Phylogeny of Immunoglobulins. Characterization of a 14S Immunoglobulin from the Gar, *Lepisosteus osseus**

Ronald T. Acton,† Peter F. Weinheimer,‡ Harry K. Dupree, E. Edward Evans, and J. Claude Bennett§

ABSTRACT: The immune macroglobulin from the longnose gar, *Lepisosteus osseus*, has been purified and physicochemically characterized. The macroglobulin was found to have a sedimentation coefficient of 13.9 S and a molecular weight of 610,000. There was a total of 4.97% carbohydrate with a residue distribution different from human macroglobins. The molecule was found to contain 82 disulfide bonds and dissociation into heavy (H) and light (L) chains could be observed following total reduction and alkylation in 7 M guanidine hydrochloride. The H and L chains could be separated by gel filtration and were found to have molecular weights of approximately 70,000 and 23,000, respectively. The macroglobulin was examined by electron microscopy and the molecule displayed a tetrameric structure. The amino acid composition was similar to mammalian IgM H and L chains. Although no free N-terminal amino acid was detected in the L chain, the N-terminal sequence of the first 4 residues of the H chain was found to be Asp-Ala-Val-Val. All of these structural data indicate a strong phylogenetic relationship among the immunoglobulins.

Immunoglobulin M (IgM) in mammals has been characterized as having a molecular weight of approximately 900,000 and is composed of five disulfide-bonded subunits, each of which consists of two heavy (μ) and two light (κ or λ) chains (Miller and Metzger, 1965; Lamm and Small, 1966; Putnam *et al.*, 1967; Bennett, 1969). The polypeptide chains were shown to have molecular weights of 66,000–75,000 for the H chain and 23,000 for the L chain. This pentameric structure of IgM suggested by physical and chemical studies has been confirmed by ultrastructural studies (Chesebro *et al.*, 1968; Feinstein and Munn, 1969; Shelton and McIntire, 1970) and serves to distinguish it from other mammalian immunoglobulin classes.

Limited physicochemical data suggest that birds, reptiles, amphibia, and elasmobranchs possess immunoglobulins resembling mammalian IgM (Marchalonis and Edelman, 1966; Clem and Small, 1967; Leslie and Clem, 1969; Acton *et al.*, 1970a; Saluk *et al.*, 1970). The need for understanding structural and functional relationships and the genetic mechanisms of immunoglobulin synthesis warrants further study from the standpoint of the phylogeny of these molecules.

The object of this investigation was to isolate and characterize the IgM-like molecules from a primitive bony fish, the gar, *Lepisosteus osseus*. The gar was felt to be ideal for these studies since one species, *Lepisosteus platyrhincus*, has been shown to produce antibodies only of the macroglobulin type to a variety of antigens (Bradshaw *et al.*, 1969).

Materials and Methods

Animals. Mature gar, weighing 2–8 kg, were captured from the Cahaba River, Perry County, Alabama, and were maintained in outdoor, artesian well-fed ponds at the Southeastern Fish Cultural Research Laboratory.

Reagents. Ultra Pure guanidine hydrochloride was obtained from Mann Research Laboratories. The optical density of a Millipore-filtered 7 M solution was less than 0.02/cm at 280 mμ. Refractive index measurements were performed on a Bausch & Lomb refractometer from which molar concentrations were determined as described by Kielley and Harrington (1960).

Sephadex G-200, Sepharose 6-B, and DEAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals and prepared for chromatography as previously described (Acton *et al.*, 1969).

Trypsin was purchased from the Worthington Biochemical Corp. reacted with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone to inactivate chymotryptic activity, and was stored at −20° in 0.001 N HCl at a concentration of 10 mg/ml until ready for use (Wang and Carpenter, 1965).

Iodoacetamide, 2-mercaptoethanol, MeSCN, pyridine, acetic anhydride, and trifluoroacetic acid were obtained from Eastman Organic Chemical. Before use iodoacetamide was twice recrystallized from hot water and MeSCN was purified by vacuum sublimation. *N*-Bromosuccinimide was purchased from K & K Laboratories, Inc., Plainview, N. J. DTNB reagent was obtained from Aldrich Chemical Co. *N,N*-Dimethylallylamine, benzene, ethyl acetate, and butyl chloride were Sequencer grade reagents purchased from Beckman. The reagents and solvents used in the sequence analysis were stored in two-neck Wolf bottles maintained under slight nitrogen pressure as described by Waterfield

* From the Department of Microbiology and the Division of Clinical Immunology and Rheumatology, Department of Medicine of the University of Alabama in Birmingham, The Medical Center, Birmingham, Alabama, and the Southeastern Fish Cultural Research Laboratory, Marion, Alabama. *Received August 28, 1970.* This investigation was supported by grants from the United States Public Health Service (AI-02693, AI-9153, 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 1970, at Atlantic City, N. J.

† Postdoctoral Research Fellow supported by U. S. Public Health Service Training Grant 5-T01-00293.

‡ Postdoctoral Research Fellow supported by U. S. Health Service Research Fellowship 1F02AM42874-01A1.

§ Recipient of a Research Career Development award (K3GM025, 386). To whom to address correspondence.

and Haber (1970). All other materials used were reagent grade according to American Chemical Society Standards.

Immunization and Bleedings. STO[1] antigen was prepared as described by Evans (1957). Human blood group A erythrocytes were obtained fresh from donors. Gar were given antigenic stimulation by injecting intraperitoneally 2×10^9 cells of STO or human A erythrocytes in 1 ml of 0.85% sodium chloride solution containing 0.3% formalin (v/v). Blood was obtained by cardiac puncture, allowed to clot, and the serum collected and stored at −25°. Agglutinin activity was assayed in Linbro trays (Linbro Chemical Co.) as described by Legler *et al.* (1970).

Preparation of Immune Macroglobulin. Immune sera were allowed to thaw at room temperature and centrifuged at 2980*g* for 15 min at 5° to remove precipitated material. Five milliliters was then 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, and 0.01% sodium azide buffer, and 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 fractions containing antibody activity from several runs on Sephadex G-200 columns were pooled and concentrated by ultrafiltration utilizing Diaflow XM-100 membranes (Amicon Corp.). Lipid and lipoproteins were then extracted from the concentrate by a modification of the method of Folch *et al.* (1951). Two volumes of the concentrate was added to one volume of chloroform–methanol (2:1, v/v), swirled, and allowed to stand for 30 min. The suspension was then centrifuged at 1560*g* for 1 hr at 5°. The upper aqueous layer was decanted and exhaustively dialyzed against 0.015 M Tris-HCl (pH 8.0). The precipitates were then removed by centrifugation at 2980*g* for 15 min at 5°. Fifty milliliters of the dialyzed extracted concentrate was then applied to a downward flow 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 NaCl gradient (0.015 M Tris-HCl, pH 8.0, to 0.6 M NaCl–0.015 M Tris-HCl, pH 8.0). The active antibody fraction from DEAE was then concentrated by ultrafiltration and 5 ml 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 final product (purified immune macroglobulin) was concentrated by ultrafiltration and used immediately or stored at 5° until studies could be completed.

