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Interaction of purified precipitating and non-precipitating (coprecipitating) antibodies with hapten and with haptenated protein. Evidence of an asymmetric antibody molecule

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Summary. The interaction of monovalent hapten dinitrophenyl ε-amino caproic acid (DNP-EACA) with purified IgG1 sheep anti-DNP precipitating and non-precipitating antibodies, and their $F(ab')_2$, F(ab') and Fab fragments, was studied by fluorescence quenching and by a radioimmunoassay. The Scatchard plots of whole non-precipitating antibody and its $F(ab')_2$ fragment showed a bi-modal curve that could be interpreted as due to the existence of two populations of sites with very different affinity for the ligand, each population representing 50% of the total number of sites. The F(ab) fragments of the non-precipitating antibody could be fractionated by immunoadsorption into two populations of high and low affinity whose association constants differed by more than 2 logs. The study of the interaction of whole antibodies with DNP-bovine serum albumin (BSA) demonstrated that each molecule of precipitating antibody can combine with two molecules of antigen but non-precipitating antibody cannot combine with more than one molecule of antigen.

It is concluded that the molecule of non-precipitating antibody is asymmetric and has a site of high affinity and another of low affinity. As a consequence of this structure the non-precipitating antibody behaves functionally as univalent and is unable to form precipitates with the multivalent antigen and to activate effector mechanisms.

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INTRODUCTION

A number of observations made in recent years has indicated that antibodies of the 'non-precipitating' or 'coprecipitating' type, first described by Heidelberger & Kendall (1935) are present in most immune sera. They have been demonstrated in IgG class antibodies of the rabbit (Margni & Binaghi, 1972), guinea pig (Margni & Hajos, 1973), sheep (Margni, Paz & Cordal, 1976), horse (Cordal & Margni, 1974), man (Perdigon *et al.*, 1982) and cow (Parma, Santisteban & Margni, 1984), and they constitute 5–10% of the total antibody population.

Non-precipitating antibodies combine with the specific antigen but do not form insoluble complexes and their biological properties are quite different to those of the precipitating antibodies. In general, they are unable, or have a very diminished capacity to mediate most effector functions. For instance, they do not fix complement by the classical or alternative pathway, they do not have opsonizing activity *in vivo* and they do not stimulate *in vitro* phagocytosis although they are cytophilic for the macrophages (Margni *et al.*,

1980). On account of these properties, non-precipitating antibodies could possibly act as blocking antibodies and evidence has been recently obtained showing that they may play a role in some chronic and parasitic diseases (Ball & Bartlett, 1969; Forget & Borduas, 1977; McCutchand *et al.*, 1978; Hajos *et al.*, 1982; Ottesen *et al.*, 1981; Iskander, Das & Aalberse, 1981).

Studies done with purified IgG1 anti-DNP sheep non-precipitating antibodies have suggested that the two combining sites of each molecule of antibody have different affinity for the antigen (Margni *et al.*, 1977). The association constant of one of the sites has a value comparable to that of the precipitating antibodies in the same serum, while the value for the other site is much smaller. Due to this fact, the molecule is functionally asymmetric and behaves practically as univalent, and that may be the reason why large complexes cannot be formed with the multivalent antigen.

In order to gain information regarding the structure and the mode of action of non-precipitating antibodies we have investigated the interaction of purified anti-DNP precipitating and non-precipitating antibodies and their $F(ab')_2$ and F(ab') fragments with a monovalent DNP hapten, and we have also studied the interaction of these antibodies with a multivalent high molecular weight antigen, DNP-BSA.

MATERIALS AND METHODS

Haptens and haptenated proteins

Dinitrophenyl ε-amino caproic acid (DNP-EACA) was prepared as described by Werblin & Siskind (1972). Dinitrophenyl 1-lysine, 2,4-ε-[phenyl-3,5-^{3}H] was obtained from New England Nuclear, U.S.A. Dinitrophenyl-human gamma globulin (DNP-HGG) and dinitrophenyl-bovine serum albumin (DNP-BSA) were prepared as described by Little & Eisen (1967). Labelling of DNP-BSA with ^{125}iodine was done by the method of Hunter & Greenwood (1962). DNP-BSA was polymerized with glutaraldehyde according to Little & Eisen (1967).

Sheep anti-DNP antibodies

A Merino sheep was immunized with DNP-HGG. Injections of 25 mg protein in Freund's complete adjuvant, total volume 5 ml, were given intramuscularly weekly for 8 weeks. The animal was bled 2 weeks after the last injection. The antiserum contained 8·7 mg/ml anti-DNP antibodies.

