Subunit Structure and Number of Combining Sites of the Immunoglobulin A Myeloma Protein Produced by Mouse Plasmacytoma MOPC-315*

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ABSTRACT: The myeloma protein produced by mouse plasmacytoma MOPC-315 was studied with respect to its subunit structure and number of ligand-binding sites per molecule. The protein, of the immunoglobulin A (IgA) class, was isolated as a 7S molecule by a procedure that entailed reduction and alkylation of five to six disulfide bonds (presumably interchain). Determination of molecular weights and amino acid compositions of the 7S protein and its separated chains showed that, like 7S immunoglobulins in general, it is composed of two heavy and two light chains. Digestion with trypsin yielded an Fab(t) fragment whose yield, molecular weight, and chain structure indicated that in the intact 7S molecule there are two indistinguishable Fab parts, each consisting of a complete light chain and 55% of a heavy chain. Similar results were obtained with protein 460, another mouse IgA-myeloma protein that binds DNP groups specifically. In accord with the duplex symmetry of its subunit structure, protein 315 has two apparently identical ligand-binding sites per 7S molecule, with one site per Fab(t) fragment. This number of sites was demonstrable by equilibrium dialysis only when the protein was tested at relatively high concentration ($\geq$0.5 mg/ml); at much lower concentrations fewer combining sites were measurable unless "protective" proteins, such as gelatin or nonspecific rabbit γ-globulin, were added.

Of the myeloma proteins[1] that bind simple substances, the one produced by mouse plasma cell tumor MOPC-315 is outstanding because of its high affinity for DNP[2] ligands, which it binds in essentially the same way as anti-DNP antibodies raised by conventional immunization procedures (Eisen *et al.*, 1968, 1970). When protein 315, a myeloma protein of the IgA class, was first identified and subjected to preliminary study there was considerable uncertainty in regard to its monomer (7S) molecular weight, and on the basis of a provisional value of 120,000 it was estimated to have fewer than two binding sites (actually 1.2) per molecule with sedimentation coefficient of 7 S (see below). As this number of sites and the provisional molecular weight were both distinctly anomalous for antibody molecules we have, in this study, attempted to clarify the subunit structure and the number of ligand-binding sites of protein 315. This seemed an especially worthwhile undertaking at this time because this protein and the tumor cells that produce it are now being studied in a number of laboratories. The present results show that the 7S form of protein 315 has (1) a molecular weight close to 150,000, (2) the same number of light and heavy chains—two of each—as other 7S immunoglobulins, and (3) two ligand-binding sites. The fewer than two sites measured previously (Eisen *et al.*, 1968) appear to be due to instability at low concentration of the mildly reduced and alkylated 7S form of this protein.

Materials and Methods

Purification of the Mildly Reduced and Alkylated 7S Monomer of Protein 315. Protein 315 was isolated from serum pools obtained from BALB/c mice bearing plasmacytoma MOPC-315 (Eisen *et al.*, 1968). In a representative purification the washed specific precipitate obtained by adding 65 mg of $DNP_{23}HSA$ to 50 ml of serum was dissolved in 15 ml of 0.1 M DNP-glycine–0.25 M Tris-Cl (pH 8.0) and reduced for 2 hr at about 23° by adding dithiothreitol (DTT, Sigma Chemical, St. Louis, Mo.) to a final concentration of 0.01 M. After reduction, the solution was chilled to 4° and made 0.022 M in iodoacetic acid (Sigma Chemical, St. Louis, Mo.) and kept at 4° for 45 min. The mildly reduced and carboxymethylated protein solution (20 ml) was applied to a column (2.5 × 40 cm) containing a bottom layer of 60 ml of Dowex 1-X8, 20–50 mesh, Cl^- form (Bio-Rad, AG-1, Richmond, Calif.) and an upper layer of 60 ml of DEAE-cellulose (Serva Gallard, Schlesinger, Long Island, N. Y.). Prior to sample application, the column was equilibrated with 0.2 M Tris-Cl–0.05 M NaCl (pH 8.0) and chromatography was carried out with the same buffer at a flow rate of 90 ml/hr. Under these conditions the antigen was retarded relative to the immunoglobulin,

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[1] Terminology: nomenclature and abbreviations for immunoglobulins correspond to those recommended by the World Health Organization (*Bull. W. H. O. 30,* 447 (1964)). The plasma cell tumors are referred to as MOPC-315 and MOPC-460 and the immunoglobulins they produce as protein 315 and protein 460, or simply 315 and 460, respectively. The tumors were carried by serial subcutaneous transplantation in BALB/c mice (Cumberland Farms, Tenn.), beginning with tumor tissue obtained originally from tumor-bearing BALB/c AnN mice kindly provided by Dr. Michael Potter.

[2] Abbreviations used are: DNP, 2,4-dinitrophenyl; HSA, human serum albumin; $DNP_{23}HSA$, human serum albumin with 23 moles of DNP (substituted on lysine residues) per mole of protein; RγG, rabbit γ-globulin; PBS, 0.15 M NaCl–0.01 M potassium phosphate (pH 7.4); DTT, dithiothreitol; A_{278}, absorbance at 278 nm; TPCK, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone; SDS, sodium dodecyl sulfate.

which eluted at 200 ml as the purified protein. Occasionally it was necessary to repeat the chromatography after concentrating the protein by freeze-drying, dissolving the precipitate in 0.05 M DNP-glycine, and passing the solution again under the same conditions through a fresh column of DEAE-cellulose and Dowex 1.

The reduction-alkylation step was necessary, as recovery of protein from the ion-exchange resins was otherwise prohibitively low. This procedure introduced one carboxymethyl substituent in the light chain, and four to five in the heavy chain (unpublished data), and converted the protein from its polydisperse state in serum (approximately 7, 9, 11, and 13 S) to a single sedimenting species ($s_{20,w}$ = 6.5 S), which is hereafter referred to as the 7S protein or 7S monomer.

The purified 7S protein was concentrated by precipitation with 40% saturated ammonium sulfate and dialyzed against PBS, or against 0.01 M phosphate (pH 7.4), and freeze-dried. In some preparations, the mildly reduced protein was alkylated with ethyleneimine (Matheson Coleman & Bell, Norward, Ohio). The 7S protein was recovered in 50–70% yield, based on the total amount of protein 315 precipitated from serum by DNP-HSA.

Protein 460, another mouse IgA myeloma protein with anti-DNP activity, was isolated in the same way with similar yield (Jaffe *et al.*, 1969, 1971). Both purified 7S proteins (460 and 315) gave single precipitin bands in gel diffusion and in immunoelectrophoresis with rabbit antisera prepared against whole (BALB/c) serum and against purified protein 315 (*e.g.*, see Figure 4, below). In both proteins the residual mole fraction of DNP-glycine was negligible (<10%).