Preparation of Antisera and Radial Immunodiffusion Analysis. Rabbit antisera to whole gar serum or the purified immune macroglobulin were prepared by intramuscular and footpad injections in Freund's complete adjuvant (Difco) at 0, 1, 3, and 6 weeks. The antisera obtained at 6 weeks after the last stimulation were used in the studies herein reported.

The immunoglobulins in gar whole sera were quantified by radial immunodiffusion as described by Alford *et al.* (1967). The concentration of total serum proteins in gar sera was determined by the biuret procedure.

Electrophoresis. Immunoelectrophoresis was conducted on film strips 12.7 × 3.5 cm, with the use of 175 V and 5 mA/strip for 1.5 hr as described by Alford *et al.* (1967). The slides were coated with 6 ml of "purified" agar (Immunology, Inc.) prepared in 0.06 M barbital buffer (pH 8.8).

Disc electrophoresis in 5% polyacrylamide gel containing 5 M urea was adapted from the procedure of Maizel (1966) as previously described (Acton *et al.*, 1969).

Reduction and Alkylation. Titration of disulfide groups in the purified macroglobulin was performed with 0.05 M 2-mercaptoethanol in 0.1 M Tris-glycine buffer (pH 7.0), containing 0.5 mg of EDTA/ml as described by Habeeb (1967). In the final reaction mixture the concentration of guanidine hydrochloride varied from 0 to 4.9 M. The number of liberated sulfhydryl groups were determined by the DTNB reagent of Ellman (1959) as modified by Habeeb (1966).

Macroglobulin H and L chains were isolated following total reduction and alkylation in guanidine hydrochloride. The macroglobulin solution (10 mg/ml) was adjusted to pH 8.4 by the addition of solid Tris and then was made 7 M with respect to guanidine hydrochloride by addition of the solid reagent. Mercaptoethanol was then added to a final concentration of 0.2 M and the solution was incubated at 37° for 4 hr. Alkylation was achieved by readjustment of the pH to 8.4 and the addition of a 1.5-fold molar excess of iodoacetamide with a reaction time of 1 hr at room temperature.

The totally reduced and alkylated material was dialyzed against 5 M guanidine hydrochloride and applied to a Sephadex G-200 upward flowing column (2.5 × 120 cm) equilibrated with 5 M guanidine hydrochloride as described by Small and Lamm (1966). The flow rate of the column was approximately 10 ml/hr. After monitoring the column effluent at 280 mμ, fractions of H and L chains were exhaustively dialyzed against distilled water and lyophilized. This same column was used to determine molecular weights of the fully reduced and alkylated heavy and light chains as described by Andrews (1964).

Physical Measurements. Sedimentation velocity measurements were performed at 20° in a Spinco Model E ultracentrifuge and sedimentation coefficients were calculated according to procedures described by Schachman (1959). Diffusion measurements were made in the same centrifuge with a synthetic boundary cell as described by Ehrenberg (1957). The maximum ordinate area method was utilized for calculation of the diffusion coefficients (Ehrenberg, 1957).

The sedimentation and diffusion measurements were carried out in 0.076 M sodium phosphate buffer (pH 7.5). All samples were exhaustively dialyzed against the buffer before analysis in the ultracentrifuge. Measurements were performed at different protein concentrations and were corrected to the reference state of water at 20°. Measured values of $s_{20,w}$ were extrapolated to zero concentration using the method of least squares to obtain $s^0_{20,w}$. The patterns were measured on a Nikon microcomparator.

The partial specific volume of gar immune macroglobulin was calculated from the amino acid and carbohydrate compositions by the method described by Cohn and Edsall (1943), and was found to be 0.712.

Extinction coefficients at 280 mμ were measured in 0.85% sodium chloride solution in a Beckman DB spectrophotometer. Protein concentration of the solutions was calculated from the nitrogen content determined with the Nessler reagent after Kjeldahl digestion. A nitrogen content of 16% was assumed. These analyses were performed in triplicate.

Carbohydrate Determinations. Before carbohydrate analyses the purified immune macroglobulin was twice precipitated by 50% $(NH_4)_2SO_4$, solubilized with 0.85% sodium chloride

[1] Abbreviations used are: STO, *Salmonella typhosa* "O"; DTNB, 5,5-dithio[bis(2-nitrobenzoic acid)].

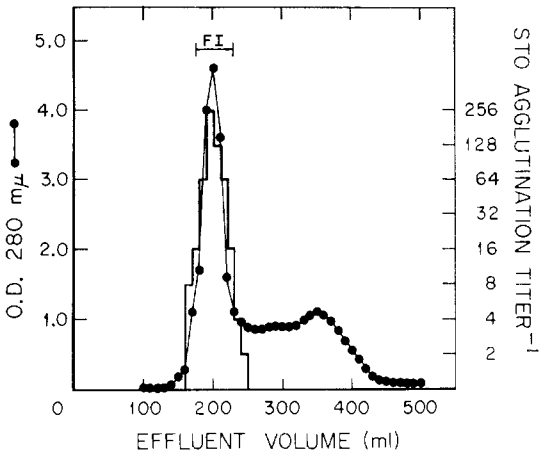

FIGURE 1: Elution pattern of gar whole immune serum from a Sephadex G-200 column (2.5 × 100 cm) equilibrated with Tris-NaCl (pH 7.4) buffer. F I indicates the fraction pooled for ion-exchange chromatography on DEAE A-50. The vertical bar represents STO antigen agglutinin titers. (●) Absorbance at 280 mμ.

