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Structure of asymmetric non-precipitating antibody: presence of a carbohydrate residue in only one Fab region of the molecule

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SUMMARY

The reactions between purified precipitating and non-precipitating anti-DNP sheep and rabbit antibodies and the antigens DNP–BSA and DNP–GABA–BSA have been studied by immunodiffusion, complement fixation and an inhibition test. Both antigens reacted identically with precipitating antibodies. On the contrary, non-precipitating antibodies did not precipitate and did not fix complement with DNP–BSA but were able to do so with DNP–GABA–BSA. A different behaviour with both antigens was also demonstrated by an inhibition test. The properties of these antibodies were also studied after treatment with endo-β-N-acetylglucosaminidase H. Non-precipitating antibody was able to give precipitin bands in gel diffusion and to fix complement with DNP–BSA after treatment with the enzyme. The treated antibody was able to agglutinate sensitized erythrocytes. Studies by fluorescence quenching showed that the affinity for the ligand DNP–GABA was significantly increased after hydrolysis of the carbohydrate residue. The properties of precipitating antibody were not modified by the endoglycosidase. Affinity chromatography of the $F(ab')_2$ and Fab fragments obtained from precipitating and non-precipitating antibodies was made with Con A–Sepharose. The Con A retained all the $F(ab')_2$ and 50% of the Fab from non-precipitating antibody, which were subsequently eluted with α-methyl-D-mannoside. The fragments from precipitating antibody were not retained at all. It is concluded that the asymmetry of the non-precipitating antibody molecule is due to a carbohydrate moiety which is present in only one of the Fab regions. This carbohydrate affects the reaction between the combining site and the antigen, and renders the molecule functionally univalent.

INTRODUCTION

The purification and the physicochemical and biological properties of IgG antibodies of the non-precipitating or coprecipitating type (Heidelberger & Kendall, 1935) have been described in previous papers (Margni & Binaghi, 1972; Margni & Hajos, 1973; Margni *et al.*, 1977. 1980; Ronco *et al.*, 1984). These studies showed that the molecule of non-precipitating antibody has two combining sites of very different affinity for the specific ligand. On account of this particular structure, the antibody molecule behaves functionally as univalent and cannot form large complexes with the plurivalent antigen. Among the hypotheses that can be made to explain the asymmetry of the molecule, it can be assumed that non-protein parts of the molecule (i.e. carbohydrate) could affect the quaternary structure in such a way as to create a steric hindrance in one of the combining sites. In the present work, we have explored these possibilities by studying the interaction of non-precipitating anti-DNP antibodies with two haptenated proteins. DNP–BSA and DNP–GABA–BSA, that differ in the position of the hapten, which in the case of DNP–GABA–BSA is separated from the protein carrier by an aliphatic chain of four carbon atoms. Furthermore, we have studied some physicochemical and biological properties of non-precipitating antibodies after treatment with endo-β-*N*-acetylglucosaminidase H, an enzyme that removes the carbohydrate attached to the Asn residue of the peptidic chain. The results obtained agree with the hypothesis that the asymmetry of the non-precipitating antibody molecule is due to a carbohydrate group.

Abbreviations: BSA, bovine serum albumin; DNP, dinitrophenyl; GABA, gamma amino butyric acid; HGG, human gamma globulin; HSA, human serum albumin.

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MATERIALS AND METHODS

Antigens

Coupling of dinitrophenyl to BSA, HSA and HGG was made according to Little & Eisen (1968). The final products were highly substituted and contained more than 30 DNP groups per

molecule. DNP–GABA was prepared as described by Werblin & Siskind (1972). Coupling of DNP–GABA to BSA was made according to Hoare & Koshland (1967) with slight modifications. To a solution of 100 mg DNP–GABA in 50 ml distilled water were added 32 mg BSA. The pH was adjusted to 4·75 and to this mixture were added 64 mg of 1-ethyl-3-(3-dimethylamino propyl) carbodiimide HCl (Sigma, St Louis, MO). The reaction was allowed to proceed for 2 hr at room temperature in the dark, and dialysed at 4° against buffered saline, pH 7. The DNP–GABA finally contained 25 groups per molecule.

