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# Symmetry of Binding Sites of a Mouse IgA Myeloma Protein (MOPC 315)†

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ABSTRACT: We have investigated the mechanism of monovalency of the 7S subunit of a mouse IgA myeloma protein (MOPC 315) against a large antigen. This subunit, although it clearly can bind two molecules of a small hapten, fails to precipitate or hemagglutinate the relevant multivalent antigen. In an equilibrium Farr assay, we have shown that the subunit has only one valence for a univalent 40 000 molecular weight antigen (dinitrophenyl-dextran). We have investigated how various levels of affinity labeling quantitatively affect (a) the valence observed in the equilibrium Farr assay against a large antigen, and (b) the binding of the MOPC 315 to an insoluble antigenic matrix. Our results indicate that the Fab regions of the 7S subunit are arranged symmetrically and that the inactivity of one of them toward a large antigen is probably due to steric hindrance caused by the antigen bound to the adjacent site.

The concept that every Fab moiety of an antibody molecule provides one antigen-binding site, and that the sites in any one molecule bind hapten identically, is clearly supported by a large body of structural and binding data with isolated fragments and intact molecules (Nisonoff et al., 1975). There are, however, curious and so far unexplained exceptions in the case of some IgM and IgA molecules: when tested against large antigens, the 7S subunits of such antibodies are functionally monovalent, although they contain two Fab fragments, both of which bind hapten identically. For example, the 7S subunit of the mouse IgA myeloma protein MOPC 315 neither precipitates multivalent dinitrophenyl (Dnp[1]) protein conjugates nor agglutinates Dnp-coated sheep red blood cells (SRBC) (Eisen et al., 1968; Potter, 1972). In addition, equilibrium binding data indicate that half of the Fab fragments in the intact 7S subunit of an IgM rheumatoid factor are not active (Stone & Metzger, 1968).

Steric hindrance of a potentially active site by a large antigen bound to a neighboring site seems the most likely explanation for this apparent monovalence, but other explanations can be imagined. Chavin and Franklin, for example, proposed (Chavin & Franklin, 1969) that it is possible for identical Fab fragments to be arrayed asymmetrically in the 7S molecule, such that the binding site of one would face out and be able to bind a large antigen, and the other would face in and would not. On the other hand, the *in* site could be sufficiently unencumbered to accept a small ligand as efficiently as the *out* site. Such a molecule would not have a twofold axis of symmetry as has been shown for a crystallized IgG molecule (Terry et al., 1968), but rather would have superimposable Fab fragments.

Another alternative explanation for the observed monovalence is an allosteric effect whereby the binding of one ligand alters the conformation of the active site of the neighboring Fab fragment such that it can no longer accept a large ligand. This explanation could further be refined by specifying whether a large or small ligand must bind to the first site in order to inactivate the second site. This third hypothesis seems to be the least appealing.

We have approached this question through the use of affinity labels and an IgA myeloma protein (MOPC 315). We have confirmed (a) that 7S MOPC 315 has two apparent binding sites for a small monovalent hapten and one for a large monovalent antigen; and we have shown (b) that blocking binding sites with a small affinity labeling hapten, bromoacetyl-Dnp-lysine (BADL), quantitatively reduces the binding of large molecules in a manner strongly favoring a model of a symmetric 7S molecule, in which the binding of a large ligand to one site inhibits an adjacent site from binding a second large ligand.

## Materials and Methods

*Proteins.* MOPC 315 was purified from reduced and alkylated ascites by affinity chromatography on Dnp-lysine agarose as described (Goetzl & Metzger, 1970). [$^{14}$C]MOPC 315 was similarly prepared by using [$^{14}$C]iodoacetamide for the alkylation step. Rabbit anti-Dnp was prepared by using

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[1] Abbreviations used: SRBC, sheep red blood cells; Dnp, dinitrophenyl; BADL, bromacetyl-Dnp-lysine; EACA, ϵ-aminocaproic acid; Pnp, *p*-nitrophenyl; HGG, human γ-globulin; Dnp-OH, dinitrophenol; NHS, *N*-hydroxysuccinimide ester; BBS, borate buffered saline; SAS, saturated ammonium sulfate.

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affinity chromatography on paranitrophenyl (PNP)-lysine agarose (Carson & Metzger, 1974), elution with 0.1 M Dnp-glycine and removal of residual hapten by dialysis against 0.1 M dinitrophenol (Dnp-OH) and passage over Agl-X8 (Bio-Rad Laboratories, Richmond, Calif.). This latter preparation was contaminated by 0.18 mol of Dnp/mol of Fab, and, in this case, probably due to the high affinity of the blocked antibodies, the occupied sites appeared to be permanently blocked. Dnp-human $\gamma$-globulin (HGG) was made by reacting HGG (Fraction II, Miles Laboratories, Kankakee, Ill.) with dinitrobenzenesulfonic acid (Eisen et al., 1959). The degree of substitution was estimated by optical density readings at 360 and 278 nm. TEPC-15, a mouse IgA, $\kappa$, myeloma protein known to bind phosphorylcholine, was a gift from Dr. Henry Metzger and was used as a control protein. Ovalbumin (5× recrystallized) was obtained from Calbiochem (La Jolla, Calif.) and used without further purification. Affinity labeling of the MOPC-315 antibody was carried out in 0.1 M $NaHCO_3$ at 37 °C as described (Haimovich et al., 1970). Approximately 1 mol of BADL was used per mol of Fab of the antibody. After varying periods of time (1–24 h), the reaction was stopped by addition of an excess of Dnp-glycine. Unbound BADL and Dnp-glycine were removed by passage over a column of Agl-X8 and dialysis.