Isolation and purification of precipitating and non-precipitating antibodies

Separation of precipitating from non-precipitating antibodies was performed by the method of Heidelberger & Kendall (1935) as modified by Margni & Binaghi (1972). Na_2EDTA was added to the antiserum (7 mg/ml) and small amounts of DNP-BSA (approximately 1/20th of the quantity required for maximal precipitation at equivalence) were added successively. After each addition the mixtures were incubated at least for 1 hr at 37° and then for 1 hr at 4° before centrifuging. Additions of antigen were continued until no more precipitate was obtained and no reaction with antigen could be demonstrated in the supernatant by Ouchterlony's tests and by counter-immunoelectrophoresis. The supernatant contained the non-precipitating antibodies. The precipitates obtained after the successive additions of antigen were pooled and used to prepare the precipitating antibodies. The methods used for the purification of both antibody types have been previously described (Margni & Binaghi, 1972; Margni & Hajos, 1973; Margni *et al.*, 1976). After purification, the antibodies were dialysed against phosphate buffer 0·01 M pH 7·6 and passed through a DEAE-cellulose column equilibrated with the same buffer. After elution of the first peak (containing IgG2 antibodies), phosphate buffer 0·1 M pH 7·6 was applied and the purified IgG1 antibodies were eluted.

Preparation of $F(ab')_2$ and F(ab') fragments

The method of Nisonoff, Wissler & Lipman (1960) was employed. To a solution containing 10 mg/ml of purified antibody in 0·1 M Na acetate buffer pH 4·5, pepsin (crystallized, Sigma, U.S.A.) 2% of the weight of protein was added. After 16 hr incubation at 37°, the reaction was stopped by adding Tris 2 M pH 8. The $F(ab')_2$ fragment was isolated by gel filtration in a G-200 Sephadex column equilibrated with PBS.

The F(ab') fragment was prepared as described by Wilder, Green & Shumaker (1975). To a solution containing 4 mg/ml of $F(ab')_2$ in PBS, was added dithiotreitol (DTT) up to a final concentration of 7 mM. After 1 hr at 37°, the mixture was alkylated with iodoacetic acid (15 mM) and incubated for a further hour at 37°, then dialysed against PBS.

Preparation of F(ab) fragments

F(ab) fragments were obtained by papain digestion

according to Porter (1959). Purified precipitating and non-precipitating antibodies, at a concentration of 10 mg/ml protein were digested for 16 hr at 37° with papain (1% w/v) in PBS containing cysteine 0·01 M and EDTA 0·002 M. After digestion, the mixture was dialysed against PBS and applied to a Sephadex G-100 column to eliminate the non-digested antibody. The F(ab) fragment was finally purified by elution from a DEAE-cellulose column at 0·01 M phosphate pH 8·0.

Fractionation of high and low affinity F(ab) and F(ab') fragments

This was performed by passage through a column containing the specific immunoadsorbent, namely polymerized DNP-BSA. The fraction not retained by the column is the 'low affinity' fragment. The 'high affinity' fragment, retained by the immunoadsorbent, was eluted with 0·1 M DNP-OH pH 7·6 and then chromatographed on IRA 400 ion exchanger to eliminate the hapten.

Analysis of antibodies

Double diffusion in agar was performed according to Ouchterlony. Immunoelectrophoresis and counter-immunoelectrophoresis were performed in agar 1% in 0·025 M veronal buffer pH 8·2. Passive haemagglutination performed with human and sheep red cells dinitrophenylated by the method of Bullock & Kantor (1965) modified by Margni & Hajos (1973). Anti-sheep antisera were prepared in rabbits.

*Determination of the average intrinsic association constant (*Ko*)*

Two methods were employed.

(i) *Fluorescence quenching.* Titration were performed as described by Eisen & McGuigan (1971) and Velick, Parker & Eisen (1960), using 2 ml of the various antibody preparations approximately 1 μM in 0·15 M Nacl, 0·01 M phosphate buffer pH 7·4 at 30°. The ligand used was 10 μM DNP-EACA. Scatchard plots were constructed to determine the r/c versus r relation (r = bound antibody; c = free antigen) and the Sips equation was used to establish *Ko* and the heterogeneity index a.