Antibodies

Sheep and rabbits were immunized with highly substituted DNP–HGG in complete Freund's adjuvant as previously described (Margni & Hajos, 1973). Precipitating and non-precipitating antibodies were separated by the method of Heidelberger & Kendall (1935) by successive additions of small amounts of antigen (DNP–BSA). The quantity of antigen added each time was 1/20 of the quantity necessary to precipitate all the antibody (equivalence point). After each addition of antigen, the mixture was incubated for 1 hr at 37° and then kept at 4° until complete precipitation (1–4 hr after the first addition of antigen, overnight after the last additions) and the precipitate was separated by centrifugation. After about 20 additions, no more precipitate was formed and the supernatant did not show any reaction with antigen in Ouchterlony tests or by counter-electrophoresis.

Precipitating antibodies were purified from the collected precipitates by solubilization with DNP–OH, 0·1 M, pH 7·5. The antibody was, therefore, recovered by passage through a column containing DEAE and IRA 400, as described by Eisen *et al.* (1968). In the case of sheep antibodies, they were further fractionated by passage through DEAE: IgG_2 was recovered by elution with phosphate buffer, 0·01 M, pH 7·6, and IgG_1 was subsequently eluted with the same buffer, 0·03 M.

Non-precipitating antibodies were obtained from the supernatant after elimination of the precipitating antibodies, by absorption with polymerized DNP–BSA, according to Avrameas & Ternyck (1969), for 18 hr at 4°, followed by elution with 0·1 M DNP–OH. The antibodies were finally separated in a column of IRA 400. In the case of sheep antibodies, IgG_2 and IgG_1 were separated as indicated before.

Antibody fragments

$F(ab')_2$ fragments from sheep IgG_1 anti-DNP antibodies were obtained by pepsin digestion as described by Turner, Bennich & Natvig (1970). Purified antibody was dialysed against 0·1 M Na acetate buffer, pH 4·5, and digested with pepsin (Sigma), enzyme/substrate ratio 1:50, for 22 hr at 37°. The digestion was stopped by the addition of Tris to pH 8. Purification of the $F(ab')_2$ fragment was achieved by Sephadex G-200 gel filtration. Fab fragments were prepared by the method of Porter (1959). Purified antibody, 12 mg/ml, was dialysed against buffered saline (0·075 M phosphate, 0·075 M NaCl, pH 7) and digested with 1% papain (Sigma) in the presence of 0·01 M cysteine and 0·200 M Na EDTA. After 16 hr at 37°, the digestion was stopped with *N*-ethylmaleimide (0·05 M). The digest was dialysed against buffered saline and was fractionated by gel filtration in G-100 Sephadex. The second peak, containing Fab and Fc fragments, was chromatographed in DEAE-cellulose in phosphate buffer, 0·01 M, pH 8, in which conditions the Fab fragment was eluted. The purity of the $F(ab')_2$ and Fab fragments was assessed by Ouchterlony and counter-immunoelectrophoresis using an antiserum specific for sheep or rabbit Fc. No precipitin lines were obtained in any case, employing protein concentration up to 5 mg/ml.

Enzymatic treatment

This was performed according to Tarentino, Plummer & Maley (1974) with endo-β-*N*-acetylglucosaminidase H (Miles Labs): an enzyme that splits oligosaccharide attached to the Asn residue of the protein. A solution containing 1·5 mg antibody protein in 0·25 M citrate buffer, pH 5·5, was made up to 0·02 M in enzyme and kept for 20 hr at 37° in conditions of sterility. The solution was then dialysed in the cold against buffered saline pH 8·2.

Radiolabelling

Labelling with ^{125}I was performed as described by Greenwood, Hunter & Grover (1963) and Kirhom & Hunter (1971).