*Antisera.* Rabbit anti-Dnp was raised by repeated immunization with $Dnp_{25}$-HGG. Sheep anti-rabbit IgG and rabbit anti-TEPC 15 were the gifts of Dr. Henry Metzger. They were partially purified by precipitation three times with half-saturated ammonium sulfate (SAS) and absorbed with Dnp-lysine–agarose for 4 h at room temperature.

*Organic Chemicals.* Dnp-lysine was obtained from Sigma Chemical Co. (St. Louis, Mo.). [$^3$H]Dnp-lysine, [$^{14}$C]iodoacetamide, and [$^{14}$C]-$\epsilon$-aminocaproic acid (EACA) were obtained from New England Nuclear (Boston, Mass.). Dnp-OH was obtained from Sigma and recrystallized from hot acidified water one or two times before use. Dnp-EACA was made from dinitrofluorobenzene and EACA (Carsten & Eisen, 1953). Dnp-EACA-*N*-hydroxysuccinimide ester (Dnp-EACA-NHS) was made as described (Martin et al., 1971). Bromoacetyl-Dnp-lysine was made as published (Weinstein et al., 1969) with the important modification that the reaction of bromoacetyl succinimide ester with Dnp-lysine was carried out for one minute rather than 60 min (Wilchek, personal communication). [$^{14}$C]Dnp-EACA-NHS and [$^3$H]BADL were made by using [$^{14}$C]EACA and [$^3$H]Dnp-lysine, respectively, in the above syntheses and carrying out the final purification by preparative thin-layer chromatography rather than recrystallization. Dextran (T40, Pharmacia Fine Chemicals, Piscataway, N.J.) was modified by the sequential addition of carboxymethyl and then diaminoethyl groups (Inman, 1975). The aminoethylcarboxymethyl-dextrans were lightly substituted with [$^{14}$C]Dnp-EACA-NHS followed by exhaustive dialysis. The level of substitution was estimated based on the known specific activity of the radioactive hapten and the starting weight of the lyophilized dextran preparation used. We used the Poisson distribution to predict the relative numbers of dextran molecules substituted with 0, 1, 2, etc., Dnp groups. For the preparations used, less than 3% of the Dnp label was bound to dextran molecules which contained more than one Dnp group. The correction factors for these multisubstituted antigen molecules were accordingly insignificant, even considering the possibility of monogamous bivalent interactions. Because of the difficulty in obtaining quantitative precipitation of rabbit anti-Dnp–Dnp-dextran complexes (see Results), the antigen was further purified by binding to insolubilized rabbit anti-Dnp and elution with hapten (Brown & Koshland, 1975).

*Assays.* Equilibrium dialysis was carried out in plastic microcells, using 0.1 mL volume on either side of the membrane (Ashman & Metzger, 1969). Results were plotted as described (Scatchard, 1949). All straight lines were fitted by the method of least squares.

The binding of the haptenated dextrans to MOPC 315 was determined in a modified Farr assay. Purified MOPC 315 (about 0.5 mg/mL) in borate buffered saline (BBS, 0.2 M borate, 0.075 M NaCl, pH 8.3) containing ovalbumin as a nonspecific protein and $^3H_2O$ as a volume marker was incubated with dilutions of the test antigen in 0.1 mL volumes at room temperature for 18–24 h. Four-tenths of a milliliter of cold SAS was then added and the tubes were incubated on ice for 30 min before centrifuging at 3000 rpm for 30 min. Precipitates were washed once with 1 mL of cold 80% SAS and redissolved in distilled water for transfer to liquid scintillation vials. Tubes were rinsed once with 100 $\mu$L of $H_2O$ in order to ensure quantitative recovery of the dissolved precipitate. In addition, approximately 100 $\mu$L of the supernatant from the final wash of the precipitate was separately transferred in order to permit correction of the precipitated counts for nonspecific entrapment. One milliliter of 1 M KOH was added to each 10 mL of scintillation fluid (Aquasol, New England Nuclear, Boston, Mass.) in order to prevent precipitation of the added dextran. Bound antigen was thus determined directly. Free antigen concentration was determined by subtracting bound antigen from antigen added. Results were plotted as for equilibrium dialysis.