(ii) *Dextran-coated charcoal radioimmunoassay (RIA).* This method could be used successfully because free DNP-lysine was readily absorbed to the dextran-coated charcoal. Different quantities of radio-labelled hapten in a total volume of 0·1 ml in 0·01 M PBS pH 7·3 were added to 0·1 aliquots of the antibody preparations and the mixtures were allowed to stand for 48 hr at 4°. Thereafter, 0·2 ml of a 5% suspension of dextran-coated charcoal in PBS was added to each tube, left at 4° for 30 min, then centrifuged. Radioactivity was measured in the supernatants Scatchard plots and Sips equations were used to establish *Ko* and the heterogeneity indexes.

Measurement of the interaction between antibody and haptenated protein

A technique based in the property of polyethylene-glycol (PEG) to precipitate the complex of antibody and DNP-BSA was used to measure the avidity of purified antibody preparations. Different amounts of ^{125}I-labelled DNP-BSA in a total volume of 0·1 ml of PBS were added to 0·1 ml of antibody solutions in 0·1 M PBS, 2% gelatine, pH 7·5 and incubated at 4° for 48 hr. After incubation, 0·05 ml normal sheep serum and 0·25 ml PEG 20% were added to the tubes and further incubated 1 hr at 4°. After centrifugation, the radioactivity was measured in the supernatants and the pellets (washed twice with 10% PEG). The radioactivity of the supernatant represents the free antigen and that of the pellet, the bound antigen.

Calculations of Ko

As will be seen in 'Results', two different patterns were obtained when antibody preparations reacted with hapten. In the case of precipitating antibodies, plots of r/c against r gave curves which on extrapolation to the abscissa gave a value of 2. But when preparations of non-precipitating antibodies were tested, bi-modal curves were obtained, suggesting the existence of two populations of sites with different affinities for the hapten. At high hapten concentration, extrapolation to the abscissa gave a value of 2, but at low hapten concentrations the extrapolation gave a value of 1. In this case, the calculation of *Ko* was made assuming that the high and low affinity populations each represented 50% of the total number of sites. Consequently, *Ko* of high and low affinity populations was calculated when 0·5 and 1·5 sites per antibody molecule, respectively, were bound to the hapten.

RESULTS

Interaction of whole antibodies and F(ab')$_2$ fragments with hapten

The results of the interaction between antibody and

DNP-EACA, using whole purified antibodies and their F(ab')$_2$ fragments and employing fluorescence quenching and radioimmunoassay is presented in Fig. 1 in the form of Scatchard plots. When precipitating antibody was used, the curves intercepted the abscissa at a value of 2, in all cases, as expected. On the other hand, when non-precipitating antibody was studied the curves were clearly bi-modal: the first part of the curve, at low hapten concentration extrapolated to $r = 1$ while at high hapten concentration the extrapolation gave a value of $r = 2$. The treatment of these data has been made, as described in 'Materials and Methods', assuming only one population of antibody sites for the precipitating antibody and two populations in the case of non-precipitating antibodies, each population representing 50% of the total number of sites. The values of the association constants *Ko* obtained for the various preparations and by the two methods employed are reported in Table 1.

Interaction of F(ab) fragments with hapten

F(ab) fragment was prepared from precipitating antibody. All the activity was retained by passage through a column containing the immunoadsorbent (polymerized DNP-BSA). When the same procedure was

