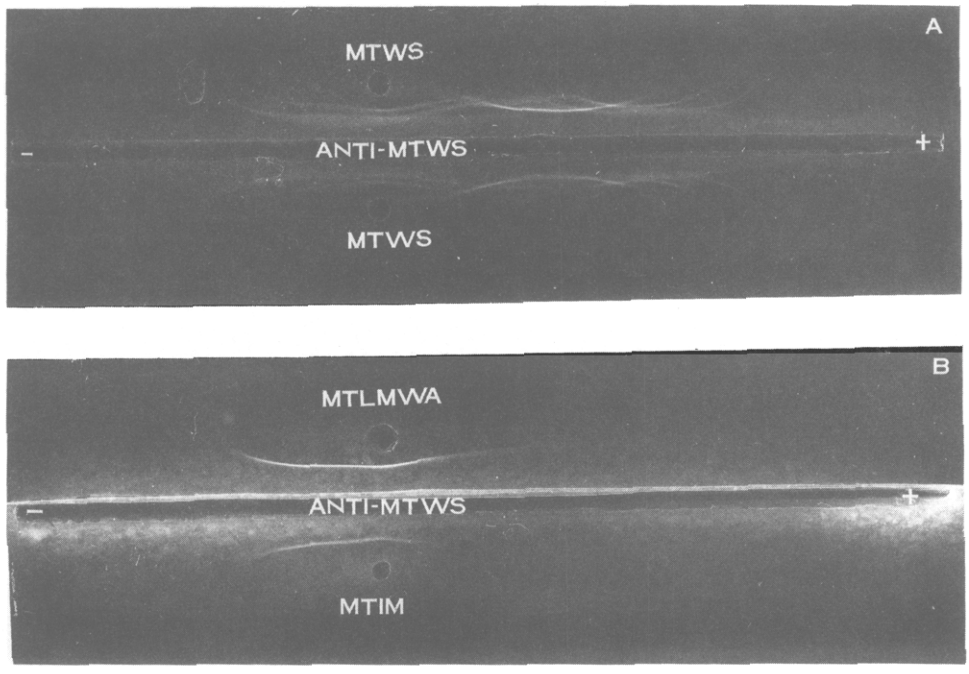

FIGURE 6: The results of immunoelectrophoretic analysis of marine toad serum and purified immunoglobulins. (A) Immunoelectrophoresis of marine toad whole sera (MTWS). (B) Top well, marine toad low molecular weight antibody (MTLWA), protein concentration 2.0 mg/ml, bottom well marine toad immune macroglobulin (MTIM), protein concentration 2.8 mg/ml. Precipitation arcs were developed, using a rabbit antiserum against marine toad whole sera (Anti-MTWS). The cathode is to the left.

TABLE I: Carbohydrate Compositions of Marine Toad Immunoglobulins.

Protein	Moles/Mole of Protein					Mannose: Galactose Ratio	% CHO
	Mannose	Fucose	Galactose	Glucosamine[a]	Sialic Acid		
Immune macroglobulin[b]	103	5	103	124	23	1:1	7.6
Low molecular weight antibody	9	0	14	10	3	1:1.6	4.2

[a] Free base. [b] Observed residues per mole were calculated on the basis of an observed molecular weight of 880,000 for the immune macroglobulin and 160,000 for the low molecular weight antibody.

Svedberg and Pedersen (1940). Utilizing calculated partial specific volumes of 0.721 and 0.725 for the immune macroglobulin and low molecular weight antibody, respectively, molecular weights were found to be 880,000 and 160,000.

Chemical Studies. The carbohydrate compositions of the marine toad purified immunoglobulins are shown in Table I. In determining the carbohydrate composition, it was found that the maximum yield of neutral sugars occurred after a 4-hr hydrolysis while a 10-hr hydrolysis was necessary to recover all the glucosamine. The sialic acid determinations were performed following 30-min hydrolysis.