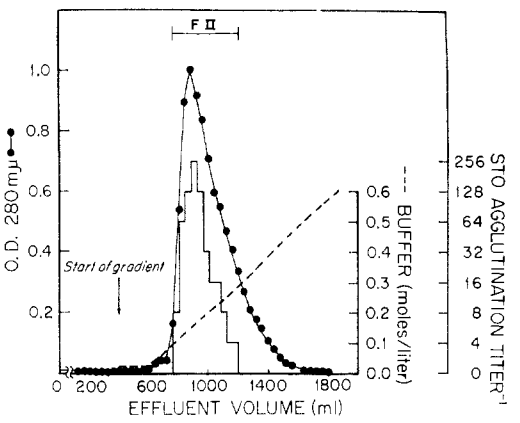

FIGURE 2: Elution pattern of the excluded active peak (F I) from Sephadex G-200 (Figure 1) from a DEAE A-50 anion-exchange column (2.5 × 45 cm). A molarity gradient (---) with 0.015 M Tris-HCl (pH 8.0) as the starting buffer and 0.015 M Tris-HCl + 0.6 M NaCl (pH 8.0) as the limit buffer was used. F II indicates the fraction pooled for gel filtration on Sepharose 6-B). The vertical bar represents STO antigen agglutinin titer. (●) Absorbance at 280 mμ.

solution and then twice precipitated by making the solution 50% with ethanol. The material was then exhaustively dialyzed against water and lyophilized. These methods were effective in removing possible carbohydrate contaminants that could arise from the Sephadex gel.

The alditol acetates of the neutral and amino sugars were identified and quantified by the gas chromatographic method of Niedermeier (1971). A Hewlett-Packard Model 404 gas chromatograph equipped with dual-flame ionization detectors, and 6-ft U-shaped, 0.25-in. glass columns packed with 1% ECNSS-M on 60–80 mesh Gas Chrom Q (Applied Science Laboratory, State College, Pa.) was utilized for these analyses. An Infotronics Model CRS 104 electronic integrator was used to quantify the results. All analyses were performed in triplicate.

Sialic acid was determined by the thiobarbituric acid method of Warren (1959). Samples were hydrolyzed in 0.05 N H_2SO_4 at 80° for 30 min.

Amino Acid Analysis. 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 on an 8-cm column for basic amino acids as described by Hubbard (1965). Routinely, samples were hydrolyzed in duplicate with 6 N HCl under nitrogen at 106° for 20 hr. After hydrolysis, HCl was removed under vacuum over NaOH. Tryptophan was determined spectrophotometrically by the *N*-bromosuccinimide method of Spande and Witkop (1967).

Peptide Maps. The two-dimensional peptide-mapping technique was performed as described by Bennett (1967). After digestion with trypsin, protein samples were subjected to chromatography followed by high-voltage electrophoresis in the second dimension. Peptides were detected by staining with ninhydrin–collidine. The Pauly stain was utilized to detect tyrosine- and histidine-containing peptides.

Amino-Terminal Sequence Analysis. The three-cycle Edman degradation method using MeSCN as described by Waterfield and Haber (1970) was followed for sequence analysis. This technique is essentially that described by Blömback *et al.* (1966) and modified by Niall and Potts (1969) except for the substitution of MeSCN for PhSCN as the coupling agent.

The methylthiohydantoin derivatives of the amino acids were resolved and identified by gas–liquid chromatography employing a Varian Aerograph Model 1840-4 dual-volume chromatograph with a Matrix temperature programmer and two flame ionization detectors in conjunction with a dual-pen Varian Model 30 recorder (Waterfield and Haber, 1970).

Electron Microscopy. Solutions of the purified immune macroglobulin were prepared for electron microscopy as previously described by Shelton and Smith (1970). The negatively stained molecules were examined and photographed at an initial magnification of 50,000 with a Siemens Elmiskop 101 electron microscope using a 50-μm objective aperture and 80-kV accelerating voltage.

Results

Purification of Immune Macroglobulin. Immune gar serum was subjected to gel filtration on Sephadex G-200 as shown in Figure 1. The largest amount of protein was eluted in the void volume and STO agglutinin activity was confined to this fraction. Serum from gar immunized with either STO or human A erythrocytes over as long as interval as 1 year consistently revealed this elution pattern. This excluded fraction contained significant amounts of residual lipid and lipoprotein. It, therefore, was pooled from several Sephadex G-200 runs as indicated by the horizontal cross bar (F I), extracted with chloroform–methanol, concentrated and dialyzed against 0.015 M Tris-HCl (pH 8.0). This fraction was then applied to a DEAE-Sephadex (A-50) column equilibrated with the same buffer. The material was eluted with an increasing salt gradient as shown in Figure 2. The elution pattern revealed a single symmetrical peak containing STO agglutinin activity. The fraction was pooled as indicated (F II), concentrated and applied to a Sepharose 6-B column (Figure 3). The eluted material contained STO agglutinin activity and was pooled as shown (F III) for examination in the ultracentrifuge. The sedimentation pattern of F III shown in Figure 4 indicated a very sharp boundary. This material had an $s_{20,w}$ value of 12.3 S and was free of high and low molecular weight contaminants. This material was found to aggregate somewhat with time. The purified immune

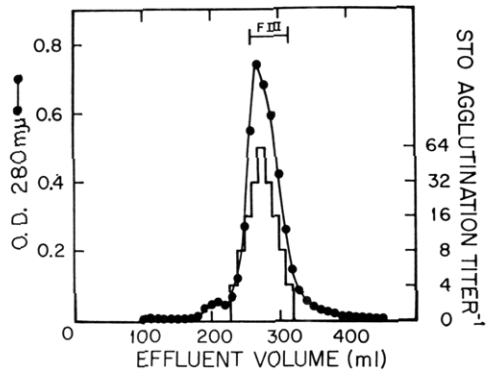

FIGURE 3: Gel filtration of F II on a Sepharose 6-B column (2.5 × 100 cm) equilibrated with Tris-NaCl (pH 7.4) buffer. F III indicates the volume pooled and will be referred to as purified immune macroglobulin. The vertical bar represents STO antigen agglutinin titer. (●) Absorbance at 280 mμ.

macroglobulin was also found to be free of contaminating proteins as determined by immunoelectrophoresis (Figure 5). Rabbit antiserum prepared against the purified gar macroglobulin resulted in a single precipitin band when reacted with whole gar serum.