As discussed in the Results, the Farr assay described failed to give interpretable data with rabbit anti-Dnp antibodies, probably because the extreme solubility of the dextran inhibited precipitation. Therefore, we combined ammonium sulfate precipitation with antibody precipitation: after the overnight incubation of antigen and antibody, 0.1 mL of sheep anti-rabbit IgG was added and incubated for 60 min at 4 °C. Then 0.4 mL of SAS was added for a further incubation of 60 min at 4 °C. The tubes were spun at 2500 rpm for 30 min at 4 °C, and, after removal of 100 $\mu$L of supernatant, the precipitates were washed twice with 60% SAS. Pellets were redissolved and counted along with first supernatant aliquots. As no volume marker was used in these experiments, counts bound to normal rabbit IgG controls were subtracted from those bound to the specific antibody. Counts from the first supernatant were used to determine the concentration of free antigen, whereas bound antigen was determined as above. MOPC-315 was processed in a parallel way, using rabbit anti-TEPC 15 as the second antibody and TEPC 15 as the nonspecific entrapment control. However, in this case 80% SAS was used, as 60% SAS caused loss of antigen during the washing stages. All liquid scintillation counting was done with correction for quenching by either internal or external standards.

Hemagglutination was carried out in microtiter plates using sheep erythrocytes coated with Dnp-EACA by reaction with Dnp-EACA-NHS (Martin et al., 1971). Double immunodiffusion was performed in 1% agarose in BBS.

Binding of [$^3$H]BADL-affinity-labeled [$^{14}$C]MOPC 315 to Dnp-lysine agarose was performed in 1-mL glass columns (Bio-Rad Laboratories, Richmond, Calif.). Bound material was eluted with 1.0 mL of 0.1 M Dnp-EACA. The eluted material was passed directly through a 3-mL Agl-X8 column to remove the highly quenching eluting hapten. The bound and unbound materials, collected as fractions of 1 mL, were counted and the counts summed. Values representing fractions bound and eluted (Table III) are presented as fractions of total recovered (about 90% in each run).

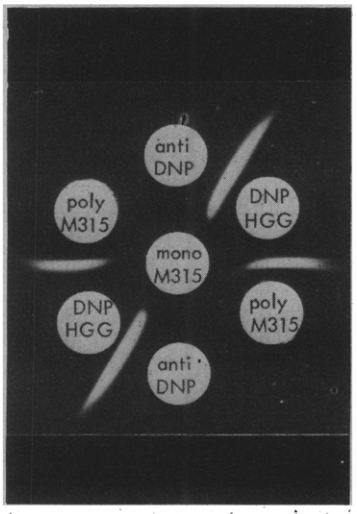


FIGURE 1: Nonprecipitating nature of reduced and alkylated MOPC 315 (center well). Peripheral wells are as follows: 12 and 6 o'clock, rabbit (IgG) anti-Dnp; 1:30 and 7:30 o'clock, $Dnp_{25}$-HGG; 4:30 and 10:30 o'clock, MOPC 315 ascites. Note the shortening of the precipitin lines toward the center well, suggesting inhibition of precipitation by the MOPC 315 7S subunit.

TABLE I: Binding Data of Unmodified and Affinity Labeled MOPC 315.

| | equilibrium dialysis | | Farr | | | |
|---|---|---|---|---|---|---|
| | | | Dnp-EACA | | Dnp-dextran T40 | |
| preparation[a] | $K_a$ ($\times 10^{-6}$) | valence | $K_a$ ($\times 10^{-6}$) | valence | $K_a$ ($\times 10^{-5}$) | valence |
| 1 | 1.5 | 2.0 | 1.4 | 1.8 | 3.5 | 1.1 |
| 2 | 1.0 | 1.7 | 1.4 | 1.5 | 2.6 | 0.99 |
| 3 | 1.2 | 1.6 | 0.8 | 1.7 | 2.3 | 0.92 |
| 4 | 1.1 | 0.83 | 1.5 | 0.89 | 2.1 | 0.58 |
| 5 | 1.4 | 0.71 | 2.3 | 0.84 | 2.0 | 0.62 |
| 6 | 0.8 | 0.56 | 0.9 | 0.49 | 1.2 | 0.38 |
| 7 | 0.9 | 0.25 | 0.8 | 0.25 | 1.5 | 0.19 |

[a] Preparation 1 represents the mean results obtained with 2–5 assays done on the unmodified (nonaffinity labeled) 7S subunits of the MOPC 315 protein. The other preparations (2–7) have been modified by affinity labeling with BADL for increasing periods of time to give increasingly substituted molecules and therefore decreasing valences.

## Results

*Monovalency of MOPC 315.* In preliminary experiments we confirmed earlier observations (Eisen et al., 1968; Potter, 1972) that the 7S subunit of the MOPC 315 protein fails to cross-link large antigens. In both a hemagglutination system using Dnp-EACA-SRBC and a double immunodiffusion plate using Dnp-HGG, we found that the 7S MOPC 315 is inactive. On the other hand, both the original ascites, presumably containing a large amount of polymeric protein, and purified 7S (IgG) rabbit anti-Dnp gave perfectly good reactions (Figure 1, hemagglutination data not shown).