Figure 7 illustrates the titration of disulfide bonds with increasing guanidine hydrochloride concentration for the marine toad immune macroglobulin. The number of disulfide bonds cleaved per mole of protein was a function of guanidine hydrochloride concentration. In the absence of guanidine hydrolchloride, a maximum of 17 disulfide bonds were titrated. With an increase in guanidine hydrochloride, there is a progressive cleavage of disulfide groups with a maximum number of 89 being titrated at a guanidine hydrochloride concentration of 4.25 to 5 M. It is of special note that the titration of the disulfide groups at the various concentrations of the denaturing solvent is not appreciably enhanced by increasing the time of reaction. Although lack of material prevented extensive studies on disulfide titrations for the low molecular weight antibody, a maximum of 17 were titrated at a guanidine hydrochloride concentration of 5.5 M.

To effect complete separation of the H and L chains, the purified immunoglobulins utilized for polypeptide chain preparations were extensively reduced and alkylated in the presence of 8 M guanidine hydrochloride. Chain separations were achieved by gel filtration on Sephadex G-200 in the presence of 5 M guanidine hydrochloride (Figure 8). The first peak in each case represents aggregated H chain, based on peptide map data and the fact that the H-chain fraction reformed an aggregate peak eluting in the same position when recycled on the same column. The polyacrylamide gel electrophoretic patterns also included in this figure demonstrate that the H and L chains were obtained in a homogeneous state. It should also be emphasized that the low molecular

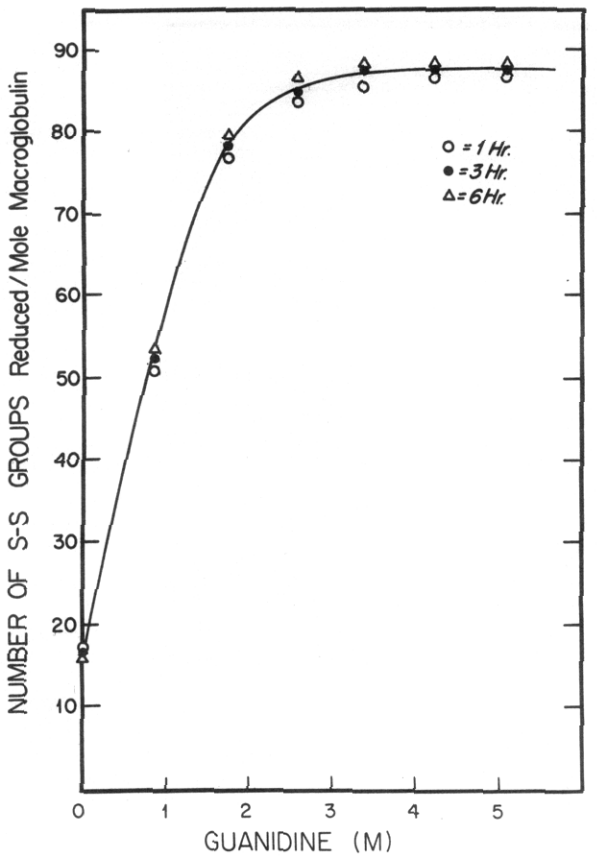

FIGURE 7: The number of disulfide bonds reduced in marine toad purified immune macroglobulin as a function of guanidine hydrochloride concentration. Reduction was carried out at pH 7 utilizing 2-mercaptoethanol.