Separation of Heavy and Light Chains. The remaining disulfide bonds of the 7S protein were reduced to facilitate complete separation of heavy and light chains. For this purpose the freeze-dried protein was dissolved in 7 M guanidine hydrochloride (Mann, Orangeburg, N. Y.)–0.5 M Tris·Cl (pH 8.2) and treated for 2 hr at 23° with 0.05 M DTT. Iodoacetic acid was then added at 2.2-fold molar excess with respect to DTT. After standing at 4° for 45 min, the reaction mixture was applied to a column of Sephadex G-100 that was equilibrated and developed with 6 M urea–1 M acetic acid. The separated chains were freeze-dried after urea was removed by dialyzing the heavy chain against water or 1 M acetic acid and by filtering the light chain through Sephadex G-25 in 1 M acetic acid. In some preparations, the fully reduced protein was alkylated with ethyleneimine instead of iodoacetic acid (for example, see Fab(t) fragment, below).

Fab(t) Fragments. The 7S protein at 15 mg/ml in 0.1 M Tris·Cl–0.02 M $CaCl_2$ (pH 8.0) was digested at 37° with TPCK-trypsin (Worthington Biochemicals Corp., Freehold, N. J.) at a total enzyme to protein ratio of 1:50 (w/w). Equal amounts of enzyme were added at 0 and 3 hr. After 6 hr the digest was passed through Sephadex G-100 in 0.05 M Tris·Cl (pH 8.0).

To resolve the Fab(t) fragment into chains, it was helpful (as in separating light and heavy chains of the 7S protein) to cleave the remaining disulfide bonds. Accordingly, lyophilized Fab(t) was dissolved in 5 M guanidine hydrochloride–0.5 M Tris·Cl (pH 8.2) and treated with 0.05 M DTT for 2 hr at 23°. After adjustment of the pH to 8.6 ethyleneimine was added in 20-fold molar excess with respect to DTT (Raftery and Cole, 1963). The aminoethylated Fab was dialyzed for 1 hr against several changes of water to remove most of the guanidine hydrochloride, and then made 1% in SDS and filtered on G-100 in 1% SDS.

Molecular Weights. High-speed equilibrium centrifugation (Yphantis, 1964) of the 7S monomer of protein 315, its heavy and light chains, and its Fab(t) fragment were carried out at 20° for 20–36 hr in a Spinco Model E analytical ultracentrifuge equipped with absorption optics (kindly made available by Dr. J. R. Little). The concentrations, solvents, and rotor speeds (revolutions per minute) were as follows: 7S monomer, 0.33 and 0.17 mg per ml in PBS, at 20,000; Fab(t) fragment, 0.15 and 0.075 mg per ml in PBS at 24,000 and 30,000; heavy chain, 0.43 and 0.1 mg per ml in 6 M guanidine hydrochloride, at 36,000; light chain, 0.4 and 0.1 mg per ml in 6 M guanidine hydrochloride, at 36,000 and 40,000. Partial specific volumes were calculated from the amino acid composition (Cohn and Edsall, 1943) and, in the case of the heavy and light chains, were corrected for binding of guanidine hydrochloride (Hade and Tanford, 1967). The values thus obtained were: 7S monomer, 0.729; heavy chain, 0.714; light chain, 0.714; Fab(t) fragment, 0.729.

Ligand Binding. Equilibrium dialysis was carried out in small Lucite chambers (Gateway Immunosera, Inc., Cahokia, Ill.); 80 μl of protein in PBS and an equal volume of the same buffer containing radioactive ligand were added to opposite sides of the membrane. After equilibration was attained (40 hr at 4°), 25-μl samples were withdrawn from each compartment and counted in 10 ml of Bray's solution (Bray, 1960) in a Packard liquid scintillation counter.

In some experiments gelatin was added to a final concentration of 1.5 mg/ml to protein 315 to minimize denaturation. The gelatin was tested along in equilibrium dialysis and shown not to bind [^{3}H]-ε-DNP-L-lysine or [^{3}H]-2,4-dinitronaphthol. In a few experiments, nonspecific rabbit γ-globulin (at 1.5 mg/ml) was used instead of gelatin, and corrections were made for its small amount of nonspecific binding of ε-DNP-L-lysine (5–7% of the free-ligand concentration). In some experiments, and to monitor column effluents, binding of ligands to protein 315 was also evaluated by the method of fluorescence quenching (Velick *et al.*, 1960; Eisen, 1964).

The following molar extinction coefficients were used to measure ligand concentrations in phosphate–saline (PBS): ε-DNP-L-lysine, 17,500 at 360 nm; 2,4-dinitronaphthol, 14,630 at 395 nm; and menadione, 20,000 at 252 nm (M. Michaelides, personal communication). The extinction coefficients for proteins 315 and 460 were 1.35 (this paper) and 1.55 cm^2 per mg (Jaffe *et al.*, 1969), respectively, at 278 nm in PBS.

Radioactive Ligands. [^{3}H]-ε-2,4-Dinitrophenyl-L-lysine was prepared by reacting [3,5,6-^{3}H]1-fluoro-2,4-dinitrobenzene (10,400 mCi/mmole) with α-*tert*-Boc-L-lysine or with poly-L-lysine followed by acid hydrolysis (Eisen *et al.*, 1968; McVeigh and Voss, 1969). After purification by thin-layer chromatography on silica gel G, developed with methyl ethyl ketone, various preparations had specific activities of 80–400 cpm/pmole. [^{3}H]-2,4-Dinitronaphthol was prepared by catalytic exchange with tritium gas (New England Nuclear) and was crystallized (three times) from glacial acid and water (2:1, v/v). After purification by thin-layer chromatography on alumina sheets (Eastman, Rochester, N. Y.) developed with propanol–H_2O–NH_3 (7.5:2.5:1, v/v), the [^{3}H]-2,4-dinitronaphthol had a specific activity of 50 cpm/pmole. [^{14}C]-2-Methyl-1,4-naphthaquinone (menadione) was obtained from Amersham–Searle (Des Plaines, Ill.); its specific activity was 23 cpm/pmole and it was used without further purification. [^{14}C]Menadione and unlabeled menadione (see below) were indistinguishable as ligands in fluorescence quenching titration with protein 315.

Other Analytical Procedures. Extinction coefficients were based on microkjeldahl analyses (kindly performed by Dr. S.

Frankel, Midwest Laboratories, St. Louis, Mo.), assuming N was 16% of protein weight. For amino acid analyses, samples were hydrolyzed under vacuum in constant-boiling HCl at 110° for 22 hr and analyzed with a Spinco automatic analyzer, Model 120B, equipped with 6.6-mm cell and a recorder modified such that the range was 4–5 mV (0.0–0.1 OD) (Spackman *et al.*, 1958). The Fd piece and the light chain isolated from the Fab(t) fragment were each precipitated with acetone (from solutions in sodium dodecyl sulfate), before being taken up in HCl for hydrolysis.

Disc electrophoresis was carried out at pH 9.5 (Ornstein, 1964 Davis, 1964) or in 1% sodium dodecyl sulfate at pH 7.2 (Weber and Osborne, 1969).

C-terminal residues were determined by hydrazinolysis (Fraenkel-Conrat and Tsung, 1967) and by digestion with carboxypeptidases A and B (Ambler, 1967). The digestions were performed at an enzyme:substrate ratio of 1:40 in 2 M urea–0.1 M NH_4HCO_3 or, when the protein was soluble in water, in 0.2 M *N*-ethylmorpholine acetate (pH 8.5). The C-terminal amino acids were measured on the amino acid analyzer.