During the purification process it became evident that a major portion of gar serum was the immune macroglobulin. To verify this the serum level of macroglobulin was quantified by radial immunodiffusion. For these assays the sera from 26 gar collected in July 1970 was pooled. The total serum protein concentration of the pooled sera was found to be 32 mg/ml with a macroglobulin level of 14 mg/ml. Thus, purified gar macroglobulin is obtained in a yield representing 40% of total serum protein. These values are similar to those reported for the levels of immunoglobulin in the

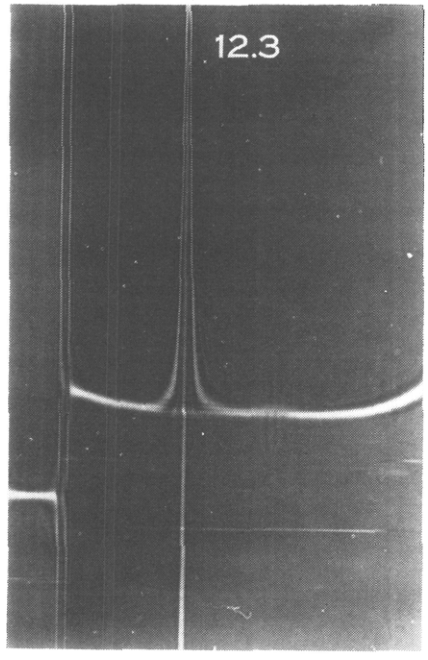

FIGURE 4: Ultracentrifugation pattern of purified immune macroglobulin (F III, Figure 3) in 0.076 M sodium phosphate buffer (pH 7.5). The photograph was taken 20 min after reaching a speed of 56,100 rpm at a phase plate-angle of 60° and a temperature of 20°. Protein concentration is 3.9 mg/ml. Sedimentation proceeds from left to right.

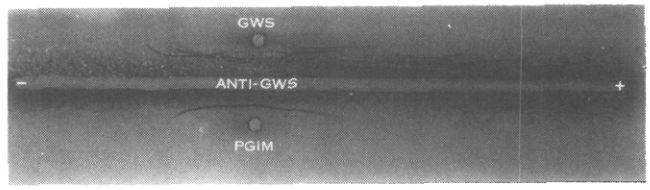

FIGURE 5: Immunoelectrophoresis of gar whole serum (GWS, top well) and gar purified immune macroglobulin (GPIM, bottom well, 5.0-mg/ml concentration). Precipitation arcs were developed using a rabbit antiserum against gar whole serum (Anti-GWS). The cathode is to the left.

nurse shark (Fidler *et al.*, 1969) and in paddlefish (Acton *et al.*, 1971a).

Physical Studies of the Immune Macroglobulin. The extinction coefficient at 280 mμ for the purified immune macroglobulin measured in 0.85% sodium chloride was 15.

Figure 6 is a plot of the sedimentation constants, $s_{20,w}$, of the purified material as a function of protein concentration. Sedimentation was slightly concentration dependent, and a coefficient of 13.97 S was obtained by extrapolation to infinite dilution. The diffusion coefficients, $D_{20,w}$, plotted as a function of protein concentration are shown in Figure 7 and at infinite dilution, $D^0_{20,w}$, was found to be 1.99.

The molecular weight of the purified immune macroglobulin was calculated from the sedimentation and diffusion coefficients at infinite dilution by use of the Svedberg equation. Using the partial specific volume of 0.712, a molecular weight of 610,000 was found.

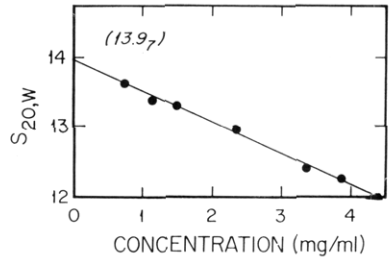

FIGURE 6: Sedimentation coefficients, $s_{20,w}$, of gar purified immune macroglobulin in 0.076 M sodium phosphate buffer, (pH 7.5) *vs.* protein concentration. The extrapolated value of $s^0_{20,w}$ at infinite dilution is indicated in parenthesis. The line is a least-squares fit of the experimental points.

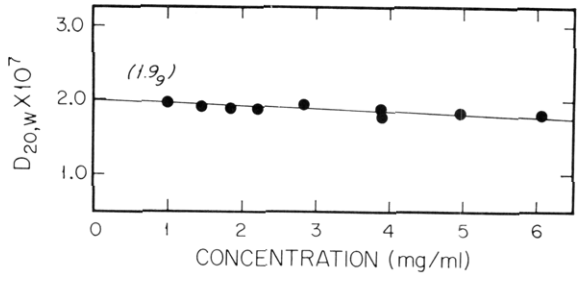

FIGURE 7: Determination of diffusion coefficient, $D^0_{20,w}$, of gar purified immune macroglobulin from a plot of $D_{20,w}$ values *vs.* concentration. Diffusion measurements were made with a synthetic-boundary cell (Ehrenberg, 1957) in 0.076 M phosphate buffer (pH 7.5). The $D^0_{20,w}$ value in parenthesis was obtained by extrapolation of the line determined by a least-squares fit of the experimental points.

TABLE I: Carbohydrate Composition of Gar Immune Macroglobulin.

	Mannose	Fucose	Galactose	Glucosamine[a]	Sialic Acid	Total Carbohydrate
Moles/moles Protein (610,000 molecular weight)	31	7	48	43	20	
% CHO ± standard deviation	0.93 ± 0.15	0.18 ± 0.02	1.45 ± 0.04	1.28 ± 0.18	1.02 ± 0.04	4.86

[a] Free Base.

Chemical Studies of the Immune Macroglobulin. The carbohydrate composition of gar purified immune macroglobulin is shown in Table I. The values reported are the averages of three determinations. It was found that the maximum yield of neutral sugars occurred after 4-hr acid hydrolysis while a 10-hr hydrolysis was necessary to hydrolyze all the *N*-acetylglucosamine from the molecule. The sialic acid determinations were carried out after 30-min hydrolysis.

The titration of disulfide bonds with increasing guanidine hydrochloride concentration is shown in Figure 8. Illustrated in the figure is the number of disulfide bonds cleaved per mole of protein as a function of time and guanidine hydrochloride concentration. A maximum of approximately 17 disulfide bonds was reduced in the absence of guanidine hydrochloride. By increasing the guanidine hydrochloride concentration there was a progressive cleavage of disulfide bonds with the maximum being obtained at a concentration of 3.5–4.9 M. At these guanidine hydrochloride concentrations a total of 82 disulfide bonds was titrated. As can be seen from the titration curve reduction was not measurably enhanced by increasing the time of reaction between 1 and 6 hr.