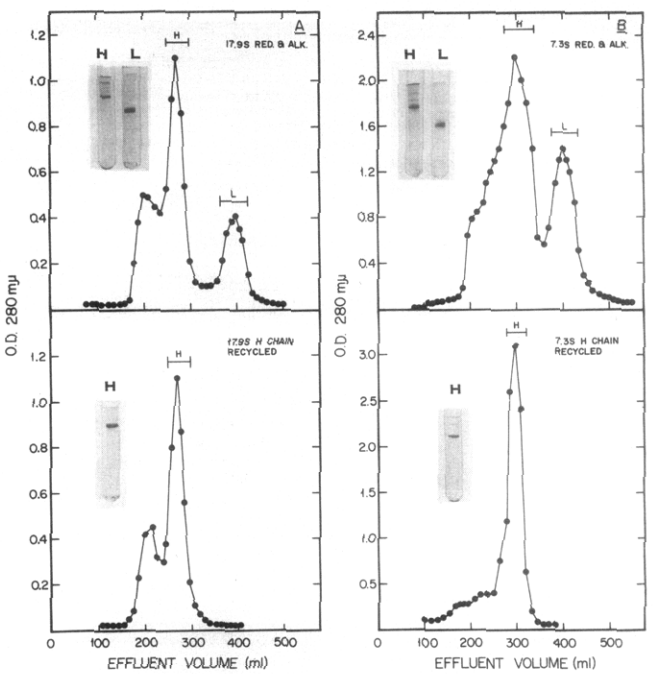

FIGURE 8: Elution pattern of totally reduced and alkylated marine toad immunoglobulins from a Sephadex G-200 column (2.5 × 120 cm). The eluting buffer was 5 M guanidine hydrochloride. (A) Purified immune macroglobulin (17.9 S). (B) Purified LMWA (7.3 S). The horizontal cross bars indicate the volume pooled of the respective H and L chains. In each case the H chains were recycled through the same G-200 column as shown. The acrylamide gel electrophoretic patterns illustrate the electrophoretic profile of the indicated pool. The anode is to the bottom of the gel pattern.

weight antibody H chain migrated further into the gel as compared to the immune macroglobulin, indicating a lower molecular weight.

The molecular weights of the recycled H and L chains from the two species of purified immunoglobulins were determined by their elution volumes from a Sephadex G-200 column equilibrated with 5 M guanidine hydrochloride. The column was calibrated with extensively reduced and alkylated known molecular weight markers as described by Andrews (1964). A plot of these data (Figure 9) reveals that the L chains from both species of purified immunoglobulins had a K_d value of 0.646 which indicated a molecular weight of 22,500 ± 2500. The H chain of the immune macroglobulin had a K_d value of 0.375 which corresponded to a molecular weight of 67,000 ± 2500, while the K_d value for the low molecular weight antibody H chain was 0.448 and the molecular weight 53,000 ± 2500.

Amino acid analyses of the chains are compared in Table II. There are distinct differences between the two types of H chains. L chains were identical within limits of the analytical method. Although the marine toad immunoglobulin H chains differ from each other in some respects, they have relatively high amounts of serine, threonine, aspartic acid, and glutamic acid which appears to be a feature common to many vertebrate immunoglobulins (Acton *et al.*, 1971c; Suzuki and Deutsch, 1967; Johnston *et al.*, 1971). There were, however, higher numbers of *S*-carboxymethylcysteine in the H chains of both the immune macroglobulin and low molecular weight antibody, respectively, than reported for human IgM and IgG (Bennett, 1969; Gall and Edelman, 1970).

Exact tracings of the tryptic peptide maps prepared using the purified H and L chains from the marine toad immunoglobulins are shown in Figure 10. The maps demonstrate that the H chains of the immune macroglobulins have unique peptides which readily distinguish these molecules from the H chains of low molecular weight antibody. Peptide map analyses did not reveal any peptides which were unique to either of the L-chain preparations.

The isolated H and L chains from the marine toad purified immunoglobulins were subjected to NH_2-terminal sequence analyses (Table III). The H chain from the immune macroglobulin had a free NH_2-terminal residue and the yield of the residue on a relative molar basis indicated that the sequence of the first two residues shown represents the principal NH_2-terminal sequence. The low molecular weight antibody H chain as well as the L chains from both types of immunoglobulins were nonreactive in the Edman procedure. The H and L chains of the low molecular weight antibody were digested with Pronase and the blocked NH_2-terminal peptides from the Pronase digests were isolated from a Dowex 50-X2 column. Analysis of the column effluent by paper chromatography (Bennett, 1968) revealed both ninhydrin positive as well as ninhydrin-negative peptides. The ninhydrin-negative peptides detected by starch–iodide staining were then eluted from paper and subjected to amino acid analysis. The amino acid compositions of the ninhydrin-negative peptides are summarized in Table III. The suggested sequences shown are based on the amino acid composition and have been arranged to afford maximum homology with mammalian immunoglobulin H and L chains (Bennett, 1968). Pyrrolidonecarboxylic acid has been assumed to be the NH_2-terminal residue since the amino acid compositions indicated significant molar

TABLE II: Amino Acid Compositions of Heavy and Light Chains of Marine Toad Immunoglobulins.