Neither of these assays, however, provide absolute evidence of monovalency, as both hemagglutination and precipitation are influenced by numerous secondary factors. We sought, therefore, to use more quantitative binding assays to investigate this phenomenon. First, we establish that our 7S MOPC 315 preparations indeed had a valence of 2 (one valence per Fab fragment) for a small antigen. This is particularly important with this myeloma protein because its valence has been a matter of some debate in the literature and has only relatively recently been shown to have a curious concentration dependence (Underdown et al., 1971). Tested numerous times at concentrations of 0.4–0.5 mg/mL, we have been able to verify a valence of 2.0 for 7S MOPC 315 interaction with Dnp-lysine (Figure 2, square symbols; Table I, preparation 1). The affinity constant we observed is in agreement with published results at room temperature (Pecht et al., 1972).

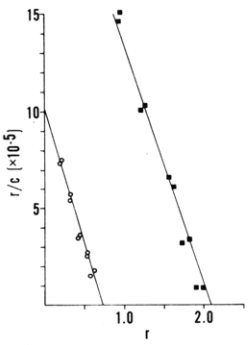


FIGURE 2: Equilibrium dialysis of MOPC 315 against [$^3$H]Dnp-lysine, Scatchard plot analysis. (■) Unmodified protein. (○) Affinity labeled protein. Points represent individual determinations and lines are fitted by least-squares analysis.

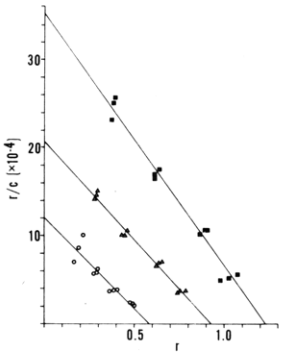


FIGURE 3: Equilibrium Farr assay of MOPC 315 against [$^{14}$C]Dnp-EACA-Dextran T40, Scatchard plot analysis. (■) Unmodified protein. (○ and △) Two affinity labeled preparations. Points represent individual determinations and lines are fitted by least-squares analysis.

We subsequently carried out a series of Farr assays with our MOPC 315 using a much larger but still monovalent antigen. Figure 3 shows the Scatchard plot of data obtained with MOPC 315 tested against 40 000 mol wt monosubstituted Dnp-dextran. In four separate experiments, an average valence of 1.1 and a $K_a$ of $3.5 \times 10^5$ were obtained (Table I, preparation 1). This monovalency did not appear to be an artifact of the Farr assay used, as a small hapten (Dnp-EACA) tested in the same assay gave an average valence of 1.8 and a $K_a$ of $1.4 \times 10^6$ (Table I, preparation 1).

As a further control on the interpretation of our assay with the 40 000 mol wt Dnp-dextran, we wished to show that an IgG antibody would be able to bind this antigen divalently. We accordingly purified rabbit anti-Dnp from the serum of rabbits hyperimmunized with $Dnp_{25}$-HGG. Figure 4 shows results of an equilibrium dialysis experiment with this antibody. We were able to demonstrate a valence of 1.6 with a small hapten (Dnp-lysine), the reduction from the expected value of 2 being due to the blocking of high affinity sites with hapten used in the purification process (see Materials and Methods). When we first tested the IgG antibody in our Farr assay against 40 000 mol wt Dnp-dextran, we found that the more antigen we added, the less binding we would observe (data not shown).

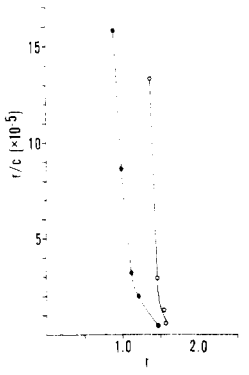


FIGURE 4: Scatchard plot of binding analyses of rabbit (IgG) anti-Dnp. (○) Equilibrium dialysis against [$^3$H]Dnp-lysine; points represent mean of two determinations. (●) Farr assay against [$^{14}$C]Dnp-Dextran T40; points represent mean of three determinations. Both lines fitted by hand.

This appeared to be due to a failure of the IgG antibody to precipitate at high antigen concentrations. We were unable to induce precipitation either with increased ammonium sulfate concentration, use of carrier protein, or by removal of the unsubstituted dextran (see Materials and Methods). We observed a similar failure to precipitate with optimal concentrations of a sheep anti-rabbit IgG. We were finally able to achieve precipitation by addition of a second antibody (sheep anti-rabbit IgG) followed by sufficient SAS to make a final concentration of 60%. The resultant data are shown in Figure 4. The IgG anti-Dnp clearly has the same valence for a 40 000 mol wt antigen as it does for one with a mol wt of 311. MOPC 315, on the other hand, when tested in this modified system, failed to give good data, probably due to dissociation occurring during the period of incubation with antiserum. In any case, the valences seen (1.2–1.5) were clearly less than two (data not shown).