Total hexose was measured by the anthrone method, with an equimolar mixture of galactose and mannose as standard (Roe, 1955). The analyses were carried out on dialyzed samples of protein or on aliquots of hydrolysates prepared in 3 N HCl for 3 hr at 100° in sealed tubes. No significant differences in results were observed.

Tryptophan content was calculated from the absorbance at 288 nm of the protein in 1 M acetic acid after correcting for the absorbance due to tyrosine; the correction was based on the tyrosine content (determined by amino acid analysis). The molar extinction coefficients of tyrosine and tryptophan at 288 nm (1 M acetic acid) were 239 and 4670, respectively.

Antisera. Rabbits were immunized by injecting hind footpads with antigens in complete Freund's adjuvant. Bleedings were taken approximately 2 weeks following the last immunization.

The antiserum to whole mouse serum was obtained from a single rabbit, injected with 0.2 ml of Swiss mouse serum followed by 0.2 ml of serum from BALB/c mice carrying MOPC-315 at 1 month and at 11 months following primary immunization.

The rabbit antiserum to both the heavy and the light chains of protein 315 was a pool obtained from two rabbits, each of which had been injected with 1 mg of the 7S form of protein 315 followed by two additional injections of partially reduced and alkylated heavy chains from protein 315, 6 and 9 months after the initial injection. As these heavy chains were not completely resolved from the light chains, the antiserum contained antibodies directed to the heavy chains (predominantly) and to the light chains of protein 315.

An antiserum to intact protein 315 was prepared by injecting 1 mg of the 7S form of protein 315 followed by a second injection 11 months after the initial injection. The antiserum specific for the light chain of protein 315 was prepared by absorbing the anti-315 serum with purified, mildly reduced and alkylated heavy chains of protein 315.

Results

Number of Chains in the 7S Monomer. From high-speed sedimentation equilibrium the molecular weights of the 7S form of protein 315 and of its heavy and light chains were calculated to be 153,000 ± 2000 (SEM), 54,500 ± 1300, and 21,000 ± 200, respectively (Figure 1). The value for the 7S protein is close to that expected (151,000) for a molecule with two heavy plus two light chains. As is shown in Table I, amino acid analyses also support the conclusion that the 7S molecule contains two heavy and two light chains. No J chains (<20 μg/4.0 mg of protein) were detected by Drs. H. L. Spiegelberg and G. C. Meinke, of the Scripps Clinic and Research Foundation, who kindly analyzed the purified 7S form of protein 315 by polyacrylamide electrophoresis at pH 9.4 in 10 M urea (Halpern and Koshland, 1970; Mestecky *et al.*, 1971; Meinke and Spiegelberg, 1970).

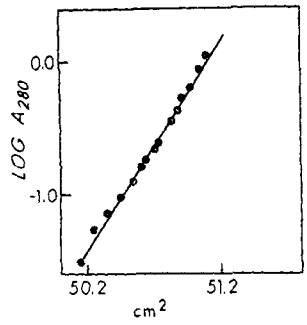

FIGURE 1: Representative plot used in the calculation of the molecular weight for the 7S monomer of protein 315 (speed, 20,000 rpm; concentration, 0.33 mg/ml; 20 hr; T 22.7°; PBS).

Gel filtration of the extensively reduced-carboxymethylated 7S protein led to the separation of heavy and light chains, with a total recovery of 83 to 88% of the optical density units applied to the column (Figure 2). The ratio of heavy to light chains was 75:25 in optical density units and 70:30 by weight, based on their determined extinction coefficients (1.5 and 1.1 cm^2 per mg, respectively, in 1 M acetic acid). These recoveries are consistent with the molecular weights of the chains and their equimolar representation in the 7S molecule.

Fab(t) Fragments. Gel filtration of the tryptic digest of protein 315 gave the elution profile shown in Figure 3. The same pattern was obtained with a tryptic digest of protein 460. Of the total A_{278} units applied to the column, over 90% was recovered and about 60% was in the major fraction, called Fab(t). Only this fraction, and the small amount of

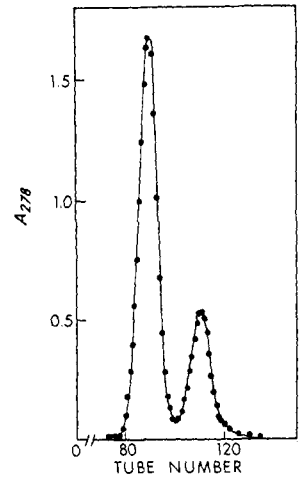

FIGURE 2: Separation of light and heavy chains. Fully reduced and alkylated protein 315 (250 mg in 15 ml) was applied to a column of Sephadex G-100 (6.5 × 130 cm) (solvent, 6 M urea–1 M acetic acid; flow rate, 50 ml/hr; 14.0 ml/tube; room temperature).

TABLE I: Amino Acid Composition of Protein 315.

Amino Acid	Heavy Chain		Light Chain		7S Monomer		
					Observed		Expected
	Mean[a]	Integer	Mean[a]	Integer	Mean[a]	Integer	2H + 2L
Lysine	18.3 ± 0.7	18	10.9 ± 0.4	11	58.7 ± 2.6	59	58
Histidine	6.3 ± 0.4	6	5.0 ± 0.2	5	24.1 ± 1.7	24	22
Arginine	11.0 ± 0.8	11	5.3 ± 0.3	5	34.5 ± 2.3	35	32
Aspartic acid	42.6 ± 1.6	43	18.4 ± 1.1	18	120.1 ± 2.5	120	122
Threonine	40.2 ± 0.9	40	25.7 ± 3.0	26	132.0 ± 2.7	132	132
Serine	45.4 ± 0.4	45	17.6 ± 3.5	18	129.0 ± 1.8	129	126
Glutamic acid	42.2 ± 4.7	42	18.9 ± 0.8	19	130.1 ± 4.5	130	122
Proline	37.6 ± 1.3	38	13.2 ± 0.9	13	102.1 ± 5.0	102	102
Glycine		38[d]	19.5 ± 0.2	20		111[d]	116
Alanine	20.4 ± 1.5	20		15[d]	70.6 ± 4.3	71	70
Cystine/2[b]	11.7 ± 0.7	12	4.6 ± 0.3	5	28.8 ± 4.5	29	34
Valine	33.0 ± 1.8	33	16.0 ± 0.3	16	93.9 ± 4.1	94	98
Methionine	3.8 ± 0.3	4	1.7 ± 0.6	2	12.1 ± 1.6	12	12
Isoleucine	14.2 ± 0.9	14	6.7 ± 0.4	7	41.1 ± 2.0	41	42
Leucine	46.0 ± 1.4	46	16.3 ± 1.1	16	119.3 ± 2.5	119	124
Tyrosine	15.3 ± 0.3	15	3.3 ± 1.3	3	39.0 ± 3.3	39	36
Phenylalanine	13.8 ± 0.2	14	10.8 ± 0.2	11	50.7 ± 3.7	51	50
Tryptophan[c]		10		4		29	28
Total		449		214		1327	1326

[a] Mean of three preparations plus or minus standard deviation. [b] With heavy and light chains, Cys/2 was the total carboxymethyl- or aminoethylcysteine observed. With the 7S monomer, Cys/2 was the sum of Cys/2 plus carboxymethylcysteine. [c] Single analyses, calculated spectrophotometrically (see Methods). [d] The values for the other amino acids was based on this number of residues and the following molecular weights: 7S monomer, 142,300 (153,000 less 6% carbohydrate); heavy chain, 50,100 (54,500 less 8% carbohydrate); light chain 23,000 (this value was used as it seemed more reasonable than the ultracentrifuge value of 21,000).

material emerging ahead of it at the void volume, bound ε-DNP-L-lysine, as shown by fluoresence quenching, and formed specific precipitates with antisera prepared against the 7S molecule. The material emerging at the void volume was probably undigested or incompletely digested 7S protein, and after 6-hr incubation with trypsin, its amount varied from about 10% (as in Figure 3) to perhaps 30% in some preparations. The small peaks that followed Fab(t) in Figure 3 were probably derived from the C-terminal half of the heavy chain (see below).