Amino Acid	Immune Macroglobulin[a] H Chain	Low Mol Wt Antibody[a] H Chain	Low Mol Wt Antibody[a] L Chain
Lysine	37	36	13
Histidine	8	9	3
Arginine	15	12	7
Aspartic acid	64	49	20
Threonine	44	37	18
Serine	50	46	22
Glutamic acid	57	44	24
Proline	33	34	10
Glycine	34	29	16
Alanine	28	15	9
Valine	43	32	15
Methionine	3	3	2
Isoleucine	27	27	10
Leucine	40	30	14
Tyrosine	25	19	10
Phenylalanine	19	12	6
S-Carboxymethylcysteine	15	13	5
Tryptophan[b]	12	7	3

[a] Observed residues per molecule were calculated on the basis of an observed molecular weight of 67,000 less the weight due to carbohydrate (5092) for the immune macroglobulin H chain, 53,000 less the weight due to carbohydrate (2226) for the low molecular weight antibody H chain and 22,500 for the L chains. The L chains were assumed to be free of carbohydrate. The residues reported are the average of duplicate determinations. [b] Determined spectrophotometrically (Spande and Witkop, 1967).

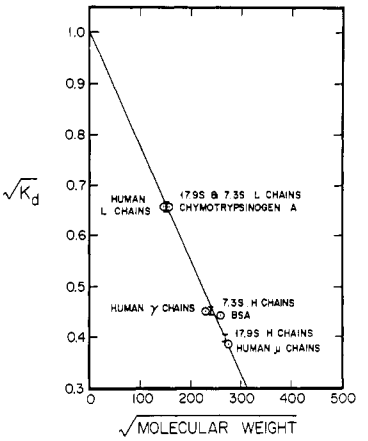

FIGURE 9: Plot of $(K_d)^{1/2}$ *vs.* (mol wt)$^{1/2}$ for totally reduced and alkylated proteins with known molecular weights eluted from a Sephadex G-200 volume (2.5 × 120 cm) with 5 M guanidine hydrochloride. The molecular weights for the standards used in this plot were 22,500 for the immunoglobulin light chains and 52,000 for the immunoglobulin γ chains (Edelman *et al.*, 1968), 66,000 for the immunoglobulin μ chains (Suzuki and Deutsch, 1967; Zikan *et al.*, 1971), and 58,000 for the paddlefish immune macroglobulin (Acton *et al.*, 1971b). The vertical bars indicate the estimate standard deviation of marine toad H and L chain K_d's obtained. $[K_d{}^a = V_e - (V_0/V_i)]^{1/2}$, where V_e is the peak elution volume (198 ml for this column as measured with Dextran-2000 (Pharmacia) and V_i the included volume. $V_0 + V_i$ = the elution volume for small molecules (702 ml for this column).

yields of glutamic acid. Also pyrrolidonecarboxylic acid has been shown to be the unreactive NH_2-terminal residue of several other immunoglobulin H and L chains (Bennett, 1968). It is possible that other blocked NH_2-terminal peptides are present in low yields.