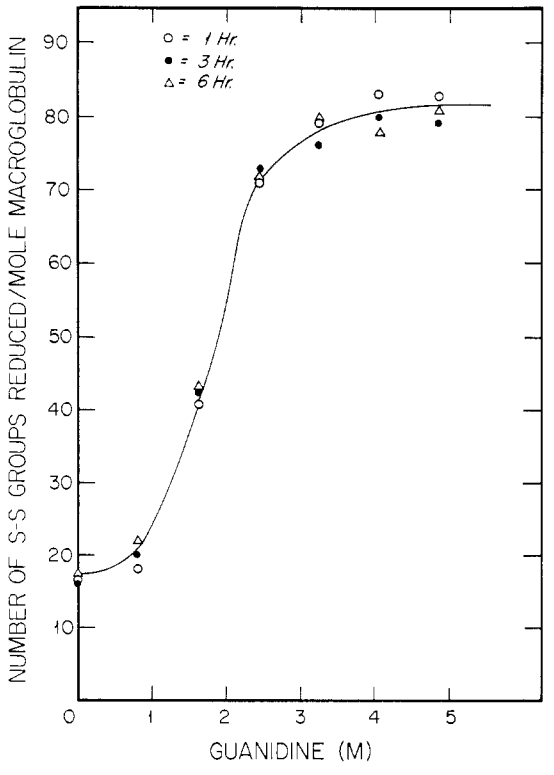

FIGURE 8: The titration of the number of disulfide bonds reduced in gar purified immune macroglobulin with increasing concentrations of guanidine hydrochloride at pH 7 with 0.05 M 2-mercaptoethanol.

For the isolation of the H- and L-polypeptide chains the purified immune macroglobulin was reduced in 7 M guanidine hydrochloride. The separation of the totally reduced and alkylated H and L chains was accomplished by gel filtration on Sephadex G-200 with 5 M guanidine hydrochloride as the eluting solvent (Figure 9). The relative yields of the H and L chains based on the optical density units was approximately 75 and 25%, respectively. The chains were pooled as indicated by the horizontal cross bars and examined by electrophoresis in acrylamide gels in the presence of 5 M urea. As can be seen from the polyacrylamide gel electrophoresis patterns, also included in Figure 9, the H- and L-chain preparations were rather homogeneous in respect to size.

The molecular weights of the H and L chains were determined from their elution volumes off the Sephadex G-200 column equilibrated with 5 M guanidine hydrochloride (Figure 9). This column had previously been calibrated with known markers also extensively reduced and alkylated. As described by Andrews (1964) a plot of $(K_d)^{1/2}$ *vs.* $(M_w)^{1/2}$

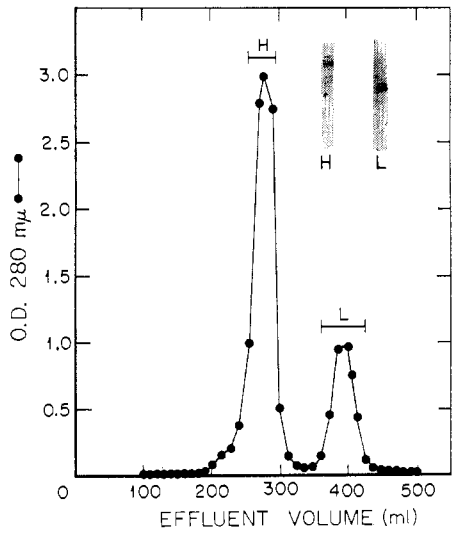

FIGURE 9: Sephadex G-200 elution pattern of totally reduced and alkylated gar purified immune macroglobulin. The eluting buffer was 5 M guanidine hydrochloride, the column size 2.5 × 120 cm. The cross bars indicate the volume pooled of the respective heavy and light chains. The acrylamide gel electrophoretic patterns illustrate the electrophoretic profile of the indicated pool.

TABLE II: Amino Acid Composition of Gar Immune Macroglobulin H and L Chains.

Amino Acid	Obsd Residues/Molecule[a]	
	H Chain	L Chain
Lysine	33	10
Histidine	9	2
Arginine	15	6
Aspartic acid	44	12
Threonine	65	20
Serine	69	30
Glutamic acid	49	21
Proline	42	12
Glycine	38	18
Alanine	36	15
Valine	45	18
Methionine	4	0
Isoleucine	18	4
Leucine	46	16
Tyrosine	21	9
Phenylalanine	23	6
S-Carboxymethylcysteine	17	5
Tryptophan[b]	18	5

[a] Observed residues per molecule were calculated on the basis of an observed molecular weight of 70,000 less the weight due to carbohydrate (3402) for the H chain and 23,000 for the L chain. The residues reported are the averages of duplicate determinations. [b] Determined spectrophotometrically.

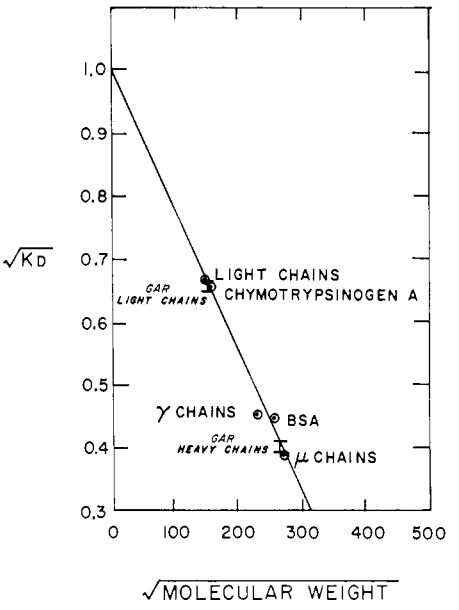

FIGURE 10: Plot of $(K_d^a)^{1/2}$ *vs.* $(M_w)^{1/2}$ for totally reduced and alkylated proteins with known molecular weights eluted from a Sephadex G-200 column (2.5 × 120 cm) with 5 M guanidine hydrochloride. The molecular weights of the standards used in this plot were 23,000 for the immunoglobulin light chains and 52,000 for immunoglobulin γ chains (Edelman *et al.*, 1968), 75,000 for immunoglobulin μ chains (Bennett, 1969), 67,000 for BSA (bovine serum albumin) (Phelps *et al.*, 1960), and 25,000 for chymotrypsinogen A (Hartley, 1964). The vertical bars indicate the estimated standard deviation of gar heavy and light chain K_d's obtained. $K_d^a = v_e - (V_0/V_i)$, where V_e is the peak elution volume, V_0 the excluded 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).

was constructed from the elution data of the known markers and is shown in Figure 10. The heavy chain was found to have a K_d value of 0.171 while the light chain had a value of 0.421. This corresponded to a molecular weight of 70,000 ± 1000 and 23,000 ± 1000 for the H and L chain, respectively.

Amino acid composition data for the purified H and L chains are shown in Table II. These data are averages of duplicate determinations. The H and L chains have a composition similar to human IgM counterparts (Bennett, 1969) with the exception of the higher number of *S*-carboxymethylcysteine in the H chain. There are relatively high amounts of serine, threonine, aspartic acid, and glutamic acid which have also been observed in mammalian immunoglobulins.