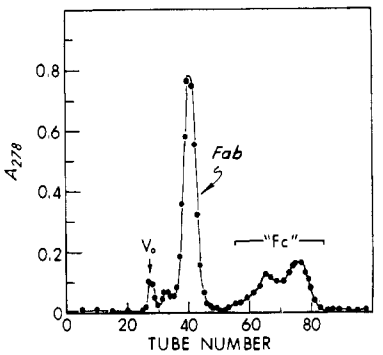

FIGURE 3: Gel filtration of a tryptic digest of the 7S monomer of protein 315. The digest (13 mg in 1.7 ml) was applied to a column (1.5 × 100 cm) of Sephadex G-100 (solvent, 0.05 M Tris·Cl, pH 8.0; flow rate 12 ml/hr; 2.7 ml/tube; room temperature). V_0, void volume.

When the Fab(t) fraction from G-100 was rechromatographed on DEAE-cellulose with a gradient from 0.0 to 0.3 M NaCl in 0.01 M potassium phosphate buffer (pH 7.4), it eluted as a single symmetrical peak. Further evidence for the purity of this fraction was provided by gel diffusion precipitin reactions (Figure 4) and acrylamide disc electrophoresis, where it usually migrated at pH 9.5 as a single band; however, in some disc electrophoresis analyses two bands were seen and may have been due to dissociation of the fragment at this pH into light chain and Fd piece (Figure 5, and see Figure 7, below). In immunoelectrophoresis the Fab(t) fragment consisted of a single component with less anodic mobility than the 7S protein (Figures 4A,B).

In gel diffusion precipitin tests, the Fab(t) fragment and the 7S protein gave reactions of identity with a rabbit antiserum specific for the light chain of protein 315 (Figure 4C). This suggested that the light chain was intact in Fab(t) and was in accord with the amino acid composition and C-terminal amino acid residue of the light chain isolated from this fragment (see below). Both protein 460 and the Fab(t) fragment from 460 failed to react with the antiserum to the light chain of protein 315 (Figure 4C).

The gel diffusion precipitin pattern obtained with a rabbit antiserum prepared against the 7S form of protein 315 is shown in Figure 4D. The substantial degree of antigenic identity between proteins 315 and 460 is expected, as both have

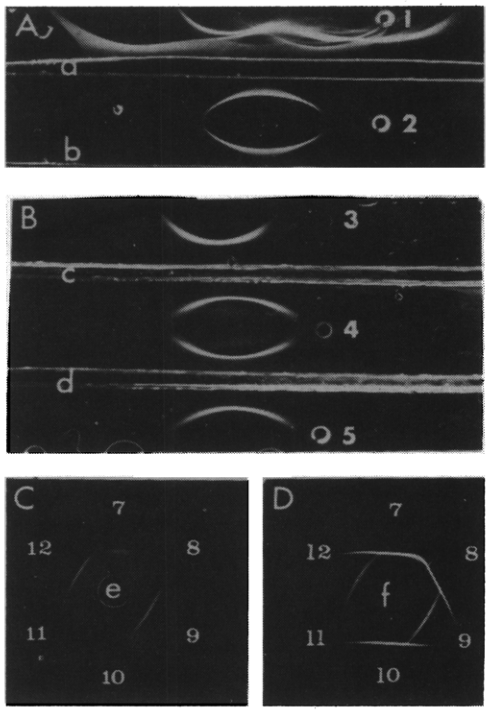

FIGURE 4: (A and B) Immunoelectrophoresis. (1) Normal BALB/c serum; (2 and 3) 7S monomer of protein 315 (5 mg/ml); (4) tryptic digest of 7S protein 315 (10 mg/ml); (5) Fab(t) fragment of protein 315 (5 mg/ml); (a and b) rabbit antiserum to whole mouse serum; (c and d) rabbit antiserum to protein 315. Anode at left. (C and D) Immunodiffusion. Antigen concentrations were 0.5–1.0 mg/ml. (7) Protein 315 (7S); (8 and 10) protein 460 (7S); (9 and 12) Fab(t) fragment of protein 315; (11) Fab(t) fragment of protein 460; (e) rabbit antiserum specific for light chains of protein 315; (f) rabbit antiserum for heavy chains and light chains of protein 315.

α heavy chains (Eisen *et al.*, 1968; Jaffe *et al.*, 1969, 1971). Though not easily seen in Figure 4D, the precipitin band of protein 315 formed a faint spur over protein 460; this was probably due to antigenic differences in their light chains (*cf.* Figure 4C). These differences were more conspicuous at the intersection of the precipitin bands due to Fab of 315 and the 7S form of 460 (8, 9, and 10 in Figure 4D). The partial

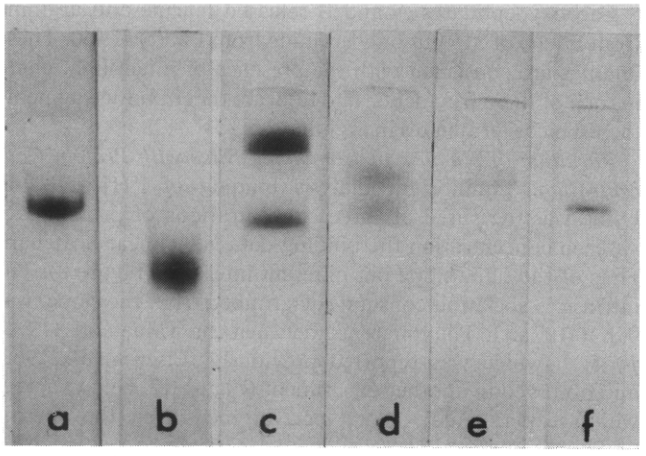

FIGURE 5: Disc electrophoresis of the purified 7S monomer (a) and Fab(t) fragment (b) of protein 315. Electrophoresis was performed at pH 9.5 in 7% acrylamide gels for 2 hr at 5 mA/tube. Protein (60 μg) was added to each gel. Electrophoresis in SDS gels. (c) Protein 315; (d) Fab(t) fragment of protein 315; (e) Fd piece; (f) light chain.