*Symmetry of the MOPC 315 Binding Sites.* Having established that MOPC 315 was monovalent toward a large antigen, we investigated the basis for this monovalency using affinity labeling techniques. Assuming that the BADL-affinity label would randomly bind the two active sites of each 7S subunit (see Discussion), we could use probability theory to predict the fraction of antibody molecules with 0, 1, and 2 sites blocked at a given level of substitution. We can then consider the theories of monovalency as follows. (a) If steric hindrance between antigen molecules, secondary to limited antibody flexibility, were occurring, we would expect symmetrical antibody molecules with 0 or 1 site blocked to be able to bind a large antigen. Molecules with 2 sites blocked, of course, would have no remaining valences. (b) If the Fab fragments of the 7S subunit were fundamentally asymmetric, we would expect all antibody molecules with 0 sites blocked and $^1/_2$ of antibody molecules with 1 site blocked to bind a large antigen. This latter estimate is based on the consideration that of the singly substituted molecules, $^1/_2$ would have their *in* site blocked and $^1/_2$ would have their *out* site blocked. (c) If binding to one antibody active site allosterically inactivated the other site, we would expect only those molecules with 0 sites blocked to continue to bind a large antigen.

Following from these theoretical considerations, we accordingly blocked the binding sites of our protein with various levels of BADL. The degree of affinity labeling was estimated by optical density at 360 and 280 nm (assuming a $17.4 \times 10^3$ extinction coefficient for the bound BADL moiety). A more accurate determination was made by equilibrium dialysis. A comparison of these two methods suggested that no more than about 8% of the bound BADL was not in the active site of the antibody. All preparations were then tested in the Farr assay against 40 000 mol wt monosubstituted dextran and Dnp-EACA. The data were analyzed in a Scatchard plot, and the valences and affinity constants so determined are collected in Table I (preparations 2–7). Results with two preparations are shown in a Scatchard plot in Figure 3, along with data from the unsubstituted preparation 1.

TABLE II: Binding of a Large Monovalent Antigen by Affinity Labeled MOPC 315.

| preparation | fraction sites blocked[a] | theor valence for large antigen[b] steric model | asymmetric model | allosteric model | obsd valence[c] |
|---|---|---|---|---|---|
| 1 | 0 | 1.0 | 1.0 | 1.0 | 1.1 |
| 2 | 0.05 | 0.98 | 0.85 | 0.72 | 0.99 |
| 3 | 0.2 | 0.96 | 0.80 | 0.64 | 0.92 |
| 4 | 0.59 | 0.66 | 0.42 | 0.17 | 0.58 |
| 5 | 0.65 | 0.58 | 0.36 | 0.13 | 0.62 |
| 6 | 0.72 | 0.48 | 0.28 | 0.08 | 0.38 |
| 7 | 0.89 | 0.23 | 0.13 | 0.02 | 0.19 |

[a] Determined from equilibrium dialysis data in Table I. [b] Calculated as follows: $F$ = fraction sites blocked; $N$ = fraction molecules with no sites blocked; $S$ = fraction of molecules with one site blocked; $T$ = fraction of molecules with two sites blocked. Then $T = F^2$; $N = (1 - F)^2$; and $S = 1 - (N + T)$. For each theory, then, valence ($V$) is calculated as follows: $V$(steric) = $N + S$; $V$(asymmetric) = $N + {}^1/_2(S)$; and $V$(allosteric) = $N$. [c] See Table I.

In Table II, the valence obtained by equilibrium dialysis of each preparation was used to calculate the fractions of antibody molecules having 0, 1, and 2 sites blocked by BADL. From this calculation, the expected binding of the 40 000 mol wt antigen is determined as discussed above for each model (see footnote of table). In the right-hand column of Table II is given the experimentally observed valence, as taken from Table I. In every case but one (preparation 6), the data favor the steric hindrance model of monovalency.

*Column Experiments.* We also explored the effective monovalency of MOPC 315 through the use of Dnp-lysine–agarose columns. As the Dnp-lysine is bound to an insoluble backbone of agarose, it is in essence a "large" antigen. We passed affinity-labeled MOPC 315 over the column and measured the fraction of molecules that failed to bind. We then eluted the column with hapten in order to determine the fraction of molecules that had bound. The MOPC 315 was radiolabeled with [$^{14}$C]iodoacetamide (see Materials and Methods), while the BADL affinity label was radiolabeled with $^3$H. In that way we could separately quantitate all antibody molecules and those antibody molecules which had at least one affinity label. Preparations that showed a great excess of unbound $^{14}$C (over $^3$H) were considered to be denatured and are not included in the data presented. The affinity label bound to the antibody molecules was quantitated by the known specific activity of the [$^3$H]Dnp-lysine used to make the [$^3$H]-BADL. Ninety-two percent of the bound BADL was assumed to be in the antibody active site, in accord with the estimations made with the unlabeled BADL-MOPC 315 preparations. The preparations tested are characterized in Table III in regard to the fraction of sites affinity labeled and the calculated distribution of the $^{14}$C and $^3$H labels on molecules that have none, one or two sites blocked.