Discussion

The experimental results presented in this paper demonstrate the presence of both high and low molecular weight antibodies in the marine toad, *Bufo marinus*. It is of particular interest that the molecular size of antibody synthesized is related to the type of antigenic stimulus. *Salmonella* H antigens elicit only high molecular weight agglutinating antibodies, even when the immunization schedule included multiple injections over a period of 241 days. However, neutralizing antibodies to T2 bacteriophage can be found in both high and low molecular weight components of serum as soon as 21 days following primary injection. Although T2 bacteriophage immunizations elicited in the marine toad antibodies of both high and low molecular weight types, a total shift to the low molecular weight variety was not observed over the interval tested. Similar findings have also been reported for other anuran amphibian species (Marchalonis and Edelman, 1966; Marchalonis *et al.*, 1970; Lykakis and Cox, 1968; Lykakis, 1969) as well as the marine toad (Diener and Marchalonis, 1970; Lin *et al.*, 1971).

The immune macroglobulin and low molecular weight antibody were found to have molecular weights of ~880,000 and ~160,000, respectively. The two molecules were also found to differ in the amount, as well as composition, of carbohydrate. The carbohydrate content of the immune macroglobulin (7.6%) is in the range of values (7.3–12.9%) reported for human macroglobulins by Davie and Osterland (1968). The low molecular weight antibody has a content of 4.2% which is significantly higher than values reported for IgG from several species of mammals by Niedermeier *et al.* (1971). This value is, however, similar to the content of 4.3% found for chicken 7S immunoglobulin (Niedermeier *et al.*, 1971). Perhaps more striking is the fact that the mannose to galactose ratio for the two types of marine toad immunoglobulins is on the order of 1:1. Several human macroglobulins (Davie and Osterland, 1968) and mammalian IgG from several species (Niedermeier *et al.*, 1971) have been found to range from 2:1 to 5:1. Recent investigations by our group (Acton *et al.*, 1972a,b) have demonstrated a 1:1 molar ratio of mannose to galactose in the carbohydrate moieties of immunoglobulins isolated from several species of vertebrates below Aves. These same studies also revealed that the majority of the carbohydrate is found associated with the H chain of both molecules from the marine toad. Less than 1% was found in the L-chain preparations.

Reduction of the immune macroglobulin under mild conditions resulted in a cleavage of 17 disulfide bonds. With increasing guanidine hydrochloride concentration, a progressive reduction of disulfide bonds resulted in a maximum number of 89 being titrated at a guanidine hydrochloride concentration of 5.04 M. The results of this titration differ from the amino acid analysis (see Table II) which would indicate 100 disulfide bonds for a pentameric molecule containing 10 H

