# Specificity and Structure of the Myeloma Protein Produced by Mouse Plasmacytoma MOPC-460*

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ABSTRACT: Protein 460, the IgA myeloma protein produced by mouse plasmacytoma MOPC-460, has been studied with respect to its subunit structure and ligand-binding activity. In its 7S form, prepared by mild reduction and alkylation, the molecular weight is 150,000 ± 4900. The 7S molecule consists of a pair of heavy chains (mol wt 55,700 ± 4200) plus a pair of light chains (mol wt 23,000 ± 1300), and has two homogeneous ligand-binding sites with substantial affinity for some nitrophenyls. The protein's affinity for ε-2,4-dinitrophenyl (DNP)-L-lysine is about the same ($3 \times 10^5$ $M^{-1}$ at 4°) as that of conventional anti-DNP antibodies isolated early after immunization with DNP proteins, but its affinity is much higher for 2,4-dinitronaphthol and 2,4-dinitro-1-naphthol-7-sulfonic acid ($5 \times 10^6$ $M^{-1}$). Specificity of the binding activity is revealed by pronounced differences in affinity of protein 460 for 2,4,6-trinitrophenyl, 2,6-dinitrophenyl, and 4-mononitrophenyl derivatives of ε-aminocaproate, and by its lack of detectable reactivity with a variety of other aromatic hydrophobic substances (ε-*p*-iodophenylsulfonyl-L-lysine, ε-toluenesulfonyl-L-lysine, ε-5-dimethylaminonaphthalenesulfonyl-L-lysine, and 1,8-anilinonaphthalenesulfonate). The protein's tryptophan fluorescence is quenched by bound nitrophenyls, whose absorption spectra undergo a red shift.

For studies of the relationship between the structure of antibody combining sites and their specific binding of ligands, it is desirable to have substantial quantities of homogeneous antibodies that bind simple ligands specifically. Since nearly all conventionally induced antibodies are heterogeneous populations of immunoglobulin molecules, considerable efforts have been made recently to identify myeloma proteins, and similarly homogeneous Waldenstrom 19S macroglobulins, with antibody-like combining sites. As a result, several of these proteins have been found to have simple ligand-binding characteristics that closely resemble those of conventional antibodies (Eisen *et al.*, 1967, 1968; Potter and Leon, 1968; Schubert *et al.*, 1968; Cohn *et al.*, 1969; Ashman and Metzger, 1969; Terry *et al.*, 1970). Among this group are a number of mouse IgA myeloma proteins with appreciable affinity for polynitrophenyls (Eisen *et al.*, 1968, 1970; Jaffe *et al.*, 1969). Produced in substantial quantities by transplantable plasmacytomas, they consitute a set of closely related proteins whose comparative properties are of interest. One of these immunoglobulins, produced by MOPC 315, was partially characterized previously (Eisen *et al.*, 1968). Here we describe some properties of the IgA produced by MOPC 460, another mouse plasmacytoma. In a preliminary report, the affinity of this protein for ε-2,4-dinitrophenyl-L-lysine was established ($3 \times 10^5$ $M^{-1}$ at 4°), and the homogeneous reactivity of its binding sites was demonstrated (Jaffe *et al.*, 1969).

## Materials and Methods

*Purification.* Plasmacytoma 460 was generously provided by Dr. Michael Potter (National Cancer Institute, Bethesda, Md.) who produced the tumor by mineral oil injection of inbred pedigreed Balb/c anN mice (Potter and Boyce, 1962). The tumor was maintained in our laboratory in Balb/c mice from Cumberland Farms, Tenn., by serial subcutaneous transplantation at about 3-week intervals. Tumor-bearing mice were exsanguinated by cardiac puncture and sera from several transplant generations were pooled and stored at −20°.

Protein 460 was isolated from serum by two methods. The first, described previously, involved precipitation with 40% saturated ammonium sulfate, followed by fractionation on DEAE-Sephadex A-25 (Eisen *et al.*, 1968; Jaffe *et al.*, 1969). The active fraction (called Fr II) was mildly reduced with dithiothreitol and alkylated with iodoacetamide and then further purified by immunoabsorbtion on DNP[1]-aminoethylcellulose,[2] from which it was eluted specifically with DNP-glycine. Purity was monitored by the effectiveness of 0.4 μmole of ε-DNP-L-lysine in quenching the protein's tryptophan fluorescence (see Figure 1).

The second procedure for isolating protein 460[3] involved its specific precipitation from serum by $DNP_{23}$-HSA. The

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[1] Abbreviations used are: DNP, 2,4-dinitrophenyl; 2,6-DNP, 2,6-dinitrophenyl; TNP, 2,4,6-trinitrophenyl; 4-MNP, 4-mononitrophenyl; HSA, human serum albumin; BγG, bovine γ-globulin; $DNP_{23}$-HSA, human serum albumin with 23 moles of DNP (substituted on lysine residues)/mole of protein; similarly, $DNP_{60}$-BγG and $TNP_{37}$-HSA have the indicated number of DNP and TNP substituents (on lysine residues) per molecule of protein; pipsyl, *p*-iodophenylsulfonyl; tosyl-*p*-toluenesulfonyl; dansyl, 5-dimethylaminonaphthalenesulfonyl; PBS, (phosphate-buffered saline), 0.15 M NaCl–0.01 M potassium phosphate (pH 7.4); Gdn·HCl, guanidine hydrochloride.