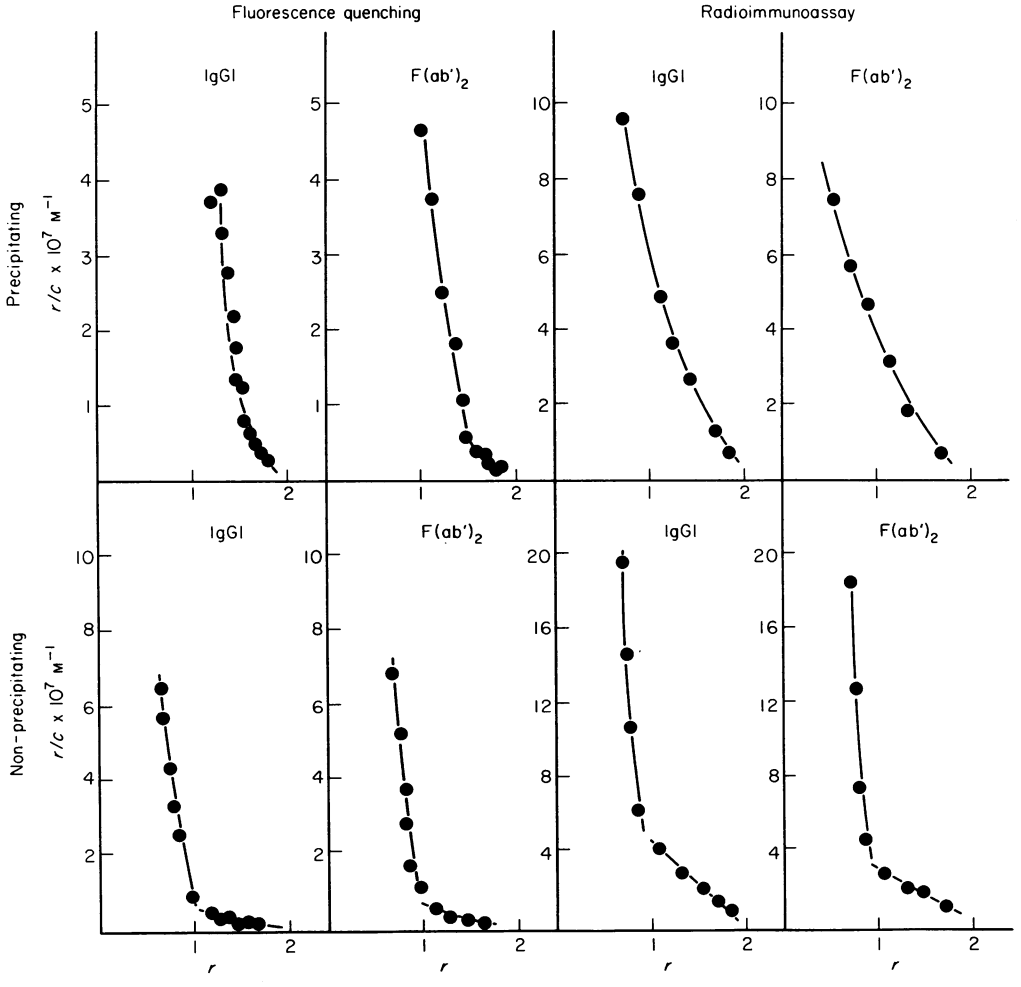

Figure 1. Scatchard plots of the interaction between DNP-EACA and IgG1 sheep anti-DNP precipitating and non-precipitating antibodies and their F(ab')$_2$ fragments, as measured by fluorescence quenching and by radioimmunoassay.

Table 1. Association constant (*Ko*) of sheep anti-DNP precipitating and non-precipitating antibodies and their F(ab')$_2$, F(ab') and F(ab) fragments

Antibody		Association constant *Ko*			
		Fluorescence quenching		Radioimmunoassay	
Precipitating	Whole	$9{\cdot}5 \times 10^7$		$6{\cdot}3 \times 10^7$	
	F(ab')$_2$	$5{\cdot}0 \times 10^7$		$3{\cdot}5 \times 10^7$	
	F(ab)	$4{\cdot}0 \times 10^6$		$5{\cdot}0 \times 10^7$	
		High affinity	Low affinity	High affinity	Low affinity
Non-precipitating	Whole	$2{\cdot}0 \times 10^6$	$1{\cdot}6 \times 10^4$	$1{\cdot}2 \times 10^7$	$0{\cdot}9 \times 10^5$
	F(ab')$_2$	$2{\cdot}1 \times 10^6$	$1{\cdot}2 \times 10^4$	$1{\cdot}2 \times 10^7$	$1{\cdot}0 \times 10^5$
	F(ab)	$3{\cdot}2 \times 10^6$	$2{\cdot}3 \times 10^4$	not done	not done
	F(ab')	not done	not done	$6{\cdot}3 \times 10^6$	$3{\cdot}2 \times 10^4$

applied to the F(ab') fragment from non-precipitating antibody, only about half of the activity was retained by the immunoadsorbent under the same experimental conditions but the eluate was still able to react with the hapten although with much lower affinity. The results obtained when these different preparation were reacted with DNP-EACA and studied by fluorescence quenching are presented in Fig. 2. It appears that the association constant of the F(ab) fragment from the precipitating antibody is comparable to that of the F(ab) fragment from non-precipitating antibody which was retained by the immunoadsorbent (high affinity) while the F(ab) fragment from the non-precipitating antibody not retained by the immunoadsorbent (low affinity) had a 2 logs lower *Ko* value (Table 1).