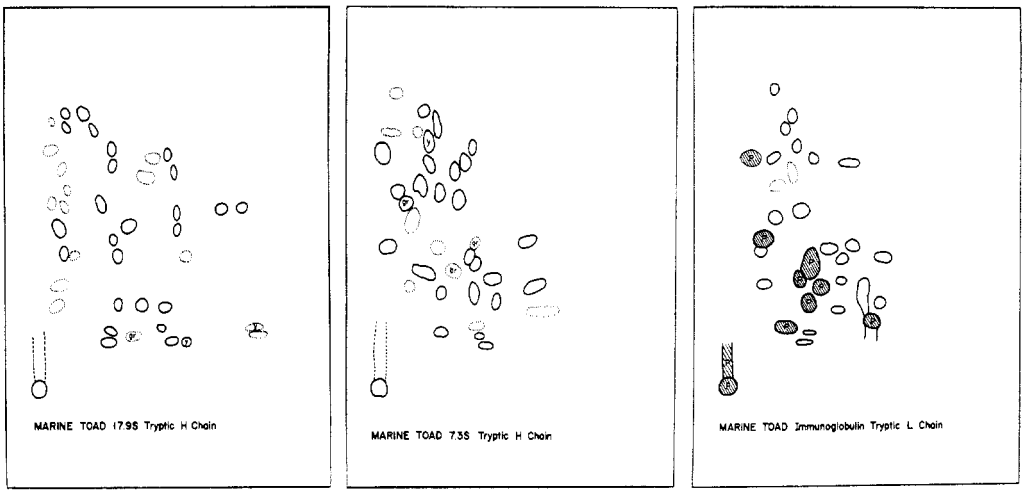

FIGURE 10: Tracings of the tryptic peptide maps of purified marine toad 17.9S and 7.3S immunoglobulin H and L chains. Chromatography was carried out in the horizontal dimension and electrophoresis was in the vertical dimension with the positive electrode on the origin side. Peptides which stain darkly with ninhydrin are outlined as solid circles. Peptides which appear only with special stains (ninhydrin negative) are drawn with a light margin. On the L chain tracing the pauli-positive peptides, used to confirm identity, are marked P and are cross-hatched. Br, Gr and Y are peptides staining brown, gray and yellow, respectively, with ninhydrin color dip.

TABLE III: Amino-Terminal Sequence Analysis of Marine Toad Immunoglobulin H and L Chains.

Immunoglobulin Type	Chain Type	1	2	3	4	5
Immune macroglobulin	Heavy		Ser	−Ile		
	Light	PCA[a]				
Low molecular weight antibody	Heavy	(PCA,	Ser,	Asx,	Glx)	
	Light	(PCA,	Ser,	Asx,	Glx,	Thr)

[a] PCA = pyrrolidonecarboxylic acid.

and 10 L chains. This difference of ~10% is consistent with the analytical limitations of these two methods, particularly since cysteine was determined as the *S*-carboxymethyl derivative rather than as cysteic acid. Although the maximum number of disulfide bonds titrated (89) agrees with the results of Habeeb *et al.* (1970) for human macroglobulin, there is a difference in the titration curve in that 90 disulfide bonds were cleaved in the human macroglobulins only after 6 hr and a guanidine hydrochloride concentration of 3–3.5 M. In the case of the marine toad immune macroglobulin there was not a substantial difference between reduction times of 1 and 6 hr. The titration of 17 disulfide groups for the low molecular weight antibody agrees with the 18 suggested from the amino acid analysis.

The H chain for the marine toad macroglobulin had a molecular weight of 67,000 while that of the low molecular weight antibody was ~53,000. The light chains from both types of immunoglobulin had a molecular weight of ~22,500. These values are in agreement with those reported for human IgM (Suzuki and Deutsch, 1967; Filitti-Wurmser *et al.*, 1970; Zikan *et al.*, 1971) and IgG (Edelman *et al.*, 1968) as well as for classes of immunoglobulins found in several species of lower vertebrates (Acton *et al.*, 1971b; Marchalonis and Edelman 1966; Clem and Small, 1967; Johnston *et al.*, 1971; Acton *et al.*, 1972a). If one assumes equal number of chains, 10 H and 10 L, for the macroglobulin and 2 H and 2 L for the low molecular weight antibody, then the aggregate molecular weight calculated from the polypeptide chain data is compatible with the total molecular weights determined by direct analysis.

The amino acid analysis of the H and L polypeptide chains from the two types of immunoglobulins also revealed that the H-chain molecules were different in many respects. The compositions for the L chains were very similar. This was further confirmed by peptide maps which also revealed differences between the H chains. The limited NH_2-terminal sequence data confirmed the fact that the H chains from the marine toad immunoglobulins were diferent.

The presence of a polypeptide chain other than the H and L chains has been observed in secretory IgA of rabbit (Halpern and Koshland, 1970) and human as well as human IgM (Mestecky *et al.*, 1971). This component of polymeric immunoglobulins has been termed "J" chain. Recent studies (Weinheimer *et al.*, 1971) have also revealed fast-moving protein bands on disc electrophoresis gels of the L-chain fractions of marine toad immune macroglobulins. The electrophoretic mobility of these bands appeared identical with those of human J chain from secretory IgA and IgM. It should be pointed out, therefore, that the difference in the amount of carbohydrate between the marine toad immune macroglobulin and low molecular weight antibody could be attributed in part to a J-chain-like component. Niedermeier *et al.* (1972) has shown purified J chain to have a total carbohydrate content of 7.6%. The contribution of J chain in this context would be very small since the ratio of L to J chain has been reported as 10:1 for pentameric macroglobulins (Wein-

heimer *et al.*, 1971). This fact, as well as the sharp cuts of the H- and L-chain fractions taken off the G-200 column in 5 M guanidine hydrochloride, precludes the significant contribution of a J-chain-like component to the physical and chemical studies of the respective chains.