The tryptic peptide map tracings of the purified H and L chains are shown in Figure 11. There are 21 major peptides in the H-chain map (spots circled with solid lines) which stained strongly with ninhydrin and/or Pauly reagent and 16 lightly stained spots (spots circled with dotted lines). Although the tryptic peptide maps never revealed the anticipated number of peptides (49) this probably is due to the presence of an insoluble core and/or tangled chains after total reduction and alkylation. As can be seen from the tracing there was always a dense origin spot with streaking in the electrophoretic dimension.

Utilizing the Edman degradation procedure of Waterfield and Haber (1970) attempts to identify a free N-terminal residue in the L chain of gar macroglobulin were unsuccessful. This method did, however, reveal a sequence for the first four residues at the N terminus of the H chain

Val-Ile

of Asp-Ala-Val-Val. The relative yields of multiple residues

Leu-Leu

at positions 2 and 3 were not accurately quantified. However, on an estimated basis the major residues (-Ala-Val-) were present at levels of about 80%.

An electron micrograph of the gar macroglobulin is shown in Figure 12. The micrograph illustrates various aspects of the gar 14S immune macroglobulin, revealing that it consists of four radially arranged subunits attached to a central region. In some of the molecules, the "pincer" of the presumptive Fab portion of the subunit can be seen.

Discussion

These studies describe the characterization of a low molecular weight immune macroglobulin from the gar, a primitive holostean fish. Of interest is the fact that a single immunoglobulin class represented the only antibody that could be detected against the two antigens tested (STO and human A erythrocytes). Utilizing STH, diptheria toxoid, and bovine serum albumin, Bradshaw *et al.* (1969) also found antibody only in the excluded fraction from Sephadex G-200 in another species of gar, *Lepisosteus plathyrhincus*. Thus, it appears that the gar is capable of synthesizing only IgM-like antibodies. Interestingly, this immunoglobulin represents about 44% of the total serum proteins.

Although the immune macroglobulin could be isolated in a highly pure form aggregation was found to occur with time. Therefore, the molecular weight of the macroglobulin

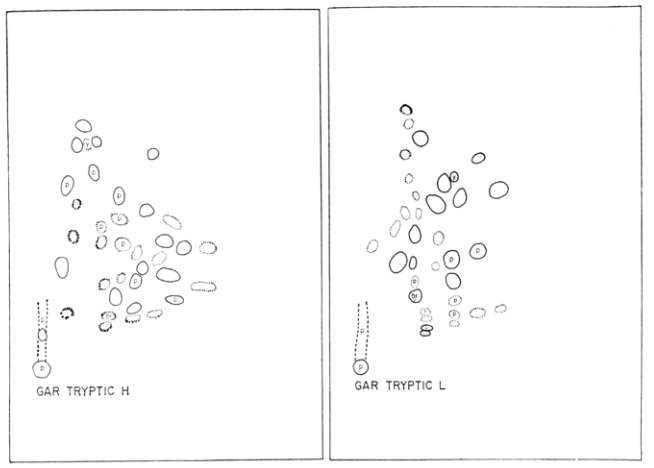

FIGURE 11: Tracings of the tryptic peptide maps of the heavy (H) and light (L) chains of gar purified immune macroglobulin. Chromatography was in the horizontal dimension and electrophoresis was in the vertical dimension with the positive electrode on the origin side. Solid circles are peptides which stain darkly with ninhydrin. Dotted circles are peptides which stain faintly with ninhydrin. Br, GR, and Y are peptides staining brown, gray, and yellow, respectively, with ninhydrin color dip. Pauly-positive peptides are marked P.

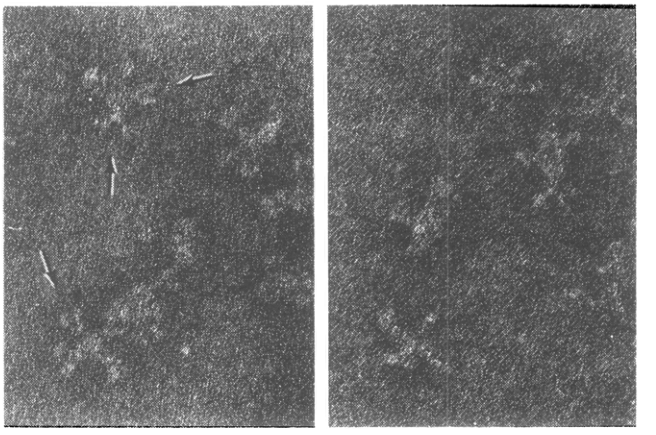

FIGURE 12: Several gar immunoglobulin molecules, each displaying four radially arranged subunits attached to a central region. In some of these molecules, the "pincer" of the presumptive Fab portion of the subunits can clearly be seen (arrows). Negatively stained with sodium phosphotungstate (pH 4.6). Magnification: 250,000×.

was determined by a combination of sedimentation and diffusion data. As pointed out by Creeth and Pain (1967) the transport method, in contrast to the equilibrium method, is almost unaffected by high molecular weight aggregates. Molecular weights derived by the sedimentation diffusion method usually are somewhat lower, however, than values of equal precision obtained by equilibrium. Even so the molecular weight of 610,000 obtained for gar macroglobulin is well below the values of 800,000–900,000 reported for macroglobulins in human, chicken, bullfrog, marine toad, and shark (Miller and Metzger, 1965; Leslie and Clem, 1969; Marchalonis and Edelman, 1966; Acton *et al.*, 1970a; Clem and Small, 1967). It should be stressed that the transport method gave a molecular weight of 850,000 for the marine toad macroglobulin (Acton *et al.*, 1970b) which agrees with the value reported by Miller and Metzger (1965) on a human macroglobulin.

Carbohydrate analysis revealed that the gar macroglobulin on a weight basis was deficient in carbohydrate as compared to human IgM. Davie and Osterland (1968) have found that the mass of carbohydrate in human IgM ranges from 7.3 to 12.9%. Other differences in the carbohydrate composition as compared to human IgM are the low level of fucose and the ratio of mannose to galactose. This molar ratio has been reported as 2:1 by Miller and Metzger (1965) and 3:1 by Davie and Osterland (1968) while gar macroglobulin has a ratio of 1:1.3. The amount of sialic acid is within the range of values reported for human IgM. Since relatively little is known about the biological function of carbohydrate or the conformational constraints the carbohydrate moiety may produce in immunoglobulins, the reason for this difference between gar and human is not clear.

The assay for the number of disulfides in gar macroglobulin resulted in a maximum of 82 bonds cleaved at a guanidine hydrochloride concentration of 3.5–4.9 M. The titration of the number of bonds cleaved was not time dependent in that a reaction time of 1, 3, and 6 hr gave similar values. The results of this titration differ from that determined from amino acid compositions (88 disulfides), but are within the limits of the method.