Interaction of total antibody with a multivalent, high molecular antigen

The interaction of whole purified precipitating and non-precipitating antibodies with highly substituted DNP-BSA was studied by a radio immunoassay technique, as described. The Scatchard plots of the

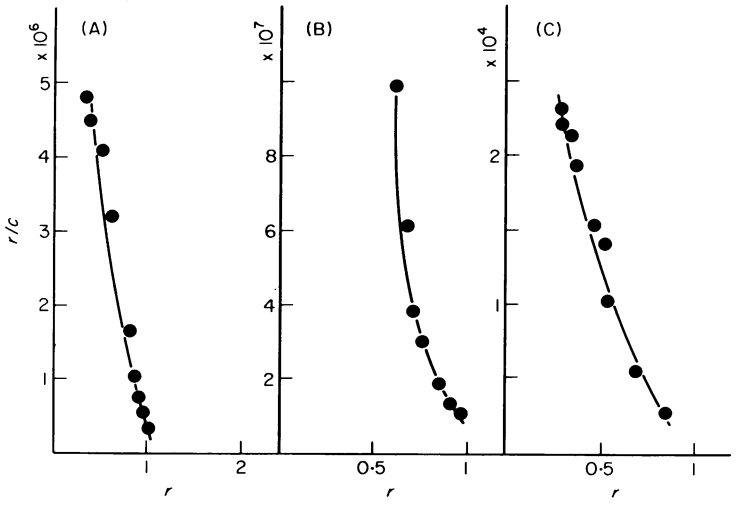

Figure 2. Scatchard plots of the interaction between DNP-EACA and sheep anti-DNP F(ab) fragments from precipitating antibody (A) and from non-precipitating antibody (B, high affinity sites; C, low affinity sites), as measured by fluorescence quenching.

results obtained are presented in Fig. 3. The precipitating antibody gave a curve that extrapolated to a value of $r=2$ but the non-precipitating antibody gave a value of $r=1$, indicating that only one molecule of antigen reacted with each molecule of antibody, even at high antigen concentration. It must be remarked that no bi-modal curves were observed with non-precipitating antibody and haptenated protein. This is in contrast to what was found when monovalent hapten

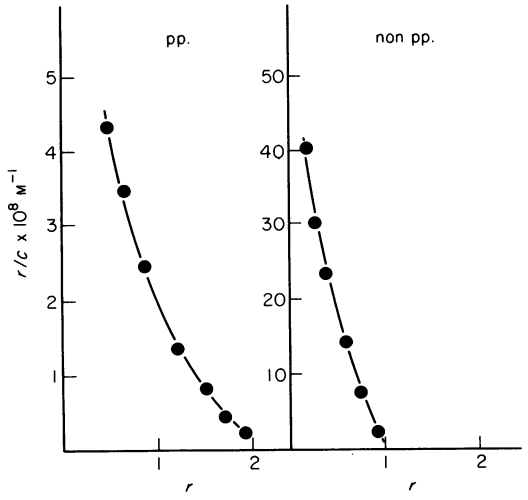

Figure 3. Scatchard plots of the interaction between DNP_{40}-BSA and IgG1 sheep anti-DNP precipitating and non-precipitating antibodies as measured by PEG precipitation.

was used and indicates that one molecule of non-precipitating antibody cannot react with more than one molecule of the high molecular weight antigen employed.

DISCUSSION

The study of the interaction of purified sheep IgG1 anti-DNP precipitating and non-precipitating antibodies with monovalent hapten DNP-EACA has indicated a striking difference in the behaviour of both antibody populations. In the case of precipitating antibody the Scatchard plot has the usual pattern and extrapolation gives a value of 2 for the valence of the molecule. The curve is very different with non-precipitating antibody: a bi-modal curve is obtained extrapolating to valence 1 at low hapten concentration and to valence 2 at high hapten concentration. This bi-modal curve has been interpreted as due to two populations of sites differing considerably in their affinity for the hapten. As shown in Table 1 the difference in affinity is of the order of 100 times, the respective *Ko* values differing in 2 logs. The fact that values 1 and 2 are obtained by extrapolation of the two parts of the curve indicated that each population of sites constitutes 50% of the total. This result confirms previous observations using a similar antibody (Margni *et al.*, 1977) and in another series of experiments, not described in the present paper, identical results have been obtained with non-precipitating rat anti-DNP IgG antibody. Therefore, it can be assumed that the bi-modal pattern shown by the non-precipitating antibody is not particular to sheep antibodies but it is characteristic of the non-precipitating population. Bi-modal curves have been obtained also with IgM antibodies (Onoue *et al.*, 1968; Oriol, Binaghi & Coltorti, 1971).