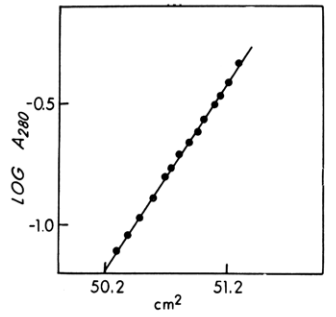

FIGURE 6: Representative plot in the calculation of the molecular weight of Fab(t) fragment of protein 315 (speed, 24,000 rpm; concentration, 0.075 mg/ml; 20 hr, T 24.7°; PBS).

identity between the 7S form of protein 315 and the Fab(t) fragment of 315 (*e.g.*, 7, 12 in Figure 4D) demonstrated that the heavy chain in the fragment was antigenically deficient (*i.e.*, Fd piece), and this is in accord with many observations of proteolytic cleavage products of IgG and IgM immunoglobulins (Fleischman *et al.*, 1963; Miller and Metzger, 1966).

Sedimentation equilibrium showed the molecular weight of the Fab(t) fragment of 315 to be 53,000 ± 1100 (SEM) (Figure 6). On the basis of molecular weight, the Fab(t) should amount to 69% of the protein digested, if there are two Fab(t) fragments per 7S molecule. The actual yield was usually 55–60%. However, a comparison by equilibrium dialysis of the whole tryptic digest with that of the undigested 7S protein showed that about 15% of the combining sites were lost on digestion. If the lost sites represented extensively digested Fab domains, the corrected recovery of Fab(t) was 65–70%, supporting other evidence (see below) that there are two Fab fragments per 7S monomer.

Gel filtration of the extensively reduced and aminoethylated Fab(t) fragment yielded two components (Figure 7). One was clearly the light chain, as its amino acid composition (Tables II and III), C-terminal amino acid residue (Table IV), and elution volume from G-100 were all the same as that of the light chain isolated from the 7S molecule. The other component, emerging before the light chain, was doubtless the Fd piece (Figure 7). Its observed amino acid composition agreed with that obtained by subtracting the composition of the light chain (Table III) from that of the Fab(t) fragment (Table II). The predominance of the Fd peak over the light-

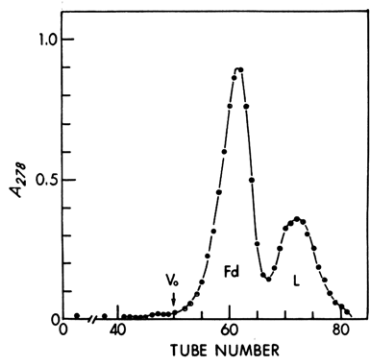

FIGURE 7: Separation of Fd piece and light chain (L) from Fab(t) fragment of protein 315. Fully reduced and alkylated Fab(t) fragment of protein 315 (29 mg in 3 ml) was applied to a column (3.0 × 105 cm) of Sephadex G-100 (conditions: solvent, 1% SDS; flow rate, 20 ml/hr; 4.6 ml/tube; room temperature).

TABLE II: Amino Acid Composition of Fab(t) Fragments.

Amino Acid	Fab(t) 315 Mean[a]	Fab(t) 315 Integer	Fab(t) 460 Mean[b]	Fab(t) 460 Integer
Lysine	19.5 ± 0.2	20	20.1 ± 2.5	20
Histidine	8.6 ± 0.2	9	7.1 ± 1.1	7
Arginine	11.4 ± 0.4	11	14.0 ± 1.0	14
Aspartic acid	43.1 ± 1.1	43	44.3 ± 1.8	44
Threonine	49.4 ± 0.8	49	41.7 ± 1.8	42
Serine	47.0 ± 1.1	47	59.0 ± 0.2	59
Glutamic acid	42.1 ± 1.4	42		43[e]
Proline	36.8 ± 1.2	37	35.5 ± 1.5	36
Glycine	41.6 ± 2.6	42	33.4 ± 0.9	33
Alanine	23.9 ± 0.6	24	18.4 ± 0.6	18
Cystine/2[c]	9.3 ± 0.8	9	10.1 ± 1.1	10
Valine		32[e]	29.8 ± 1.4	30
Methionine	3.9 ± 0.5	4	6.2 ± 0.7	6
Isoleucine	16.5 ± 1.8	17	18.4 ± 0.6	18
Leucine	40.1 ± 2.9	40	40.4 ± 4.6	40
Tyrosine	12.2 ± 1.5	12	22.9 ± 1.1	23
Phenylalanine	18.3 ± 0.5	18	14.5 ± 0.9	15
Tryptophan		13[d]		11[d]
Total		469		469

[a] Mean of three different preparations plus or minus standard deviation. [b] Mean of two different preparations plus or minus standard deviation. [c] Total Cys/2 plus carboxymethylcysteine. [d] Spectrophotometric method; a single determination. [e] Values for the other amino acids in this column were based on this number of residues and a molecular weight of 53,300 less 6% carbohydrate.

TABLE III: Amino Acid Composition of Light (L) Chains and Fd Pieces Derived from Fab(t) Fragments.

Amino Acid	Protein 315 Expected Fd[a]	Protein 315 Expected L[b]	Protein 315 Observed Fd[c]	Protein 315 Observed L[d]	Protein 460 Expected Fd[e]	Protein 460 Expected L[f]
Lysine	9	11	10	11	7	13
Histidine	4	5	4	5	2	5
Arginine	6	5	6	5	6	8
Aspartic acid	25	18	28	18	21	23
Threonine	23	26	25	27	24	18
Serine	29	18	27	19	32	27
Glutamic acid	23	19	22	19	24	19
Proline	24	13	22	12	25	11
Glycine	22	20	23	20	19	14
Alanine	9	15	10	16	10	8
Cystine/2	5	5	6	5	5	5
Valine	16	16	17	15	17	13
Methionine	2	2	2	2	4	2
Isoleucine	10	7	9	7	11	7
Leucine	24	16	21	16	23	17
Tyrosine	9	3	7	2	16	7
Phenylalanine	7	11	8	11	5	10
Tryptophan[g]	9	4	9	4	7	4
Total	256	214	256	214	258	211

[a] Difference between Fab(t) and light chain (Tables I and II). [b] Light chain from 7S form of 315 (Table I). [c] Single analysis of Fd from Fab(t) normalized to 30,000 molecular weight. [d] Single analysis of light chain from Fab(t) normalized to 23,000 molecular weight. [e] Difference between Fab(t) (Table II) and values reported for light chain of the 7S form of 460 (Jaffe *et al.*, 1971). [f] Reported by Jaffe *et al.* (1971). [g] Based on Tables I and II.

chain peak in the elution profile of Figure 7 is in accord with the higher molecular weight of the Fd piece (*i.e.*, 30,000 based on difference between molecular weight of Fab(t) and light chain), and the relatively low extinction coefficient of the light chain (1.1 cm^2/mg at 278 nm). Disc electrophoresis in polyacrylamide gels provided additional evidence that the chains isolated from Fab(t) fragment were the Fd piece and light chain (Figure 5). The Fd pieces of 315 and 460 differed appreciably in amino acid composition; *e.g.*, in histidine, methionine, and tyrosine (Table III).