The data presented in this paper on the immunoglobulins of the marine toad as well as comparable physical and chemical data on other species of anuran Amphibian by Marchalonis and Edelman (1966) and Marchalonis *et al.* (1970) suggest a close analogy between the immunoglobulins of Amphibia and the IgG and IgM molecules of mammals.

Acknowledgments

The authors acknowledge the technical assistance of Barbara Painter, Dianne Bentley, Martha Moyer, Karin Acton, Freda Moore, and Jim Griggs. We are also indebted to Joanna Schaefer of the Clinical Pathology Electrophoresis Laboratory for immunoelectrophoretic and antigenic analysis, and to Drs. Ahmed Habeeb and Ralph Schrohenloher for many helpful discussions and criticisms.

References

Acton, R. T., Bennett, J. C., Evans, E. E., and Schrohenloher, R. E. (1969), *J. Biol. Chem. 244*, 4128.

Acton, R. T., Niedermeier, W., Weinheimer, P. F., Clem, L. W., Leslie, G. A., and Bennett, J. C. (1972b), *J. Immunol.* (in press).

Acton, R. T., Weinheimer, P. F., Dupree, H. K., Evans, E. E., and Bennett, J. C. (1971a), *Biochemistry 10*, 2028.

Acton, R. T., Weinheimer, P. F., Dupree, H. K., Russell, T. R., Wolcott, M., Evans, E. E., Schrohenloher, R. E., and Bennett, H. C. (1971b), *J. Biol. Chem. 246*, 6760.

Acton, R. T., Weinheimer, P. F., Shelton, E., Neidermeier, W., and Bennett, J. C. (1972a), *Immunochemistry 9*, 421.

Adinolfi, M., and Wood, C. B. S. (1969), *in* Immunology and Development, Adinolfi, M., Ed., Lavenham, The Lavenham Press Ltd., p 27

Alford, C. A., Schaefer, J., Blankenship, W. G., Straumford, J. V., and Cassady, G. (1967), *New Eng. J. Med. 277*, 437.

Andrews, P. (1964), *Biochem. J. 91*, 222.

Bennett, J. C. (1967), *Methods Enzymol. 11*, 330.

Bennett, J. C. (1968), *Biochemistry 7*, 3340.

Bennett, J. C. (1969), *Arch. Biochem. Biophys. 131*, 551.

Clem, L. W., and Leslie, G. A. (1969), *in* Immunology and Development, Adinolfi, M., Ed., Lavenham, The Lavenham Press Ltd., p 62.

Clem, L. W., and Small, P. A., Jr. (1967), *J. Exp. Med. 125*, 893.

Cohn, E. J., and Edsall, J. T. (1943), *in* Proteins, Amino Acids and Peptides, Cohn, E. J., and Edsall, J. T., Ed., New York, N. Y., Reinhold Publishing Corp., p 370.

Davie, J. M., and Osterland, C. K. (1968), *J. Exp. Med. 128*, 699.

Diener, E., and Marchalonis, J. (1970), *Immunology 18*, 279.

Edelman, G. M., Gall, W. E. Waxdal, M. J., and Konigsberg, W. H. (1968), *Biochemistry 7*, 1950.

Ehrenberg, A. (1957), *Acta Chem. Scand. 11*, 1257.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Evans, E. E., Kent, S. P., Bryant, R. E., and Moyer, M. (1966), *in* Phylogeny of Immunity, Smith, R. T., Meischer, P. A., and Good, R. A., Ed., Gainesville, Fla., University of Florida Press, p 218.

Filitti-Wurmser, S. M., Tempete-Gaillourdet, M., and Hartman, L. (1970), *Immunochemistry 7*, 443.

Gall, W. E., and Edelman, G. M. (1970), *Biochemistry 9*, 3188.