[2] F. S. Farah and H. N. Eisen, unpublished observations.

[3] B. J. Underdown, E. S. Simms, and H. N. Eisen, in preparation.

washed specific precipitate was dissolved in 0.1 M DNP-glycine and then treated with 0.005 M dithiothreitol, followed by a 3-fold molar excess of iodoacetamide (three-times recrystallized) to convert 460 into its monomeric 6.6S form (hereafter referred to as 7S monomer). Monomer 460 was separated from the hapten and the dissolved antigen by chromatography on DEAE-cellulose and Dowex 1-X8, as described (Eisen *et al.*, 1967). In order to remove the antigen completely, it was usually necessary to concentrate the protein recovered from the DEAE-cellulose–Dowex column, to add DNP-glycine, and to repeat the mixed-bed column chromatography. The protein obtained was finally dialyzed against PBS and used directly or dialyzed against 0.01 M potassium phosphate (pH 7.6) and freeze-dried.

Protein purified by both procedures had the same affinity for ε-DNP-L-lysine, as measured by both equilibrium dialysis and fluorescence quenching.

*Precipitin Assays.* In order to determine the serum concentration of protein 460, and to establish the optimal amount of $DNP_{23}$-HSA required for its specific precipitation, precipitin analyses were carried out with (per tube) 0.05 ml of serum, varying amounts of $DNP_{23}$-HSA, and a total volume of 0.15 ml. After incubation at 37° for 1 hr and at 4° overnight, precipitates were washed five times in PBS, dried, dissolved in 1.0 ml of 0.5% sodium dodecyl sulfate (Matheson Coleman & Bell) and absorbency at 278 and 360 nm was determined. Double-diffusion precipitin reactions and immunoelectrophoresis were carried out in 0.05 M barbital (pH 8.6) in 1% agar (Bausch & Lomb, Rochester, N. Y.), unless otherwise specified.

*Separation of Light and Heavy Chains.* Lyophilized protein 460 was dissolved in 7 M guanidine in 0.7 M Tris-Cl (pH 8.2), and reduced with 0.01 M dithiothreitol for 2 hr at room temperature. After the solution was chilled, a 2.2 molar excess of iodoacetic acid was added and the reaction mixture was maintained at 4° for 45 min. The protein was then chromatographed at room temperature on Sephadex G-100 (3 × 110 cm), equilibrated, and developed with 6 M urea—1 M acetic acid. The separated light and heavy chains were freed of urea by filtration on Sephadex G-25 in 1 M acetic acid and freeze-dried.

*Molecular Weights.* High-speed equilibrium ultracentrifugation (Yphantis, 1964) was carried out with the 7S monomer (at 0.05, 0.15, and 0.50 mg per ml) in PBS, and with the isolated light and heavy chains (each at 0.1 and 0.4 mg per ml) in 6 M Gdn·HCl (Mann, Orangeburg, N. Y., Ultra Pure grade). Samples were centrifuged at 17,000, 48,000, and 34,000 rpm for the 7S monomer, the light, and the heavy chains, respectively, for 20–36 hr in a Spinco Model E ultracentrifuge equipped with absorption optics. For purposes of standardization, the molecular weights were similarly measured for BRY, an IgG-1 myeloma protein (Eisen *et al.*, 1967), and for its light chain and found to be 150,000 and 22,000, respectively. Partial specific volumes were calculated from amino acid analyses (Cohn and Edsall, 1943) of the 7S monomer ($\bar{v}$ = 0.732) and of the reduced, carboxymethylated light and heavy chains ($\bar{v}$ = 0.727 for each). For experiments in 6 M Gdn·HCl, $\bar{v}$ was reduced by 0.015 to correct for Gdn·HCl binding (Hade and Tanford, 1967). The density of 6 M Gdn·HCl was taken as 1.143 (Kawahara and Tanford, 1966).

*Amino Acid Analyses.* Samples were hydrolyzed under vacuum in constant-boiling HCl at 110° for 22 hr. Analyses were performed with a Spinco Model 120B (automatic analyzer) by the method of Spackman *et al.* (1958). The values reported are not corrected for destruction.

*Carbohydrate.* Total hexose was determined by the anthrone method (Roe, 1955), using an equimolar mixture of galactose and mannose as standard. 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.

*Fluorescence Quenching.* Protein fluorescence (activation at 290 nm, emission at 345 nm) and its quenching by nitrophenyl ligands was measured at 4° in an Aminco-Bowman spectrofluorometer (Eisen, 1964). When high concentrations of ligand were used, nonspecific attenuation of fluorescence was corrected for by parallel titrations of tryptophan with the same ligand solution.