Carbohydrate Content. Only preliminary data were obtained. Total hexose in protein 315 was 2.6 g/100 g of protein. A similar value was found for protein 460, another mouse IgA, in which all the hexose is present in the heavy chain (Jaffe *et al.*, 1971). As no amino sugars were noted in amino acid analyses of the 315 light chain, we assume that in this protein, also, the carbohydrate is associated exclusively with heavy chain. We have also assumed the total carbohydrate content of 315 is 6%, the value found by Clamp and Putnam (1965) for a human IgA myeloma protein whose hexose content was 2.56%, or essentially the same as that of protein 315.

C-Terminal Amino Acid Residues. The results of digestion with carboxypeptidases A and B and of hydrazinolysis are summarized in Table IV. The heavy chains of proteins 315 and 460 had C-terminal tyrosine, in agreement with previous findings on human and mouse α chains (Abel and Gray, 1968; Seki *et al.*, 1968). The C terminus of the light chain of 315 was leucine, as also found by Goetzl and Metzger (1970). No C-terminal residue was obtained by carboxypeptidase A digestion of the carboxymethylated light chain of protein 460, probably because this chain is κ type with C-terminal carboxymethylcysteine.

Carboxypeptidases A and B released leucine and arginine from Fab(t) of 315 but only arginine from Fab(t) of 460. These findings are consistent with the presence of intact light chain in each of these fragments, and indicate that in both fragments the Fd piece terminates in arginine.

Variation of Number of Combining Sites with Protein Concentration. Equilibrium dialysis binding of [^{3}H]-ϵ-DNP-L-lysine was measured at three concentrations of protein 315. At each concentration the binding data gave linear Scatchard plots of the same slope but different intercept (Figure 8). The intrinsic association constant, determined from the slope, was 0.8×10^7 M^{-1}. The difference between this value and $1\text{–}2 \times 10^7$ M^{-1}, which was reported previously (Eisen *et al.*, 1968) and observed in another experiment (Figure 12, below) agrees within experimental error; *e.g.*, in measuring the specific activity of the radioactive ligands. Though the association constant was independent of protein concentration, the number of combining sites per 7S protein molecule (n) increased from 1.4 at 0.03 mg/ml to 1.9 at 0.52 mg/ml. At higher protein concentration (1.9 mg/ml) 1.9 moles of ligand was bound per mole protein at a saturating level of free ligand (1×10^{-5}

TABLE IV: C-Terminal Amino Acids.[a]

	Hydrazinolysis	CPA	CPB
Protein 315	Nd	Leucine (0.43) Tyrosine (0.50)	Tyrosine (0.67)
Heavy chain, 315	Tyrosine (0.38)	Tyrosine (0.70)	Nd
Light chain, 315	Leucine (0.43)	Leucine (0.52)	Nd
Fab(t), 315	Nd	Leucine (0.48)	Arginine (0.48)
Heavy chain, 460	Tyrosine (0.48)	Tyrosine (0.60)	Nd
Light chain, 460	Nd	–	Nd
Fab(t) 460	Nd	–	Arginine (0.55)

[a] The yield of the C-terminal residue is given in parentheses (moles per mole of protein, chain, or fragment). In all cases the amino acid listed was present in amounts greater than 10× background; Nd, not done; –, no C-terminal amino acid found. CPA, carboxypeptidase A; CPB, carboxypeptidase B.

M ε-DNP-L-lysine). In the presence of gelatin, protein 315 at low concentration (0.05 mg/ml) also had close to two sites per 7S molecule for [^{3}H]-ε-DNP-L-lysine (Figure 9). It seems clear therefore that the limiting value for n is 2.0 (±0.2).

The presence of two combining sites per 7S molecule was confirmed with menadione, a ligand with low affinity for protein 315. Though structurally quite dissimilar from DNP, menadione appears to be bound at the same sites as ε-DNP-L-lysine (M. Michaelides, and H. Eisen, in preparation; Eisen *et al.*, 1970). With protein 315 at 1.04 mg/ml, n was 1.9 for menadione (Figure 10).

Like 315, protein 460 also showed variation in n with protein concentration. At 1.5 mg/ml the reduced-alkylated 7S form of 460 was previously shown to have two sites at which it bound ε-DNP-L-lysine (Jaffe *et al.*, 1969). Because its affinity for this ligand was low (3×10^5 M^{-1} at 4°), protein 460 at low concentration had to be examined with 2,4-dinitronaphthol, for which it has about 10-fold greater affinity (Jaffe *et al.*, 1971). In the absence of gelatin at low protein concentration (0.03 mg/ml), 460 had less than two combining sites per 7S molecule (n = 1.68). In the presence of gelatin, with protein 460 at low concentration (0.036 mg/ml) the value of n increased to 2.16. The ratio for n in the presence and absence of gelatin was essentially the same for dilute solutions (0.04–0.05 mg/ml) of proteins 315 and 460 (compare Figures 9 and 11).

The protective effect of gelatin and nonspecific RγG suggested that, at low concentration, the 7S forms of 315 and 460 undergo appreciable surface denaturation. To determine if protein was actually lost from solution, the recovery of [^{125}I]-protein 315 was measured after standing 40 hr in dialysis chambers. (Dr. E. W. Voss, Jr., kindly prepared the [^{125}I]protein 315, which contained 2 I atoms/molecule of protein.) With the labeled protein initially at 0.5 and 0.05 mg per ml, 90 and 80%, respectively, were recovered. The difference (11%) seemed too small to account for the 30–35% reduction in n at the lower protein concentration.

Equilibrium dialysis with the Fab(t) fragment of protein 315 showed that its affinity for [^{3}H]-ε-DNP-L-lysine was essentially the same as the 7S monomer. At saturation, one mole of ligand was bound per mole of fragment (mol wt 53,000) in the presence of nonspecific rabbit γ-globulin in (RγG) and 0.75 mole of ligand in the absence of RγG (Figure 12). The single site on the Fab fragment is in accord with the

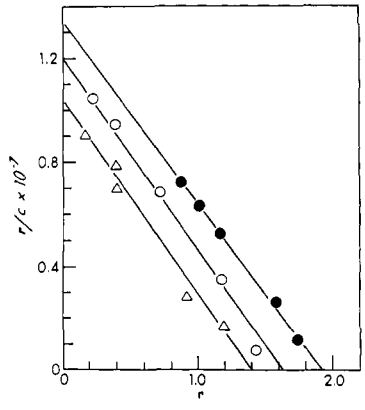

FIGURE 8: Equilibrium dialysis of protein 315 with [^{3}H]-ε-DNP-L-lysine at 4°. Protein concentrations were (●) 0.52 mg/ml, (○) 0.13 mg/ml, and (△) 0.03 mg/ml. $K = 0.8 \times 10^7$ M^{-1} for all three protein concentrations.

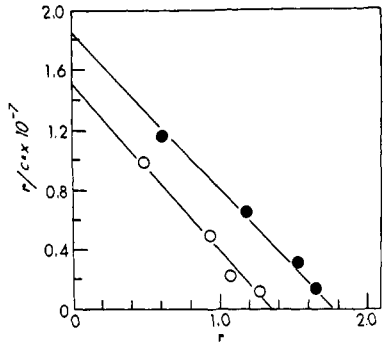

FIGURE 9: Equilibrium dialysis of protein 315 with [^{3}H]-ε-DNP-L-lysine at 4° in the presence and absence of gelatin. Protein 315 concentration = 0.048 mg/ml. (●) + gelatin (1.5 mg/ml) and (○) no gelatin. $K = 1.0 \times 10^7$ M^{-1}. Under these conditions no ligand binding was attributable to gelatin.