Although the gar macroglobulin differs in various physical and chemical aspects from human IgM the polypeptide chains appear to be similar. The H and L chains could be separated by gel filtration following extensive reduction and alkylation in relative yields of 75 and 25%, respectively. The chains have molecular weights of 70,000 for the H and 23,000 for the L which agrees with the molecular weights reported for rabbit IgM H and L chains (Lamm and Small, 1966) bullfrog (Marchalonis and Edelman, 1966) and for lemon shark (Clem and Small, 1967). The amino acid composition of gar H and L chains are in general similar again to values reported for human (Bennett, 1969; Putnam *et al.* ,1967), bullfrog (Marchalonis and Edelman, 1966), lungfish (Marchalonis, 1969), and dogfish shark (Marchalonis and Edelman, 1966). The number of *S*-carboxymethylcysteine residues in gar H chain is slightly higher than reported for human IgM H chain, while the gar L chain has the same number as in the human.

The molecular weight of the H chain may be too high by as much as 10,000 as determined from standardized gel filtration columns. The H chain from a closely related animal, paddlefish, similarly had a molecular weight of 70,000 by the column method, but was found to be ~60,000 by sedimentation equilibrium in guanidine hydrochloride employing ultraviolet optics (Acton *et al.*, 1971a). The lower H-chain molecular weight is in fact more compatible with a total molecular weight of 610,000 (assuming eight H and eight L chains).

The N-terminal sequence of $\text{Asp-Ala-}\begin{matrix}\text{Val-Ile}\\ \text{Leu-Leu}\end{matrix}\text{-Val}$ for the first four residues suggests a great deal of sequence heterogeneity for the immune H-chain population. This limited sequence is not only homologous to μ-chain N-terminal sequences reported for human (Bennett, 1968; Köhler *et al.*, 1970), horse (Montgomery *et al.*, 1970), paddlefish (Acton *et al.*, 1970a), and leopard shark (Suran and Papermaster, 1967) but is similar to the N-terminal sequence of rabbit immunoglobulin L chains (Doolittle, 1966; Hood *et al.*, 1969). This similarity between immunoglobulin H and L chains from species as diverse as bony fish and mam-

mals is further evidence that these chains had a common ancestral origin. Most of the heavy chains sequenced from mammals have been shown to have a blocked N-terminal residue (pyrrolidonecarboxylic acid). There has now been reported a group of heavy chains characterized by the presence of a free N-terminal group (Köhler *et al.*, 1970; Pink *et al.*, 1970). Although the gar heavy chain was found to contain a free N terminus, there is the possibility that a minor population of H chain may be present with a blocked N-terminal residue as found in the leopard shark by Goodman *et al.* (1970).

The 14S gar immune macroglobulin appears to be similar in many aspects to IgM from other species. The molecule does, however, show significant differences. A molecular weight of 610,000 with an equal number of H and L chains (assuming the extinction coefficients of these chains to be similar) and only 82 disulfide bonds/molecule suggests the presence of fewer subunits than is found in the usual pentameric form of IgM. This has been confirmed by examination in the electron microscope (Figure 12) which clearly demonstrates a tetrameric subunit structure. This same molecular form has also been demonstrated in the paddlefish (Acton *et al.*, 1970a), of the order Chondrostei and in the carp, a Teleostei which is the more advanced of the bony fish (Shelton and Smith, 1970). There is now evidence for a distinct tetrameric form of an IgM-like protein in all three orders of the bony fishes (Acton *et al.*, 1971b). The evolutionary significance of these tetrameric IgM immunoglobulins is not presently clear. These molecules could, however, serve as models in understanding subunit assembly, genetics, and the structural basis for antibody specificity in the IgM class of immunoglobulins.

Acknowledgments

The authors acknowledge the many helpful discussions and criticisms of Drs. Ahmed Habeeb and Ralph Schrohenloher. We are also indebted to Dr. Emma Shelton of the Biosynthesis Section, National Cancer Institute for the electron micrograph, to Dr. William Niedermeier for carbohydrate analysis, to Joanna Schaefer of the Clinical Pathology, Electrophoresis laboratory for immunoglobulin quantitation, and to Lanier Green for his aid in the husbandry of the gar. The excellent technical assistance of Karin Acton, Freda Moore, and Jim Griggs is also appreciated.

Added in Proof

The H and L chains from a closely related animal, paddlefish, were found to have molecular weights of 58,100 ± 1500 and 21,000 ± 800, respectively, as determined by sedimentation equilibrium studies in 5 M guanidine hydrochloride employing ultraviolet optics. When the H and L chains of the gar were cochromatographed with those of the paddlefish on standardized gel columns the chains eluted together, suggesting that their weights are identical. These lower H- and L-chain molecular weights are more compatible with the total molecular weight of 610,000 obtained for the tetrameric gar macroglobulin.

References

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

Acton, R. T., Evans, E. E., Weinheimer, P. F., Dupree, H. K., and Bennett, J. C. (1970a), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 772.

Acton, R. T., Weinheimer, P. F., Dupree, H. K., Russel, T. R., Wolcott, M., Evans, E. E., and Bennett, J. C. (1971a), *J. Biol. Chem.* (in press).

Acton, R. T., Weinheimer, P. F., Hall, S. J., Niedermeier, W., Shelton, E., and Bennett, J. C. (1971b), *Proc. Nat. Acad. Sci. U. S.* (in press).

Acton, R. T., Weinheimer, P. F., Wolcott, M., Evans, E. E., and Bennett, J. C. (1970b), *Nature (London) 228*, 992.

Alford, C. A., Schaefer, J., Blankenship, W. J., Straumfjord, 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.

Blömback, B., Blömback, M., Edman, P., and Hessel, B. (1966), *Biochim. Biophys. Acta 115*, 371.

Bradshaw, C. M., Clem, L. W., and Sigel, M. M. (1969), *J. Immunol. 103*, 496.

Chesebro, B., Bloth, B., and Svehag, S.-E. (1968), *J. Exp. Med. 127*, 399.

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, Coh, E. J., and Edsall, J. T., Ed., New York, N. Y., Reinhold Publishing Corp., p 370.

Creeth, J. M., and Pain, R. H. (1967), *in* Progress in Biophysics and Molecular Biology, Butler, J. A. V., and Haxley, H. E., Ed., New York, N. Y., Pergamon Press, p 273.

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

Doolittle, R. F. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1195.

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

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

Evans, E. E. (1957), *in* Manual of Microbiological Methods, Conn, H. J., and Pelczar, M. J., Ed., New York, N. Y., McGraw-Hill Book Co., p 199.