Immunodiffusion tests

These were made in 1·5% agar in veronal buffer, pH 8·6. Counter-immunoelectrophoresis was carried out for 45 min at 35 mA.

Passive haemagglutination

Human and sheep red cells were labelled with DNP as described by Hirata & Brandiss (1968). The tests were performed in microplates using 50 μl antibody dilutions and 50 μl of 2% labelled red cells, and readings were made after overnight incubation in the cold.

Complement fixation

The method described by Kabat & Mayer (1961), at 50% lysis, was used. Mixtures of 20 μg antibody, 1 μg antigen and 0·5 ml guinea-pig serum diluted to 4 U CH_{50}/ml in a total volume of 1·8 ml were incubated for 1 hr at 0°, and then 1·2 ml of 2% sensitized red cells was added and incubation was continued for 30 min at 37°. After centrifugation, the lysis was estimated by measuring the optical density at 540 nm. The weight ratio of antibody to antigen of 20 to 1 used in all cases was found to be optimal.

Inhibition test

The binding capacity of precipitating and non-precipitating antibodies to DNP–BSA and DNP–GABA–BSA was compared by a radioinhibition test. Mixtures containing 4 ng labelled DNP–BSA and varying quantities of cold DNP–BSA or DNP–GABA–BSA were incubated with 70 ng antibody in a total volume of 1 ml. Polyethyleneglycol 1000 was added to a final concentration of 10%, and the precipitate obtained was washed three times with 20% polyethyleneglycol 1000. The radioactivity measured in the supernatant and in the washed precipitate allowed the calculation of bound and free antigen. The results were expressed as radiolabel bound against cold competing antigen.

Fluorescence quenching

The affinity for the univalent ligand DNP–GABA of the various purified antibody preparations was measured by quenching. This measurement is based on the fact that the fluorescence of the antibody molecule (induced by excitation at 280 nm) is

quenched when it combines with an hapten that absorbs light of the same wavelength of the fluorescent emission (360nm), which is the case for DNP–GABA. The extent of quenching is proportional to the amount of combined hapten, and therefore the association constant K can be calculated. The technique described by Eisen (1964) was adopted, using an Aminco-Bowman spectrophotofluorometer. The antibody concentration was 0·1 mg/ml. The results are presented in the form of Scatchard plots of r/c against r, where r is the combined and c the free hapten per mol of antibody.

Concanavalin A–Sepharose affinity chromatography

Con A–Sepharose 4 B was obtained from Pharmacia, Uppsala, Sweden. The gel was equilibrated with buffer pH 7·2 (HCl–Tris 0·025 M; NaCl 0·22 M and 3 mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$. Small columns with 2 ml gel and 0·5 ml containing Fab or $F(ab')_2$ fragments were allowed to rotate end-over-end for 24 hr at 4°. Thereafter, the same buffer was applied to the columns, and after elimination of the protein not retained, the material bound to the Con A was eluted with 0·1 M α-methyl-D-mannoside in the same buffer.

RESULTS

Interaction of precipitating and non-precipitating antibodies with DNP–BSA and DNP–GABA–BSA

Three different techniques were employed to study the behaviour of the purified antibodies with the two haptenated proteins, with the following results.

Immunodiffusion tests. The results of Ouchterlony's tests made with rabbit- and sheep-purified antibodies indicated that precipitating antibody gave positive reactions with both antigens, while non-precipitating antibody gave positive reactions with DNP–GABA–BSA only. One example is presented in Fig. 1.

Complement fixation. As reported in Table 1, non-precipitating rabbit anti-DNP antibody fixed complement when reacted with DNP–GABA–BSA, but not with DNP–BSA. Precipitating antibody showed the same complement consumption with both antigens. Similar results were obtained with sheep antibodies (not shown in Table 1).