Grey, H. M. (1963), *Proc. Soc. Exp. Biol. 113*, 963.

Grey, H. M. (1969), *Advan. Immunol. 10*, 51.

Habeeb, A. F. S. A. (1966), *Biochim. Biophys. Acta 115*, 440.

Habeeb, A. F. S. A., Schrohenloher, R. E., and Bennett, J. C. (1970), *J. Immunol. 105*, 846.

Halpern, M. S., and Koshland, M. E. (1970), *Nature (London) 228*, 1276.

Hubbard, R. W. (1965), *Biochem. Biophys. Res. Commun. 19*, 679.

Johnston, W. H., Jr., Acton, R. T., Weinheimer, P. F., Niedermeier, W., Evans, E. E., Shelton, E., and Bennett, J. C. (1971), *J. Immunol. 107*, 782.

Legler, D. W., Evans, E. E., Weinheimer, P. F., Acton, R. T., and Attleberger, M. H. (1969), *in* Biology of Amphibian Tumors, Mizel, M., Ed., New York, N. Y., Springer-Verlag, p 169

Leslie, G. A., and Clem, L. W. (1969), *J. Exp. Med. 130*, 1337.

Lin, H. H., Haywood, B. E., and Rowlands, D. T., Jr. (1971), *Immunology 20*, 373.

Lykakis, J. J. (1968), *Immunology 14*, 799.

Lykakis, J. J. (1969), *Immunology 16*, 91.

Lykakis, J. J., and Cox, F. E. (1968), *Immunology 15*, 429.

Maizel, J. V., Jr. (1966), *Science 151*, 988.

Marchalonis, J., and Edelman, G. M. (1965), *J. Exp. Med. 122*, 601.

Marchalonis, J., and Edelman, G. M. (1966), *J. Exp. Med. 124*, 901.

Marchalonis, J. J., Allen, R. B., and Saarni, E. S. (1970), *Comp. Biochem. Physiol. 35*, 49.

Marchalonis, J. J., Ealey, E. H. M., and Diener, E. (1969), *Aust. J. Exp. Biol. Med. Sci. 47*, 367.

Mestecky, J., Zikan, J., and Butler, W. T. (1971), *Science 171*, 1163.

Niedermeier, W. (1971), *Anal. Biochem. 46*, 465.

Neidermeier, W., Kirkland, T., Acton, R. T., and Bennett, J. C. (1971), *Biochim. Biophys. Acta 237*, 442.

Niedermeier, W., Tomana, T., and Mestecky, J. (1972), *Biochim. Biophys. Acta 257*, 527.

Parkhouse, R. M. E., Askonas, B. A., and Dourmashkin, R. R. (1970), *Immunology 18*, 575.

Romer, A. S. (1968), The Vertebrate Body, Philadelphia, Pa., W. B. Saunders Co., p 57.

Saluk, P. H., Krauss, J., and Clem, L. W. (1970), *Proc. Soc. Exp. Biol. Med. 133*, 365.

Schachmann, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic Press.

Small, P. A., Jr., and Lamm, M. E. (1966), *Biochemistry 5*, 259.

Spande, T. F., and Witkop, B. (1967), *Methods Enzymol. 11*, 498.

Sterzl, J., and Riha, I. (1970), Developmental Aspects of Antibody Formation and Structure, New York, N. Y., Academic Press.

Suzuki, T , and Deutsch, H. F. (1967), *J. Biol. Chem. 242*, 2725.

Svedberg, I., and Pedersen, K. D. (1940), The Ultracentrifuge, Oxford, Clarendon Press.

Warren, L. (1959), *J. Biol. Chem. 234*, 1971.

Waterfield, M., and Haber, E. (1970), *Biochemistry 9*, 832.

Weinheimer, P. F., Mestecky, J., and Acton, R. T. (1971), *J. Immunol. 107*, 1211.

Zikan, J., Niedermeier, W., Hurst, M. M., and Bennett, J. C. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 467.