Using reasoning similar to that used above, we may make

TABLE III: Characteristics of [$^3$H]BADL Afffinity Labeled [$^{14}$C]-MOPC-315.

| | distribution of isotope label[b] | | | | | |
|---|---|---|---|---|---|---|
| | $^{14}$C | | | $^3$H | | |
| $F$[a] | $N_C$ | $S_C$ | $T_C$ | $N_H$ | $S_H$ | $T_H$ |
| 0 | 1 | 0 | 0 | | | |
| 0.017 | 0.97 | 0.03 | 0.0003 | 0 | 0.98 | 0.02 |
| 0.057 | 0.89 | 0.11 | 0.0003 | 0 | 0.95 | 0.05 |
| 0.51 | 0.24 | 0.50 | 0.26 | 0 | 0.49 | 0.51 |

[a] $F$ = fraction of sites labeled (see text). [b] $N$ = fraction of isotope found on non-affinity-labeled molecules. $S$ = fraction of isotope found on singly affinity labeled molecules. $T$ = fraction of isotope found on doubly affinity labeled molecules. C(subscript) = $^{14}$C; H(subscript) = $^3$H. Computed as follows: For $^{14}$C (representing all MOPC 315 molecules), $N_C = (1 - F)^2$; $T_C = F^2$; $S_C = 1 - (N + T)$. For $^3$H (representing affinity label), $N_H = 0$; $T_H = (2)(T_C)/((2)(T_C) + S_C)$; $S_H = S_c/((2)(T_C) + S_C)$.

the following predictions: (a) by the steric hindrance model, only doubly affinity labeled molecules would fail to bind to the column; (b) by the asymmetric model, all doubly affinity labeled molecules, plus one-half of singly labeled molecules would fail to bind to the column; (c) by the allosteric model, all affinity labeled molecules would fail to bind to the column. Calculations based on these predictions as well as the actual data obtained are presented in Table IV. In all cases the predictions made by the steric hindrance model come closest to the observed ratios.

## Discussion

The data we have obtained by both the direct binding Farr assay and the column binding experiments strongly favor the steric hindrance model of MOPC 315 monovalency. Such a model implies that the Fab binding sites are symmetrically arranged in the 7S subunit, but that they are too close together to permit two large antigens to bind simultaneously. It is in accord with the twofold axis of symmetry known to exist in the IgG molecule that has been studied (Terry et al., 1968). In addition, it is relevant that primary sequence data (Kratzin et al., 1975) indicate that there is a disulfide bridge between the CH1 domain and the hinge region of the human monoclonal IgA studied. Such work suggests that this bridge, along with two other nearby disulfide links, could contribute to the stabilization of the hinge region. It seems possible that such "stabilization" could well limit the flexibility of the attached Fab fragments with respect to one another. On the other hand, fluorescence polarization studies with human IgA myeloma proteins have yielded contradictory results in regard to hinge region flexibility (Weltman & Davies, 1970; Zagyansky & Gavrilova, 1974). Furthermore, although electron microscopic studies done on IgA proteins (including MOPC 315) do suggest that the Fab regions on a single 7S molecule can be quite widely separated (Dourmashkin et al., 1971; Green et al., 1971; Bloth & Svehag, 1971; Munn et al., 1971), we find it difficult to interpret such investigations. The examined proteins are not visualized in solution and, therefore, the configurations observed could well be an artifact of the fixation procedure.

Others (Edberg et al., 1972) have also studied the monovalence of 7S subunits in depth, using rabbit polyclonal anti-dextran antibodies of the IgM and IgG classes. They demonstrate how increasing the size of the antigen used from a mol wt of 342 to a mol wt of 7100 decreased the observed valence of the intact IgM antibodies from 10 to 5, while the valence of the IgG antibodies remained unchanged. These authors assumed that steric hindrance caused by bound antigen molecules prevented other antigen molecules from binding to nearby sites, but they did not consider the alternative explanations that we have discussed. We would suspect that the mechanism of 7S subunit monovalence is the same for IgM as it is for our IgA protein, but we cannot assume that our results can be extended to IgM antibodies or even to other antibodies of the IgA class. In this regard it should be noted that MOPC 315 is an unusual myeloma protein, in its relatively high affinity, its rare light chain subclass (λ 2) and the curious concentration dependence of its valence. In addition, it assumably has the interlight chain disulfide bonds found in IgA myelomas of the BALB/c origin (Abel & Grey, 1968). This bond, along with a lack of light-heavy disulfide bonds, is also characteristic of a particular subgroup of human IgA proteins (Jerry et al., 1970; Grey et al., 1968), and may well put some limitation on the motion of the Fab regions relative to one another.

It is important to keep in mind the assumptions that are essential to our interpretation of the data employing the affinity labeling reagent. We think that it is justified to consider the BADL affinity labeling reagent as a "small" antigen: (a) its molecular weight (517) is much closer to that of Dnp-lysine (311) than that of the 10 000 mol wt monosubstituted dextran that appears to be on the borderline between large and small for MOPC 315 (our unpublished data); (b) it clearly can bind to both sites of the 7S molecule simultaneously, as evidenced by its ability to block more than 50% of the available sites. We have also assumed that affinity labeling with the tritiated reagent is as specific as with the nonradioactive compound. We

TABLE IV: Dnp-Lysine-Agarose Binding of [$^3$H]BADL Affinity Labeled [$^{14}$C]MOPC 315.