Inhibition test. The results obtained are presented in Fig. 2. It can be seen that precipitating antibody reacts identically with both antigens, indicating that the competition for the combining sites between DNP–BSA or DNP–GABA–BSA and the tracer DNP–BSA is the same. On the contrary, when non-precipitating antibody was tested, different curves were obtained with DNP–BSA and with DNP–GABA–BSA. The quantity of DNP–GABA–BSA necessary to displace the labelled tracer was much higher than the corresponding quantity of DNP–BSA.

Interaction of precipitating and non-precipitating antibodies and their fragments with antigen after treatment with endo-β-*N*-acetylglucosaminidase H

The various tests performed gave the following results.

Immunodiffusion tests. Non-precipitating antibody was unable to precipitate with the specific plurivalent antigen DNP–BSA. However, after treatment with the enzyme, precipitin arcs were formed in Ouchterlony's tests and in counter-immunoelectrophoresis. The same results were obtained with the $F(ab')_2$ fragment. The enzymatic treatment did not affect the reactions of precipitating antibody or its $F(ab')_2$ fragment. An example is shown in Fig. 3.

Passive haemagglutination. The results are reported in Table 2. Positive reactions were obtained with precipitating antibody and with its $F(ab')_2$ fragment in all cases. When whole non-precipitating antibody was tested, positive reactions were observed with sheep red cells but not with human red cells, as expected (Hajos *et al.*, 1978). After treatment with endoglycosidase, the non-precipitating antibody agglutinated both sheep and human sensitized cells. The $F(ab')_2$ fragment was unable to agglutinate the sensitized red cells but, after treatment with the enzyme, both sheep and human cells were agglutinated.

Complement fixation. In Table 3 are presented the results obtained in complement fixation tests at 50% lysis, when

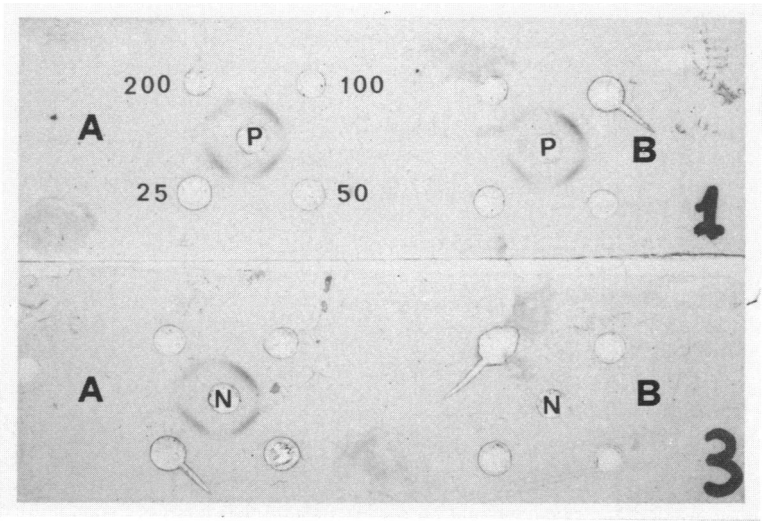

Figure 1. Ouchterlony's tests between precipitating (P) and non-precipitating (N) purified sheep anti-DNP antibodies and DNP–GABA–BSA (A) and DNP–BSA (B). Antibody concentration 1·6 mg/ml. The antigen concentration is indicated in μg/ml.

Table 1. Complement fixation by precipitating and non-precipitating purified rabbit anti-DNP antibodies using DNP–BSA and DNP–GABA–BSA

Tube	Precipitating antibody (μg)	Non-precipitating antibody	DNP–BSA (μg)	DNP–GABA–BSA (μg)	% lysis
1	20	—	1	—	0·02
2	20	—	—	1	0·01
3	—	20	1	—	45·0
4	—	20	—	1	0·07
5	—	—	1	—	45·4
6	—	—	—	1	43·8