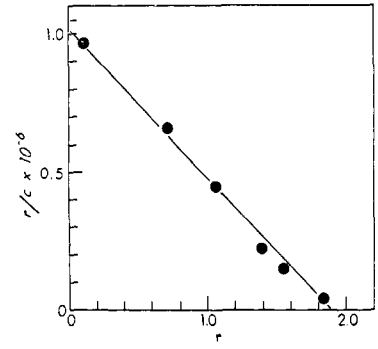

FIGURE 10: Equilibrium dialysis of protein 315 with [^{14}C]menadione at 4°. Protein 315 concentration = 1.04 mg/ml; $K = 5.4 \times 10^5$ M^{-1}.

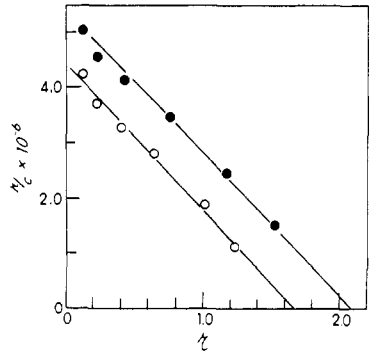

FIGURE 11: Equilibrium dialysis of protein 460 with [^{3}H]-2,4-dinitronaphthol at 4° in the presence and absence of gelatin. Protein 460 concentration = 0.036 mg ml. (●) + gelatin (1.5 mg ml) and (○) no gelatin; $K = 2.5 \times 10^6$ M^{-1}. Under these conditions no ligand binding was attributable to gelatin.

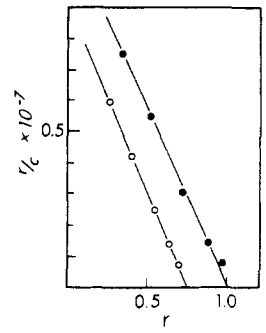

FIGURE 12: Equilibrium dialysis of Fab(t) fragment of protein 315 with [^{3}H]-ε-DNP-L-lysine at 4° in the presence and absence of nonspecific rabbit γ-globulin. Fab(t) concentration = 0.43 mg/ml. (●) + rabbit γ-globulin (1.5 mg/ml) and (○) no nonspecific rabbit γ-globulin; $K = 1.0 \times 10^7$ M^{-1}; corrected for nonspecific binding by rabbit γ-globulin.

evidence for two ligand-binding sites and two Fab fragments per 7S molecule.

Discussion

The foregoing results show that the 7S form of protein 315 has the same general structure as other 7S immunoglobulins: *i.e.*, its molecular weight is 150,000 to 155,000[3] and it consists of two heavy plus two light chains (Fleischman *et al.*, 1963). Similar results were obtained previously with the IgA myeloma proteins produced by two other mouse BALB/c plasmacytomas, Adj PC-6A (Seki *et al.*, 1968) and MOPC-460 (Jaffe *et al.*, 1971).

Besides heavy and light chains a third type, called J chain, has been found in those immunoglobulins (IgM and IgA) that occur as multimers of 7S monomers (Halpern and Koshland, 1970; Mestecky *et al.*, 1971; Meinke and Spiegelberg, 1971). Accordingly, it was of interest that Drs. Spiegelberg and Meinke could detect no J chains (<20 μg/4.0 mg of protein) in the 7S form of 315, though it was present in the 315 multimers they isolated from serum (personal communication). It appears therefore that J chains were eliminated during isolation of the 7S monomer (see Methods).

In accord with the duplex symmetry implied by the four-chain structure, tryptic digestion of protein 315 and 460 yielded the equivalent of two Fab(t) fragments per 7S molecule. The digestion also yielded a mixture of smaller peptides; these were apparently derived from the C-terminal 40% of the heavy chains, as the Fab(t) fragment of 315 had an intact light chain and its heavy-chain component had arginine as the C-terminal residue, rather than the tyrosine C terminus of the intact heavy chain. Proteolytic cleavage of a variety of other native immunoglobulins has also split their Fc domain into several small peptides (Bernier *et al.*, 1965; Miller and Metzger, 1966).

Unlike most immunoglobulins, the IgA myeloma proteins of BALB/c mice appear to lack disulfide bonds between light and heavy chains (Abel and Grey, 1968; Seki *et al.*, 1968). Though it also seems to lack these bonds (H. Grey, personal communication), protein 315 did not appear to be particularly unstable (except, perhaps, as indicated by the dependency of n, the number of combining sites per molecule, on protein concentration; see below). Indeed separation of the light and heavy chains of proteins 315 and 460 by gel filtration demanded more stringent conditions than are ordinarily required for IgG and other immunoglobulins: *e.g.*, it was necessary to use a more powerful dissociating solvent (6 M urea–1 M acetic acid, rather than simply 1 M acetic or 1 M propionic acid), and essentially all SS bonds had first to be cleaved in order to achieve complete separation of chains. This suggests that noncovalent bonds between the native chains may be unusually stable in these proteins, possibly as compensation for the lack of light–heavy interchain SS bonds.

The Fab(t) fragment of protein 460 resembled that of protein 315 in size (it had the same elution volume on G-100) but the two fragments differed in amino acid composition; and composition differences were found between their respective light chains and Fd pieces. Differences between the light chains of 315 and 460 are expected, as they differ in type. The 460 light chain is κ (F. Richards, personal communication) and the 315 light chain (L^{315}) resembles λ more than κ but has certain distinguishing features. For example, in both L^{315} and in λ chains the most readily reduced half-cystine residue is penultimate to the C-terminal residue, which is leucine in L^{315} and serine in λ; and the C-terminal nonapeptide of L^{315} differs in two additional residues from the corresponding sequence in mouse λ chains (Schulenburg *et al.*, 1971; Appella *et al.*, 1967). Whether L^{315} represents an allotypic variant of λ chains or another type (isotype) of light chain is currently under investigation.

Since proteins 315 and 460 differ in affinity for diverse ligands (Eisen *et al.*, 1969; Jaffe *et al.*, 1971), they should have different amino acid sequences in their V_H regions, and this is in accord with the differences in composition between their respective Fd pieces. However, the V_H region involves, approximately, only the N-terminal half of the Fd piece and we cannot rule out differences between the C-terminal halves, though these should be identical, as both chains are of the α class.

On the basis of equilibrium dialysis experiments with immunoglobulin at low concentration (0.05 mg/ml), protein 315 was previously reported to have 1.2 ligand-binding sites per 7S molecule, whose molecular weight was incorrectly thought to be 120,000 (Eisen *et al.*, 1968). With the correct molecular

[3] The provisional value of 120,000, reported previously (Eisen *et al.*, 1968), was probably in error because it was based primarily on osmometry, which is readily biased by low molecular weight contaminants (buffer salts, haptens), and was supported by fluorescence quenching titrations which, with further experience, now appear to be too imprecise for accurate determination of numbers of binding sites. Because the high-speed sedimentation equilibrium result previously reported was based on a single run, it was probably due to experimental error.

weight (150,000–155,000), the previous data correspond to 1.5 sites (per 7S molecule), which is still significantly less than two. In the present study we have verified the earlier results, but also found that the 7S molecule actually has two sites if the measurements are made at sufficiently high concentration of protein ($\geq$0.5 mg/ml), or if a "protective" protein like gelatin is present when the immunoglobulin is at low concentration (*e.g.*, 0.05 mg/ml).