Feinstein, A., and Munn, E. A. (1969), *Nature (London) 224*, 1307.

Fidler, J. E., Clem, L. W., and Small, P. A., Jr. (1969), *Comp. Biochem. Physiol. 31*, 365.

Folch, J., Ascoli, I., Lees, M., Meath, J. A., and deBaron, F. N. (1951), *J. Biol. Chem. 191*, 833.

Goodman, J. W., Klaus, H. G., Nitecki, D. E., and Wang, A.-C. (1970), *J. Immunol. 104*, 260.

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

Habeeb, A. F. S. A. (1967), *Arch. Biochem. Biophys. 121*, 692.

Hood, L., Lackland, H., Eichman, K., Kindt, T. J., Braun, D. G., and Krause, R. M. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 890.

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

Kielley, W. W., and Harrington, W. F. (1960), *Biochim. Biophys. Acta 41*, 401.

Köhler, H., Shinizu, A., Paul, C., and Putnam, F. W. (1970), *Science 169*, 56.

Lamm, M. E., and Small, P. A., Jr. (1966), *Biochemistry 5*, 267.

Legler, D. W., Acton, R. T., Weinheimer, P. F., and Dupree, H. K. (1970), *Immunology* (in press).

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

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

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

Marchalonis, J. J. (1969), *Aust. J. Biol. Med. Sci. 47*, 405.
Miller, F., and Metzger, H. (1965), *J. Biol. Chem. 240*, 3325.
Montgomery, P. C., Bello, A. C., and Rockey, J. H. (1970), *Biochim. Biophys. Acta 200*, 258.
Niall, H. D., and Potts, J. I., Jr. (1969), *Proc. 1st Amer. Peptide Symp., New York, N. Y.*
Niedermeier, B. (1971), *Anal. Biochem.* (in press).
Pink, J. R. L., Buttery, S. H., DeVries, G. M., and Milstein, C. (1970), *Biochem. J. 117*, 33.
Putnam, F. W., Kozuru, M., and Easley, C. W. (1967), *J. Biol. Chem. 242*, 2435.
Saluk, P. H., Krauss, J., and Clem, L. W. (1970), *Proc. Soc. Exp. Biol. Med. 133*, 365.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic Press.
Shelton, E., and McIntire, K. R. (1970), *J. Mol. Biol. 47*, 595.
Shelton, E., and Smith, M. (1970), *J. Mol. Biol. 54*, 197.
Small, P. A., Jr., and Lamm, M. E. (1966), *Biochemistry 5*, 259.
Spande, T. F., and Witkop, B. (1967), *Methods Enzymol. 11*, 498.
Suran, A. A., and Papermaster, B. W. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1619.
Wang, S.-S., and Carpenter, F. H. (1965), *J. Biol. Chem. 240*, 1619.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Waterfield, M., and Haber, E. (1970), *Biochemistry 9*, 832.

On the Functional Organization of the Respiratory Chain at the Dehydrogenase–Coenzyme Q Junction*

M. Gutman,† C. J. Coles,‡ Thomas P. Singer, and John E. Casida

ABSTRACT: Coenzyme Q_{10} (CoQ_{10}) was reincorporated into CoQ_{10}-depleted membrane preparations from heart mitochondria by two methods reported to achieve full restoration of reduced nicotinamide–adenine dinucleotide (NADH) oxidase activity. Reincorporation in aqueous medium or in an anhydrous one were compared on the basis of restoration of NADH and succinoxidase activities, effect on NADH dehydrogenase activity, CoQ_{10} content, and reactivity with piericidin A. While reversible removal of CoQ_{10} from the succinoxidase system is readily achieved without obvious damage to the dehydrogenase in the reconstituted particles, NADH dehydrogenase is significantly modified even though there is extensive restoration of NADH oxidase activity. Contrary to previous evidence, reincorporation of CoQ_{10} to the level originally present does not result in full restoration of NADH oxidase activity. Comparison of NADH and succinoxidase activities on titration of the depleted particles with increasing amounts of CoQ_{10} further suggests a compartmentation of CoQ_{10} at the flavoportein junction, a proposal supported by the findings of Ernster and coworkers from experiments involving gradual depletion of the particles by serial pentane extractions.

The observation that in reconstituted particles the inhibition by piericidin A is competitive with respect to CoQ_{10} is discussed in relation to the mechanism of action of this inhibitor.

The functional organization of the NADH dehydrogenase–CoQ_{10}[1] juncture in the respiratory chain is poorly understood despite the obvious importance of this knowledge to the definition of many reaction mechanisms. This locus appears to be the rate-limiting step in the NADH oxidase system because both NADH dehydrogenase activity and the turnover rate of the cytochromes exceed the overall NADH oxidase or NADH–CoQ reductase activities (Cremona and Kearney, 1964; Singer and Gutman, 1971). It is also a distribution point where CoQ_{10} molecules, in large molar excess, accept electrons from or donate electrons to a few but more rapidly reacting NADH dehydrogenase molecules, and it is the point at which reducing equivalents from a variety of substrates (*i.e.*, succinate, α-glycerophosphate, NAD-linked substrates) enter a common pathway (Klingenberg, 1968). Finally, this juncture is in the immediate vicinity of energy conservation site I (Schatz and Racker, 1966; Gutman *et al.*, 1970a) and of the inhibition sites of rotenone, piericidin A, and barbiturates (Horgan *et al.*, 1968a,b).

There are many technical difficulties and few unambiguous techniques for studying the interaction of the flavoprotein with endogenous CoQ_{10}. Kinetic studies have been hampered, until recently (Gutman and Singer, 1970), by the lack of an appropriate method for following the oxidation–reduction of NADH dehydrogenase without serious interference by other

* From the Molecular Biology Division, Veterans Administration Hospital, San Francisco, California 94121, the Department of Biochemistry and Biophysics, University of California Medical Center, San Francisco, California 94122, and the Division of Entomology, University of California, Berkeley, California 94720. *Received November 10, 1970.* Paper XXI in the series: Studies on the respiratory chain-linked reduced nicotinamide–adenine dinucleotide dehydrogenase. This work was supported by grants from the American Cancer Society (P-531), the National Science Foundation (GB 8248), the National Institutes of Health (HE 10027 and ES 00049), and the American Heart Association (67 706).

† To whom correspondence should be addressed. Present address: Veterans Administration Hospital, San Francisco, California 94121. On leave of absence from Tel-Aviv University, Israel.

‡ Fellow of the Bay Area Heart Association.

[1] Abbreviations used are: CoQ, coenzyme Q; ETP, nonphosphorylating preparation of the inner membrane; ESP and ETP_H, phosphorylating inner membrane preparations.