*Equilibrium Dialysis.* In small lucite chambers 50 μl of protein solution was dialyzed to equilibrium (40 hr) at 4° against 50 μl of [$^3$H]ε-DNP-L-lysine at various concentrations (Eisen *et al.*, 1968). The binding of various unlabeled ligands was estimated from their competitive reduction of the binding of [$^3$H]ε-DNP-L-lysine (Karush, 1956). Aliquots (25 μl) from each side of the dialysis membrane were counted in Bray's solution (Bray, 1960) in a Packard liquid scintillation counter. In a few experiments, large dialysis chambers were used (1.0 ml in each compartment) to determine, in the absence of a competitive ligand, the binding of 2,4-dinitronaphthol, whose equilibrium concentration in the protein-free chamber was measured by absorbancy at 395 nm (molar extinction coefficient in PBS, 14,630). All equilibrium dialysis measurements were made at 4° with protein concentrations of 1.2–1.4 mg/ml.

*Difference Spectra.* The difference in absorbance spectra of free ligands and ligands bound to protein 460 were determined in rectangular tandem quartz cells at about 7° in a Cary 14 recording spectrophotometer.

*Ligands.* [$^3$H]ε-DNP-L-lysine was the preparation used previously (Eisen *et al.*, 1968). Recrystallized menadione and 2,4-dinitronaphthol had melting points as reported in the literature (105 and 138°, respectively). 2,4,6-TNP-aminocaproate, 2,6-DNP-aminocaproate, and 4-MNP-aminocaproate were kindly provided by Dr. J. R. Little. Dr. B. J. Underdown provided purin-6-oyl aminocaproate and 5-acetouracil caproate. ε-Pipsyl-L-lysine, ε-dansyl-L-lysine, ε-tosyl-L-lysine, and 1,8-anilinonaphthalenesulfonate were gifts from Dr. C. W. Parker.

## Results

*Purification.* Fluorescence quenching of protein 460 at several stages in its purification are compared in Figure 1, where ε-DNP-L-lysine was added to a final concentration of $6 \times 10^{-5}$ M. Based on the association constant of the purified 7S protein (Jaffe *et al.*, 1969; and see below) this concentration was sufficient to saturate over 90% of the binding sites, and the final quenching values shown should be close to the maximum obtainable ($Q_{max}$).

Fraction II of Figure 1 refers to the protein retarded on DEAE-Sephadex chromatography (Eisen *et al.*, 1968; Jaffe *et al.*, 1969); it was polydisperse in the ultracentrifuge (boundaries of approximately 7, 9, 11, and 13 S were noted), and only 25% of its fluorescence was quenched. After mild reduction and alkylation of fraction II, the protein sedimented with a single symmetrical boundary, $s_{20,w}$ = 6.6 S, and its quenching increased to 35%. Called the 7S protein, it appeared to contain at this stage about 20% impurity, as 80% of it was retained on a column of DNP-aminoethylcellulose. The retained protein was specifically eluted with DNP-

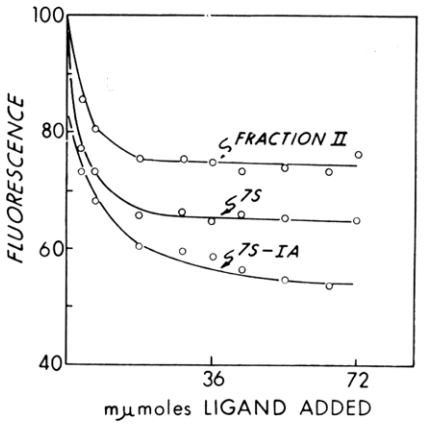



FIGURE 1: Fluorescence quenching of protein 460 at various stages of purification. Protein (1 ml; $A_{278}$ 0.065) was titrated with ε-DNP-L-lysine at $3.6 \times 10^{-4}$ mole/l. at 4°. The fluorescence values shown were adjusted for nonspecific attenuation by applying correction factors based on titration of tryptophan with the same ligand solution. Fraction II is the polydisperse fraction isolated on DEAE-Sephadex (Jaffe *et al.*, 1969; Eisen *et al.*, 1968). 7 S is mildly reduced carboxymethylated fraction II ($s_{20,w}$ = 6.6 S). 7S-1A is the 7S protein after further purification by immunoadsorption.

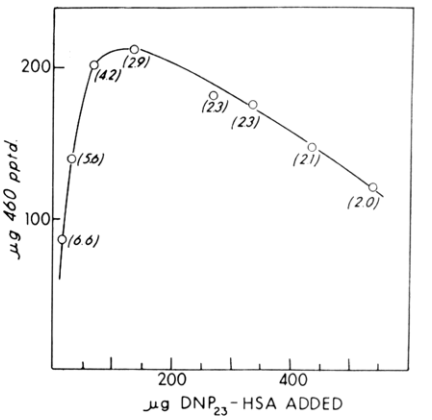



FIGURE 3: Quantitative precipitin reaction with $DNP_{23}$-HSA and serum from mice bearing MOPC 460. The numbers in parentheses are antibody–antigen weight ratios.

glycine and designated "immunoabsorbed 7 S" (7S-1A); the eluted protein had an unchanged sedimentation coefficient (6.6 S), and its $Q_{max}$ increased to 46%, in agreement with the removal of approximately 20% inactive protein. The final yield of the purified immunoabsorbed protein amounted to 2.8 mg/ml of serum (about 60% of the amount in serum, see below).