Dependence of *n*, the apparent number of combining sites per molecule, on protein concentration was also observed with protein 460, but has not hitherto been reported for conventional antibodies. We do not know if the concentration effect observed with the 7S forms of 315 and 460 is due to their having been (mildly) reduced and alkylated, or represents inherent instability of BALB/c IgA myeloma proteins, possibly related to their lack of light–heavy interchain disulfide bonds. The concentration dependency could also be a general property of all antibodies, and escaped notice previously because these proteins are usually employed in equilibrium dialysis at relatively high concentration (*i.e.*, well above 0.05 mg/ml).

A fundamental feature of antibodies of the IgG class (and of IgG myeloma proteins with antibody activity) is the consistent correlation between their chain structure and ligand-binding sites: each molecule has a pair of identical heavy chains plus a pair of identical light chains and two identical binding sites, each associated with a light–heavy pair. The present findings, in conjunction with studies of two other IgA myeloma proteins with anti-DNP activity, one produced in BALB/c mice (Jaffe *et al.*, 1969) and the other in a human (Terry *et al.*, 1970), establish that immunoglobulins of the IgA class also have the same general structure.

It is only with antibodies of the IgM class that the valency of the 7S subunit remains in doubt. Conventional antibodies of this class have been found to have fewer than two combining sites per 7S subunit or to have an apparent inequality, with one-half the sites having much higher affinity for ligand than the other half (Onoue *et al.*, 1968). However, the conventional IgM antibody preparations so far studied have been highly heterogeneous with respect to affinity, and for most of them the average affinity has been low (*e.g.*, $\leq 10^5$ M^{-1}). Accordingly, it has been difficult to establish with certainty their number of combining sites; and it has also been difficult to distinguish between heterogeneity of combining sites within a molecule and among molecules. These difficulties have been obviated by Ashman and Metzger (1969) who studied a homogeneous Waldenstrom macroglobulin, that bound *p*-nitrophenyl ligands specifically, and demonstrated ten identical combining sites per 19S molecules, or two per 7S subunit. If their results and those of the present study are generally representative of IgM and IgA antibodies, respectively, as seems likely, there appear to be no remaining exceptions to the rule that immunoglobulins of all classes have two identical ligand-binding sites per 7S molecule or subunit.

Acknowledgment

We are grateful to Mr. Walter Gray for technical assistance in various phases of this work and to Mr. Richard Pinkston for amino acid analyses.

Added in Proof

Since completing this work, additional reports have appeared on the number of combining sites per molecule of IgA immunoglobulins. In a study of mouse IgA myeloma proteins (S63 and S107) that bind phosphorylcholine, Sher and Tarikas (1971) found the equivalent of two combining sites per 156,000 g of myeloma protein when unfractionated antisera were analyzed, but 1.0–1.5 sites were found with the isolated myeloma proteins; they suggest that denaturation during purification led to loss of combining sites. Green *et al.* (1971) obtained evidence for two DNP-binding sites on purified protein 315 (mol wt 150,000) by fluorescence quenching titrations of the protein at relatively high concentrations (0.15 mg/ml) with a strongly bound bifunctional DNP ligand.

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Preferential Interaction of Manganous Ions with the Guanine Moiety in Nucleosides, Dinucleoside Monophosphates, and Deoxyribonucleic Acid*

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ABSTRACT: Manganous ions were found to decrease the melting temperatures of several DNAs as the concentration of Mn^{2+} was increased from a Mn^{2+}:DNA-P molar ratio of 3.7 to 15.5. The destabilizing effect of the metal ion increased as the (G + C) content of the DNA increased. When Mn^{2+} was present during the melting of DNA, the hypochromic spectra were altered and these changes were more apparent in DNAs with 50% or greater (G + C) content. These results suggest that the metal ion interacts with the base moieties of the DNA molecule, apparently with some preference for sites on guanine and/or cytosine. Nuclear magnetic resonance (nmr) spectra of the dinucleoside monophosphates TpT, ApA, GpA, ApG, and CpA reveal that the Mn^{2+} possesses a very marked preference for association with guanine over the other three bases. The interaction of Mn^{2+} with nucleosides was then studied by nmr spectroscopy. The results again showed a marked preference for Mn^{2+} association with the guanine ring, providing a good indication that the dinucleoside monophosphate results reflected the existence of a discrete metal ion binding site on the guanine ring.

Divalent cations are essential components of many enzymatic reactions involving DNA. Most such reactions can proceed in the presence of either Mg^{2+} or Mn^{2+}, but changes in the kinetics and specificity of some of these reactions have been described when Mn^{2+} is substituted for Mg^{2+}. Some of the effects of Mn^{2+} subsitution on the reactions catalyzed by RNA polymerase (2.7.7.6) from *Eschericha coli* and DNase I (3.1.4.5) are as follows. When Mn^{2+} was substituted for Mg^{2+}, the rate of *in vitro* RNA synthesis catalyzed by RNA polymerase increased when DNA from calf thymus or various bacterial sources was used as the template but decreased when DNA from several bacteriophages was used as the template (Furth *et al.*, 1962). Substitution of Mn^{2+} for Mg^{2+} in this reaction also was found to lead to a decrease in the rate and yield of chain initiation by ATP (Maitra *et al.*, 1967) and an increase in the amount of enzyme bound to native DNA (Sternberger and Stevens, 1966). The rate of hydrolysis of DNA by DNase I increased upon Mn^{2+} substitution for Mg^{2+} (Wiberg, 1958), and the mechanism of hydrolysis has been found to change from single-chain scission to double-chain scission (Melgar and Goldthwait, 1968). Further, the presence of Mn^{2+} allows DNase I hydrolysis of the poly(dC) chain of dI:dC polymer which does not occur in the presence of Mg^{2+} (Bollum, 1965). The substitution of Mn^{2+} for Mg^{2+} also stimulates the rate and extent of DNA synthesis catalyzed by *Micrococcus luteus* DNA polymerase when either *M. luteus* DNA (72% G + C) or poly(dG:dC) is used as template while with DNAs or polymers of high (A + T) content no differences in cation effect are observed (Litman, 1971).

These results, as well as others not discussed here, suggest that Mn^{2+} may alter the interaction between DNA and the enzyme proteins. Eichhorn and Shin (1968) have presented data indicating that while Mg^{2+} binds primarily to the phosphate groups of DNA, Mn^{2+} interacts with the base rings as well as with the phosphate groups. The work presented here describes a further investigation of the interaction of Mn^{2+} with DNA. Two types of experiments were employed. The first type concerned the effect of Mn^{2+} on the denaturation and hypochromic spectra of DNAs of varying base composition; the second type utilized proton nuclear magnetic resonance (nmr) spectroscopy to examine the interaction of Mn^{2+} with nucleosides and dinucleoside monophosphates in order to identify the site of metal ion complex formation with the bases. The results of the first type of study indicate that Mn^{2+}

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