| | | fraction unbound/fraction bound | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | expected by theory:[b] | | | | | | | |
| | | steric model | | asymmetric model | | allosteric model | | obsd | |
| fraction act. sites blocked | $N$[a] | $^{14}$C | $^3$H | $^{14}$C | $^3$H | $^{14}$C | $^3$H | $^{14}$C | $^3$H |
| 0 | (1) | 0/1.00 | | 0/1.00 | | 0/1.00 | | 0/1.00[c] | |
| 0.017 | (3) | 0/1.00 | 0.02/0.98 | 0.02/0.98 | 0.51/0.49 | 0.03/0.97 | 1.00/0 | 0/1.00 | 0.08/0.92 |
| 0.057 | (2) | 0/1.00 | 0.05/0.95 | 0.06/0.94 | 0.53/0.47 | 0.11/0.89 | 1.00/0 | 0/1.00 | 0.20/0.80 |
| 0.51 | (2) | 0.26/0.74 | 0.51/0.49 | 0.51/0.49 | 0.76/0.24 | 0.76/0.24 | 1.00/0 | 0.30/0.70 | 0.55/0.45 |

[a] $N$ = number of times experiment was done. [b] Calculated as follows (see also Table III, footnote 2): $(U/B)_C$ = fraction $^{14}$C unbound/fraction $^{14}$C bound; $(U/B)_H$ = fraction $^3$H unbound/fraction $^3$H bound. Steric: $(U/B)_C = T_C/(N_C + S_C)$; $(U/B)_H = T_H/S_H$. Asymmetric: $(U/B)_C = (T_C + 1/2S_C)/(N_C + 1/2S_C)$; $(U/B)_H = (T_H + 1/2S_H)/(1/2)S_H$. Allosteric: $(U/B)_C = (T_C + S_C)/N$; $(U/B)_H = (T_C + S_C)/0 = 1.0/0$. [c] By definition. All percentages are corrected for the 6.4% of counts that were unbound with the non-affinity labeled MOPC 315 preparation.

have no reason to doubt this assumption, although we did not test it directly.

Several aspects of the Farr assay bear discussion. We feel that it is valid to use it as an equilibrium method, based on previous experiments (Seppala, 1975) and on the fact our results in this assay with a hapten (Dnp-EACA) are comparable to our results in equilibrium dialysis (Table I). With the MOPC 315 protein, however, it was found that (a) some carrier protein (ovalbumin) had to be added to ensure complete precipitation; and (b) if 60% SAS was used instead of 80%, bound antigen would be lost during the washes. The difficulty in precipitating the rabbit anti-Dnp in our assay is quite curious. This does not appear to be due merely to solvent effects caused by the large concentration of dextran used, as no change was noted when >95% of the (unhaptenated) dextran was removed by affinity chromatography. We were also unable to overcome this problem with high concentration of SAS (80%) or with a precipitating anti-rabbit IgG alone. At low concentrations of Dnp-dextran, however, we could get reasonable amounts of precipitation and antigen binding. As the Dnp-dextran concentration was increased, a peak of binding occurred well below 1 mol of Dnp/mol of antibody and then increasing amounts of antigen gave decreasing amounts of binding as well as decreasing amounts of visible precipitate. Our assumption is that a complex of two Dnp-dextran T40 molecules and one rabbit IgG anti-Dnp is extremely soluble, even in high concentrations of SAS. However, if more protein is added to the complex, in the form of sheep anti-rabbit IgG, then it becomes possible quantitatively to salt it out of solution. It was this combined approach that allowed us to obtain interpretable data with our rabbit anti-Dnp. This same method gave us great difficulty with the MOPC 315 protein, probably due to the lower affinity of this preparation.

An objection to the modified Farr assay is that by including a wash after precipitation of the antigen–antibody complexes we could be losing some of the bound antigen by dissociation. We found this wash step to be necessary, because in those tubes with high antigen concentrations, the amount of antigen in the first supernatant was large compared with the amount bound, such that applying the correction factor based on our volume marker meant subtracting two large numbers to get a smaller one. Using the wash step, we were able to compare the antigen left in the second (i.e., wash) supernatant in those tubes that had MOPC 315 and those tubes that had only ovalbumin (which precipitated under the ammonium sulfate concentrations used). Based on these data, it is apparent that about 10% of the bound antigen could be lost from the antibody pellet in the tubes with the higher amounts of antigen. Slightly more might be lost in the lower antigen tubes, because there is less free antigen, but in those cases the numbers of counts involved were too small to permit any direct calculations. If a correction were made for such antigen loss, it would shift the Scatchard plots to the right and upward, and tend to make the overall curve slightly steeper. The resultant valences would probably tend to be slightly higher. Among the three models, the steric model predicts the highest valences in all cases. Therefore, although we do not have a more quantitative way to deal with this limitation, correcting the data for antigen washed off would favor this model and further support our overall conclusion.

It would be of interest to extend our studies to other proteins, but we found that we were limited due to the requirements for a homogeneous antibody of reasonably high affinity for a defined hapten in order to permit quantitative investigations of the type described. Similar studies with a monoclonal IgM anti-Pnp-EACA ($IgM_{wag}$, Ashman & Metzger, 1969) proved impossible, assumably due to the protein's low affinity, as we were unable to demonstrate binding to a large, monovalent antigen. In this regard, we should note that in the case of MOPC 315 and the rabbit anti-Dnp that we used, the affinity measured for the large antigen was lower than that against the small hapten (Table I, Figure 4).