In the alternate method of purification, in which protein 460 was precipitated from serum with $DNP_{23}$-HSA, the mildly reduced, carboxymethylated 7S protein finally isolated was obtained in higher yield (an average of 3.95 mg/ml of serum in repeated isolations) and in an equal state of purity; *i.e.*, its $Q_{max}$ was 46% under the titration conditions given in Figure 1, and it gave a single precipitin band in immunoelectrophoresis when tested with a rabbit antiserum prepared against whole serum from Balb/c mice (Figure 2).

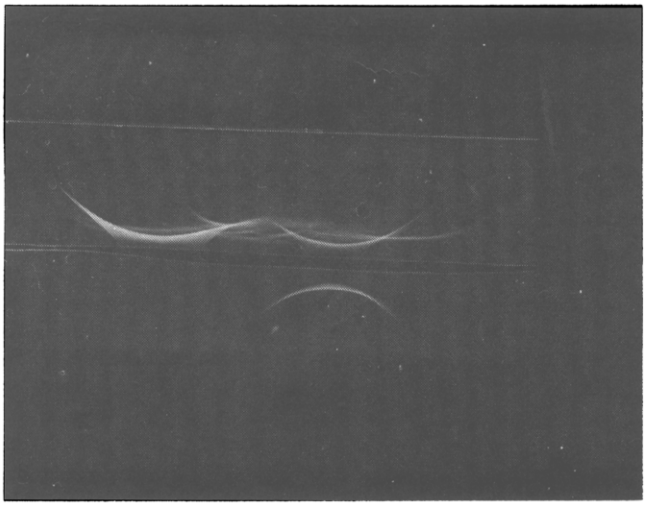


FIGURE 2: Immunoelectrophoresis of protein 460. Top well: ascites fluid from mice bearing MOPC 460. Lower well: purified protein 460 (3 mg/ml). Electrophoresis was carried out for 105 min, 230 V, 6.5 mA, 0.05 M barbital buffer, pH 8.6. Antiserum in trough: rabbit antiserum prepared against whole serum from BALB/c mice bearing another IgA producing plasmacytoma (MOPC 315).

*Precipitin Reactions.* The precipitin reaction between $DNP_{23}$-HSA and serum from mice bearing MOPC 460 is shown in Figure 3. Antibody:antigen weight ratios decreased progressively as more antigen was added, as is observed with conventional antibodies. At the point of maximal precipitation, 4.4 mg of protein was precipitated per ml of serum in the representative pool shown in Figure 3.

In agar diffusion precipitin reactions, fraction II (protein 460 in polydisperse form) formed a precipitin band with $DNP_{60}$-BGG and with $TNP_{37}$-HSA. Under identical conditions (fraction II added at 1.3–1.5 mg/ml and the antigens added at 0.2–1.0 mg/ml) no precipitin bands were obtained with 5-acetouracil-BSA or purin-6-oyl-BSA (Schubert *et al.*, 1968). When agar plates contained ε-DNP-L-lysine at $1 \times 10^{-4}$ M, the reactions with DNP-BGG and TNP-HSA were completely inhibited.

In contrast to fraction II, the 7S form of protein 460 failed to precipitate with DNP- or TNP-substituted proteins. Because some chicken 7S antibodies only precipitate with antigens at high salt, but not at low salt concentrations (1.5 M *vs.* 0.15 M NaCl; see Benedict *et al.*, 1963), the 7S form of 460 was tested by gel diffusion in 0.15 M and in 1.5 M NaCl and

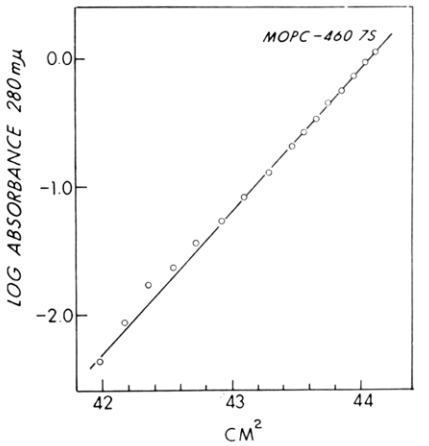
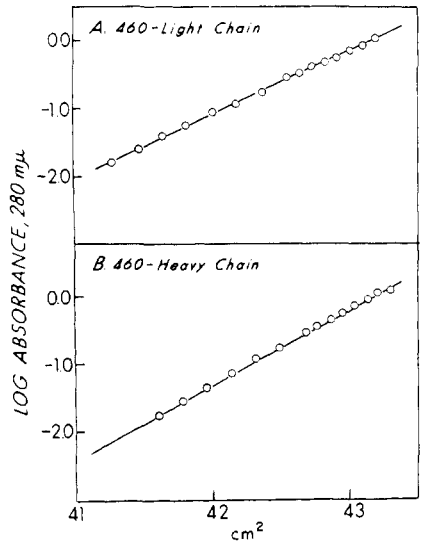



FIGURE 4: Representative plot used in calculation of molecular weight for 7S monomer of protein 460. (Speed 17,000 rpm; concentration 0.15 mg/ml; 17 hr; $T$ = 24°; PBS.)