Analysis of the interaction between the $F(ab')_2$ fragments and hapten has equally shown two populations of sites. The Scatchard plot obtained with non-precipitating $F(ab')_2$ is similar to that obtained with whole non-precipitating antibody. This result indicates that the Fc portion of the molecule does not play a role in the observed differences between precipitating and non-precipitating antibodies.

In order to better characterize the existence of the two populations of sites, F(ab') and F(ab) fragments have been prepared and, in the case of fragments obtained from non-precipitating antibody, they have been fractionated by immunoadsorption. With this procedure the fragments of high affinity, retained by the immunoadsorbent, were separated from the fragments of low affinity, not retained, and it has been possible to determine the association constant of each subpopulation. It was found that the low affinity population has a *Ko* value more than 100 times smaller than that of the high affinity population. The value obtained for the high affinity population is essentially the same as that of the F(ab) fragment from the precipitating antibody. It is to be remarked that the procedure employed to fractionate the populations of fragments demonstrates that the $F(ab')_2$ fragment of the non-precipitating antibody is formed by one F(ab') or high affinity and another of low affinity. In effect, the $F(ab')_2$ fragment was completely retained by immunoadsorption while only half of the F(ab') fragments was retained by the same method. That would not be the case if the two F(ab') of the $F(ab')_2$ fragment were of low affinity. Evidence that each

molecule of non-precipitating antibody is assymmetrical has been previously obtained from experiments showing that non-precipitating antibodies isolated by serial adsorptions with small amounts of immunoadsorbent gave bi-modal curves in all cases and also by experiments of hybridization where precipitating antibodies were produced by random hybridization of half molecules of non-precipitating antibody (Margni *et al.*, 1977).

It seemed interesting to study the behaviour of precipitating and non-precipitating antibodies in their interaction with an antigen of high molecular weight. The antigen employed was haptenated bovine serum albumin containing 40 DNP groups per molecule. Given the size of the carrier and the number of hapten groups per molecule, it should be expected that a great number of determinants would be available and at close proximity so that combination with antibody would not be hindered by topological reasons. Both precipitating and non-precipitating antibodies gave similar plots and no bi-modal curve was observed with the non-precipitating antibody. But, when compared in terms of molecules of antigen bound per molecule of antibody, in antigen excess, it was found that the combining capacity of the precipitating antibody was twice that of the non-precipitating antibody. This fact indicates that the non-precipitating antibody acts as univalent, even at great antigen excess, in its interaction with a multiple-determinant, high molecular size antigen.

In general, the equilibrium curves obtained with the different preparations tested are very comparable and the values of *Ko* obtained by fluorescence quenching or by the dextran-coated charcoal radioimmunoassay method are similar, as seen in Table 1. The differences observed in some cases may be explained by various technical reasons. In particular the data obtained from fluorescence quenching is subjected to some theoretical limitations derived from the uncertainty of the adopted value of maximum quenching and from the physicochemical nature of the quenching itself which makes difficult the comparison of quenching in molecules with different structure and size. It is also possible that some denaturation has taken place during the multiple manipulations necessary to purify the antibodies and to prepare the fragments.

An important, and yet unsolved problem, is the reason why the two combining sites of the same molecule of non-precipitating antibody have different affinity. Repeated efforts to detect any structural difference by immunochemical or chemical methods have consistently failed (Margni & Hajos, 1973; Cordal & Margni, 1974; Margni *et al.*, 1976). Moreover, the accumulated data on amino acid sequences of H and L chains, as well as genetic considerations, support unanimously the existence of a symmetrical molecule. It could be argued that combination of one site of the molecule with the ligand could affect the affinity of the other site but this possibility is eliminated by the fact that the populations of different affinity can be fractionated by immunoadsorption. Another possibility, under study in our laboratories, is that one of the combining sites is hindered by a carbohydrate moiety present only in one of the F(ab) regions.

In summary, it appears that non-precipitating antibodies have a peculiar molecular structure, with two combining sites of quite different affinity. On account of this structure, they behave functionally as univalent antibodies, and are unable to form large size insoluble complexes and to activate most of the physiological effector functions, like complement fixation or opsonization of bacteria. These non-precipitating antibodies represent 5–10% of the total antibody population in most antisera and have been found in many animal species. It is then reasonable to assume that they play some important physiological role. Because of the fact that they combine with antigen but do not trigger effector mechanisms, they may act as 'blocking' or 'facilitating' antibodies and may compete with precipitating antibodies. Evidence recently obtained suggests that this is the case in a number of chronic and parasitic diseases.

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