It also would have been nice to have had a high affinity anti-Dnp IgG myeloma to compare with MOPC 315 in our system. We feel, however, that the rabbit anti-Dnp preparation does provide a valid control. In our preliminary cross-linking experiments, involving immunoprecipitation and hemagglutination, one could argue that the multivalent antigens used could show some antigenic heterogeneity which might favor interaction with a mixed antibody population. In our quantitative binding studies, however, we used a univalent antigen to measure directly the valence of individual molecules. Therefore, the heterogeneity of the antibody population should have no fundamental effect, except insofar as it distorts the Scatchard plots from a straight line. The monovalency of the MOPC 315 7S subunit toward a 40 000 mol wt antigen is not just an artifact of the antigen used, but rather results from a basic structural aspect of this protein that differentiates it from IgG molecules.

Teleologically, it is not surprising that an antibody that is monovalent in its 7S subunit would normally be found in polymeric form. Naturally occurring antigens are generally "large". The effectiveness of an antibody in dealing with such an antigen would be limited if it could not cross link. On the other hand, it is difficult to understand why the nonfunctional "active" sites should be present at all, particularly if we assume that IgM is the most primitive immunoglobulin phylogenetically (Good & Papermaster, 1964), and therefore would have developed evolutionarily before the advent of IgG.

Our data have permitted us to draw conclusions about the basic three-dimensional structure of an antibody protein molecule as it must exist in solution. Our "structural" evidence is of necessity indirect, as we must go through several steps of reasoning from the raw observations and must accept certain assumptions. Nevertheless, we feel that such an approach provides important information that should be considered along with more direct studies done on molecules no longer in solution by such methods as electron microscopy and X-ray crystallography.

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# Surface-Specific Iodination of Membrane Proteins of Viruses and Eucaryotic Cells Using 1,3,4,6-Tetrachloro-3$\alpha$,6$\alpha$-diphenylglycoluril†

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ABSTRACT: The use of the iodinating reagent 1,3,4,6-tetrachloro-3$\alpha$,6$\alpha$-diphenylglycoluril (chloroglycoluril) to selectively label membrane surface proteins was investigated with the following systems: enveloped viruses (Sendai and Newcastle disease viruses), human erythrocytes, and nucleated cells propagated both in suspension (EL-4) and in monolayer culture (BHK-21). Conditions are described for specifically iodinating surface proteins while maintaining full virus integrity or cell viability. Comparison of the chloroglycoluril method with the lactoperoxidase and chloramine-T methods for labeling surface membrane proteins shows that the chloroglycoluril method has a number of advantages: It routinely produces a 3- to 17-fold greater specific radioactivity without sacrificing viral or cellular integrity, it is technically simpler to use, it does not require the addition of extraneous protein to initiate the reaction nor a strong reducing reagent to terminate it. Chloroglycoluril also proved to be an effective substitute for chloramine-T in the nonvectorial labeling of viral and cellular proteins. Membrane protein samples were solubilized with the detergent sodium dodecyl sulfate before iodination or labeled in the presence of high iodide concentrations without prior solubilization. The resulting specific radioactivities generated by the use of chloroglycoluril were equal to or greater than those generated by the chloramine-T method. The effectiveness, simplicity of use, and versatility of chloroglycoluril recommend it as an iodinating reagent for both surface-specific and nonvectorial labeling of membrane systems.

The currently expanding interest in membrane architecture and surface phenomena such as hormonal action, cellular recognition, and chemotaxis demands methodology to label selectively externally disposed proteins, i.e., surface-specific (vectorial) methods. One of the most popular methods, lactoperoxidase-catalyzed iodination (LPO,[1] Philips and Morrison, 1971), using $H_2O_2$ alone or generated by the glucose-glucose oxidase system (GO, Hubbard and Cohn, 1972), requires the addition of extraneous protein to the system. This

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[1] Abbreviations used: LPO, lactoperoxidase; GO, glucose oxidase; chloramine-T, *N*-chloro-*p*-toluenesulfonamide; Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; PBS, 150 mM NaCl in 5 mM sodium phosphate buffer at pH 8.0; DPBS, Dulbecco's phosphate-buffered saline (Dulbecco and Vogt, 1954); EL-4, C57 BL leukemia cells; BHK, baby hamster kidney cells; DPBI, Dulbecco's phosphate-buffered iodide (0.8% NaCl of DPBS replaced by 0.8% NaI); *Paramxyovirus* proteins; P, minor nucleocapsid protein; HN, major envelope protein associated with hemagglutinating and neuraminidase activities; NP, major nucleocapsid protein; $F_1$, envelope protein with role in hemolysis, cell fusion, and infectivity (possibly with $F_2$); M, matrix protein of the viral membrane; $F_2$, small envelope protein of unknown function; LETS, a large, external transformation-sensitive cellular glycoprotein.

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