FIGHRE 5: Representative plots used in the evaluation of molecular weight for 460 light and heavy chains. (A) Speed 48,000 rpm; 0.4 mg/ml; 6 M Gdn·HCl; 26.4°; 24 hr. (B) Speed 34,000 rpm, 6 M Gdn·HCl; 26.4°; 0.4 mg/ml; 23 hr.

still failed to precipitate with $DNP_{23}$-HSA under either condition.

*Evidence for Four Chains in the 7S Monomer.* Representative data for the high-speed sedimentation equilibrium experiments are given in Figures 4 and 5. The molecular weight of the 7S monomer was calculated to be 150,000 ± 4900 (std dev) (range 141,000–155,400). For the light and heavy chains the molecular weights were 23,000 ± 1300 (range 21,100–24,200) and 55,700 ± 4200 (range 49,200–60,500), respectively. The 7S molecule thus appears to consist of two light plus two heavy chains. This conclusion is also supported by the elution profile of heavy and light chains from G-100 where, on the basis of $A_{278}$ absorbency, about 73% of the reduced, carboxymethylated protein was heavy chain and about 27% was light chain (Figure 6). The amino acid compositions of the individual chains and of the 7S molecule are also consistent with a four-chain structure for the 7S monomer (Table I).

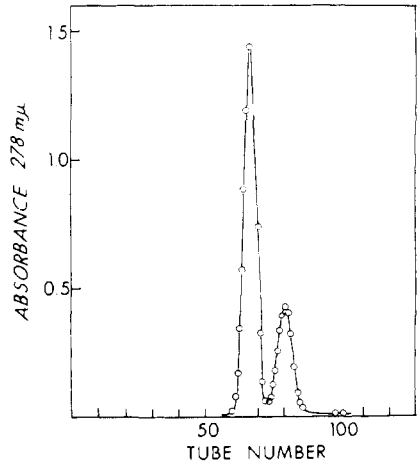



FIGURE 6: Separation of light and heavy chains on Sephadex G-100 in 6 M urea–1 M acetic acid. Extensively reduced and carboxymethylated protein 460 (30 mg in 2.1 ml) was applied to the column at room temperature (3 × 110 cm; 2.7 ml/tube).

TABLE I: Amino Acid Analyses of Protein 460.[a]

| | Light Chain[b] | Heavy Chain[b] | 7S Monomer | |
|---|---|---|---|---|
| | | | Expected[c] | Obsd[d] |
| Lysine | 13 | 20 | 66 | 61.8 ± 4.2 |
| Histidine | 5 | 6 | 22 | 22.0 ± 1.2 |
| Arginine | 8 | 14 | 44 | 40.0 ± 3.3 |
| CM-cysteine | 5 | 17[h] | | |
| Aspartic acid | 23 | 37 | 120 | 120 ± 6.3 |
| Threonine | 18 | 42 | 120 | 109 ± 12 |
| Serine | 27 | 48 | 150 | 141 ± 19 |
| Glutamic acid | 19 | 46 | 130 | 130 ± 4.5 |
| Proline | 11 | 40 | 102 | 105 ± 3.3 |
| Glycine | 14 | 36 | 100 | 96.6 ± 4.4 |
| Alanine | 8 | 22 | 60 | 60.3 ± 2.5 |
| Valine | 13 | 34 | 94 | 98.5 ± 4.7 |
| Methionine | 2 | 6 | 16 | 16.8 ± 0.6 |
| Isoleucine | 7 | 17 | 48 | 45.3 ± 3.9 |
| Leucine | 17 | 44 | 122 | 118 ± 7.4 |
| Tyrosine | 7 | 21 | 56 | 54.1 ± 3.4 |
| Phenylalanine | 10 | 11 | 42 | 39.4 ± 1.7 |
| Tryptophan | (4) | (11) | (30) | |
| Molecular weight | 23,000[e] | 51,100[f] | | 146,000[g] |

[a] Amino acid composition of the light and heavy chains were computed on the basis of ultracentrifuge molecular weights, corrected for carbohydrate content (light chain 23,000, no neutral sugar detected; heavy chain 55,700 less 8% carbohydate, or 51,100). For the 7S monomer the number of amino acid residues observed were moles found (uncorrected for losses) divided by moles of 7S monomer hydrolyzed; the latter was based on extinction coefficient ($E^{1\%}_{1\,cm}$ 15.5, Jaffe *et al.*, 1969), and the carbohydrate-free ultracentrifuge molecular weight (150,000 less 6% carbohydrate, or 141,000). Tryptophan values, in parentheses, are those found in protein 315 (B. J. Underdown *et al.*, in preparation), the IgA anti-DNP myeloma protein produced by a different mouse plasma cell tumor (MOPC-315). [b] Average values for duplicate analyses, given to nearest integer. [c] For two light plus two heavy chains per molecule of 7S monomer. [d] Mean value ± standard deviation for five analyses. [e] Ultracentrifuge molecular weight. [f] Ultracentrifuge molecular weight less 8% carbohydrate. [g] Sum of observed residue weights. [h] Analysis of chains from aminoethylated protein 460 indicates that the correct value for half-Cys in the heavy chain is 11 (R. G. Q. Leslie, personal communication).

*Carbohydrate Content.* Only preliminary data were obtained. Total hexose in protein 460 was 2.66 g/100 g of protein. A similar value was found for protein 315, another mouse IgA, in which all the hexose is present in the heavy chain. As no amino sugars were noted in amino acid analyses of the 460 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 460 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 460.

TABLE II: Association Constants for Ligands Bound by Protein 460.[a]

| Ligand | [$^3$H]ε-DNP-L-Lysine: Total Concn of Inhibitor (M) | $r'$ | $c$ (mole/l. × $10^5$) | $K_i$ (l./mole × $10^{-5}$) |
|---|---|---|---|---|
| None | | 1.00 | 0.263 | |
| 2,4-Dinitronaphthol | $3.92 \times 10^{-4}$ | <0.016 | 0.720 | >10.0 |
| 2,4,6-TNP-aminocaproate | $8.2 \times 10^{-5}$ | 0.34 | 0.582 | 1.2 |
| Menadione | $3.6 \times 10^{-4}$ | 0.45 | 0.519 | 0.16 |
| 2,6-DNP-aminocaproate | $8.9 \times 10^{-5}$ | 0.81 | 0.317 | 0.09 |
| 4-MNP-aminocaproate | $9.1 \times 10^{-5}$ | 0.92 | 0.331 | 0.07 |
| Purin-6-oyl aminocaproate | $1.1 \times 10^{-2}$ | 0.16 | 0.654 | 0.03 |

[a] Measured by inhibition of binding of [$^3$H]ε-DNP-L-lysine in equilibrium dialysis at 4°. $K_i$, the inhibiting ligands intrinsic association constant, was determined from (Karush, 1956): $K_i = (r/(r') - 1))(1 + Kc)/(I)$, where $r$ and $r'$ refer to moles of DNP-lysine bound per mole of protein in the absence and in the presence of inhibitor, respectively, $c$ is the free DNP-lysine concentration, and $K$ is the intrinsic association binding constant for ε-DNP-lysine ($4 \times 10^5$ M$^{-1}$). Inhibitor was added in large molar excess over protein and free inhibitor concentration, (I), was assumed to be the same as total inhibitor. The affinity found in this experiment ($4 \times 10^5$ M$^{-1}$) and the $3 \times 10^5$ M$^{-1}$ value reported previously (Jaffe *et al.*, 1969) are within experimental error. The following ligands, at concentrations given in parentheses, caused no inhibition of binding of ε-DNP-L-lysine: 5-acetouracil caproate ($5.4 \times 10^{-4}$ M), ε-(*p*-iodophenylsulfonyl)-L-lysine ($1.8 \times 10^{-4}$ M), ε-(5-dimethylaminonaphthalenesulfonyl)-L-lysine ($3.7 \times 10^{-4}$ M), ε-(*p*-toluenesulfonyl)-L-lysine ($4.5 \times 10^{-4}$ M), and 1,8-anilinonaphthalenesulfonate ($7.3 \times 10^{-4}$ M).

*Ligand Binding.* Protein 460 was previously shown to bind ε-DNP-L-lysine at two sites per 7S molecule with intrinsic affinity for this ligand of $3 \times 10^5$ M$^{-1}$ (Jaffe *et al.*, 1969). The affinity for several other ligands, determined by inhibition of the binding of [$^3$H]ε-DNP-L-lysine, are given in Table II. The competitive effect of 2,4-dinitronaphthol was especially pronounced. At low concentrations, dinitronaphthol was also more effective than ε-DNP-lysine in quenching the proteins's tryptophan fluorescence (Figure 7). Titration with both of these ligands at high concentration showed that about 46% of the protein's fluorescence was quenched at saturation ($Q_{max}$). On the basis of this value, the association constant for the binding of dinitronaphthol was calculated to be $5 \times 10^6$ M$^{-1}$ (Figures 7 and 8).

Because radioactive dinitronaphthol was not available, the use of equilibrium dialysis to confirm the protein's affinity for this ligand was limited by the necessity of using absorbency at 395 nm to measure the concentration of unbound ligand. Relatively high concentration was therefore required. The single measurement made under these conditions was consistent with the intrinsic association constant of $5 \times 10^6$ M$^{-1}$ found by fluorescence quenching (Figure 8).

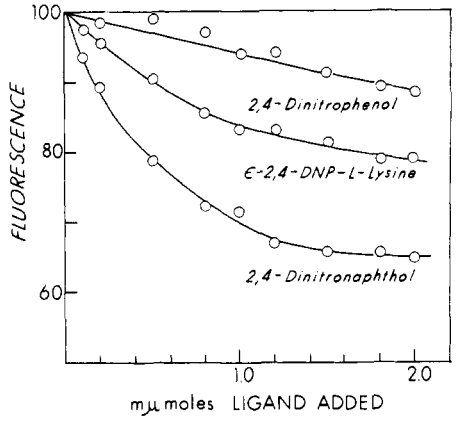



FIGURE 7: Fluorescence quenching of protein 460 by several DNP-ligands. One ml of protein (40 μg) was titrated at 4° by successive additions of up to a total of 0.2 ml of each of the ligands shown. No correction for nonspecific attenuation was made.

The binding of ε-DNP-lysine and dinitronaphthol are compared in Figure 8. Although there was appreciable scatter in the fluorescence quenching data, it was clear that protein 460 has about a 10-fold higher affinity for dinitronaphthol than for ε-DNP-L-lysine. Fluorescence quenching titrations also showed that the protein had essentially the same affinity for 2,4-dinitro-1-naphthol-7-sulfonic acid as for dinitronaphthol.

As shown in Table II, the protein's affinity for 2,4,6-TNP-aminocaproate was only slightly less than for ε-DNP-L-lysine, but it bound other nitrophenyls much less well; *e.g.*, 2,6-DNP-aminocaproate was bound about $^1/_{40}$th as well. It is especially notable that protein 460 did not bind to a detectable extent ($K_i < 10^3$ M$^{-1}$) a number of hydrophobic aromatic molecules, *e.g.*, pipsyllysine, dansyllysine, tosyllysine, and 1,8-anilinonaphthalenesulfonate (Table II).

*Spectral Shift of Bound Dinitronaphthol.* When polynitrophenyl ligands are specifically bound by antibodies, the li-

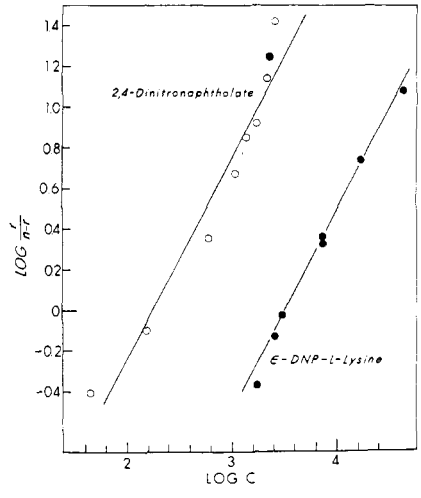



FIGURE 8: Sips plot of the binding of ε-DNP-L-lysine and 2,4-dinitronaphtholate by protein 460 (fluorescence quenching (○) and equilibrium dialysis (●). The ε-DNP-lysine binding data are from Jaffe *et al.*, 1969. $r$ is moles of ligand bound per mole of protein; $n$ (= 2.0) is the number of combining sites per protein molecule; $c$ is the free concentration of ligand in μμmoles/ml.

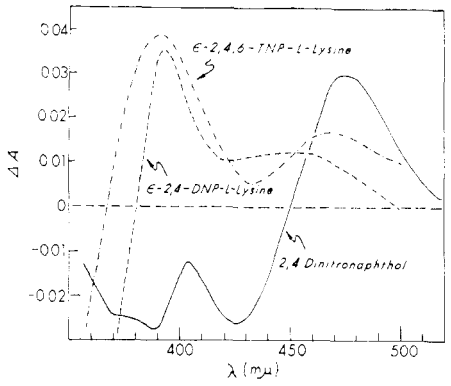



FIGURE 9: Difference absorption spectra between free ligands and ligands bound to protein 460. Protein concentration, $7 \times 10^{-6}$ M. Total ligand concentrations were: ε-DNP-L-lysine, $2.5 \times 10^{-5}$ M; ε-TNP-L-lysine $2.6 \times 10^{-5}$ M; 2,4-dinitronaphthol $2.5 \times 10^{-5}$ M. Spectra were determined at about 7°.

gand's absorbtion spectrum undergoes a red shift and the difference spectrum between free and bound ligand has a maximum at about 470 nm (Little and Eisen, 1967). A similar effect is shown in Figure 9, where the difference spectrum between free and protein-bound dinitronaphthol shows a maximum at 470 nm. The increase in molar absorbtivity ($\Delta E_m$) was estimated to be 2100, which is somewhat higher than for DNP- and TNP-ligands bound by conventionally prepared anti-DNP and anti-TNP antibodies (Little and Eisen, 1967).

## Discussion

The molecular weight and amino acid analyses show that the 7S form of protein 460 has the same fundamental four-chain structure as other immunoglobulins; *i.e.*, it consists of a pair of heavy chains and a pair of light chains (Fleischman *et al.*, 1962). In accord with this duplex structure, the 7S monomer has two combining sites (Jaffe *et al.*, 1969).

In their binding reactions, these sites seem to be as specific as those of antibodies in general. While they do not react to a detectable extent with a variety of aromatic hydrophobic substances (ε-pipsyl-L-lysine, ε-dansyl-L-lysine, ε-tosyl-L-lysine, or 1,8-anilinonaphthalenesulfonate), they bind some nitrophenyl ligands with affinities that approximate those of conventional antibodies. The affinity for ε-DNP-L-lysine, for example, is $3\text{–}4 \times 10^5$ $M^{-1}$, which corresponds to the average affinity for this ligand of antibodies obtained early after immunization with DNP-protein immunogens (Eisen and Siskind, 1964). Like conventional antibodies moreover, the tryptophan fluorescence of protein 460 is quenched by bound nitrophenyl ligands, and absorbtion spectra of the bound ligands are shifted to higher wavelengths, with a maximum at 470 nm in the difference spectrum between bound and free dinitronaphthol. Similar difference spectra between free and protein bound nitrophenyls have been observed with anti-DNP and anti-TNP antibodies formed in the immunized rabbit, guinea pig, goat, horse (Little and Eisen, 1967), chicken (E. Voss, A. Benedict, H. Eisen, in preparation), and shark (Voss *et al.*, 1969), and with other myeloma proteins having antinitrophenyl activity (Eisen *et al.*, 1967, 1968; Ashman and Metzger, 1969). Quite different spectral changes appear when nitrophenyls are bound by serum albumin (Carsten and Eisen, 1952).

The similarity between combining sites of protein 460 and conventional antibodies is also evident in the capacity of the sites to discriminate among the members of a homologous series. Thus, ε-2,4,6-TNP-aminocaproate was bound almost as well as ε-2,4-DNP-L-lysine, but ε-2,6-DNP-aminocaproate and 4-MNP-aminocaproate were bound only about $^1/_{50}$th as well.

Of all ligands tested, 2,4-dinitronaphthol and 2,4-dinitro-1-naphthol-7-sulfonic acid were bound most strongly by protein 460. Indeed, its affinity for these ligands is probably a good deal higher than that of representative antibody preparations formed against DNP and TNP proteins (Eisen *et al.*, 1970). With respect to the "strange" cross-reactions exhibited by some other antinitrophenyl antibodies and myeloma proteins it is notable that protein 460 has exceedingly weak affinity for menadione ($1 \times 10^4$ $M^{-1}$), and did not bind 5-acetouracil caproate (Schubert *et al.*, 1968; Eisen *et al.*, 1970).

The differences in specificity between proteins 315 and 460 can be contrasted by ordering their affinities for some representative ligands: for 460 the order is 2,4-dinitronaphthol > ε-DNP-L-lysine > menadione, whereas for 315 the order is ε-DNP-L-lysine > menadione > 2,4-dinitronaphthol.

In accord with their differences in function, there are also differences in primary structure between these IgA myeloma proteins. For example, the heavy chain of 460 has 40% more tyrosine than the heavy chain of 315; and the C-terminal residue of 315 light chain is leucine (Underdown *et al.*, 1970; Goetzl and Metzger, 1970), whereas the 460 light chain (reduced and carboxymethylated) has no C-terminal residue removed by carboxypeptidase A, because it is a K chain (F. Richards, personal communication) and probably has C-terminal carboxymethylcysteine.

The extent of the differences in structure and in function between 460 and 315 seems to correspond to those observed among different sets of antihapten antibodies produced in response to immunization with 2,4-DNP proteins (McGuigan *et al.*, 1968).

DNP and TNP proteins do not give precipitin reactions with the 7S form of 460, though they specifically precipitate with serum from mice bearing MOPC-460, and with protein isolated from DEAE-Sephadex as "fraction II" (Eisen *et al.*, 1968; Jaffe *et al.*, 1969). Because the latter is polydisperse, with various oligomers of the 7S monomer, we assume protein 460 is similarly polydisperse in serum, as are so many other mouse IgA myeloma proteins (Fahey, 1961; Potter and Kuff, 1961; Lieberman *et al.*, 1968). Why does the 7S monomer fail to give a precipitin reaction? Though it was subjected to mild reduction and alkylation in the course of isolation, the 7S molecule contains two combining sites whose reactivity with ligand is unimpaired, at least as indicated by the fluorescence quenching assay (see Figure 1 and corresponding experiments with protein 315 in Eisen *et al.*, 1968). Nevertheless it remains possible that the reduction and alkylation eliminates, by an as yet unknown mechanism, the ability of the 7S molecule to participate in precipitin reactions. It would be desirable to evaluate the precipitability of the "native" 7S molecule, isolated directly from serum without chemical modification. This has not yet been achieved, in part because only a small proportion of the polydisperse protein in fraction II (and presumably in serum) occurs in 7S form.

A more interesting possibility arises from the finding that certain IgG (7 S) antibodies, produced by conventional immunization, also do not form precipitates with antigen, even though these antibodies have two high-affinity combining sites per molecule (Klinman *et al.*, 1964; Klinman and Karush, 1967). An antibody molecule of this type may form cyclic ("monogamous bivalent") complexes with one antigen mole-

cule (Klinman and Karush, 1967; Warner and Schumaker, 1970), rather than cross-link two antigen molecules as in the classical "lattice" of the precipitin reaction (Heidelberger and Kendall, 1934). The nonprecipitability of the 7S form of 460 (and of 315) might also be somehow related to the curious light–heavy-chain association in Balb/c IgA myeloma proteins, in which the two light chains per 7S monomer appear to be SS bonded to each other, rather than to heavy chains as in other immunoglobulins (Abel and Grey, 1968).

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