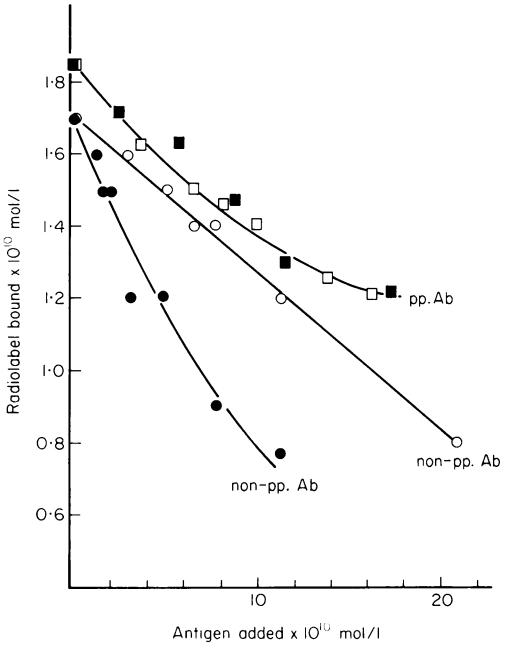

Figure 2. Competition between DNP–BSA or DNP–GABA–BSA and the labelled tracer DNP–BSA, for the combining sites of precipitating and non-precipitating anti-DNP antibodies. (■) and (●), DNP–BSA; (□) and (○), DNP–GABA–BSA.

precipitating and non-precipitating antibodies were reacted with DNP–BSA before and after enzymatic treatment. It is clear that the endoglycosidase has modified the behaviour of the non-precipitating antibody, which was then able to fix complement. It must be noted that the quantity of treated antibody added to each tube was four times more than the corresponding quantity of non-treated antibody.

Fluorescence quenching. The Scatchard plots obtained are presented in Fig. 4. Treatment with endoglycosidase did not produce any change in the affinity of the precipitating antibody or its $F(ab')_2$ fragment. On the contrary, the affinity of the non-precipitating antibody preparations was significantly increased.

Affinity chromatography on Con A–Sepharose. $F(ab')_2$ and Fab fragments obtained from precipitating and non-precipitating sheep-purified antibodies were chromatographed on Con A–Sepharose. The fragments obtained from precipitating antibody were not retained by the Con A. The $F(ab')_2$ fragment from non-precipitating antibody was totally retained and could be subsequently eluted with α-methyl-D-mannoside. When the Fab fragments from non-precipitating antibody were used, 51% of the protein was not retained by the column. The fraction that reacted with Con A could be eluted with the mannoside. These two fractions were tested against the ligand DNP–GABA by fluorescence quenching. The values of Ko obtained were $3{\cdot}4 \times 10^5$ M^{-1} for the fraction not retained by Con A, and

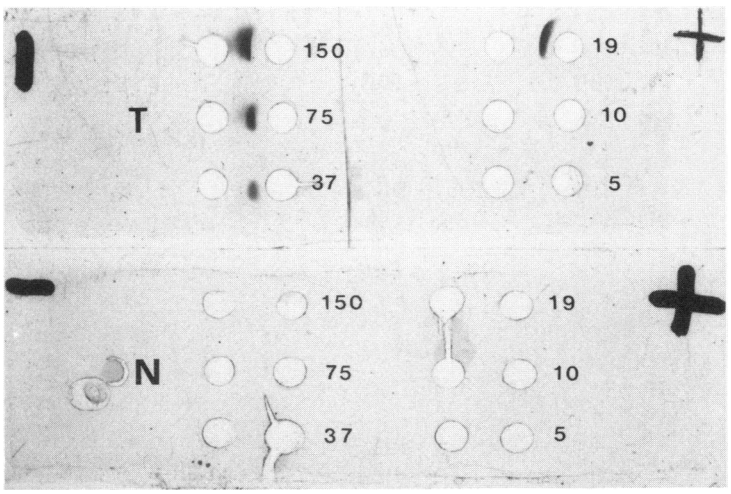

Figure 3. Counter-immunoelectrophoresis of $F(ab')_2$ fragments from sheep anti-DNP non-precipitating antibody, before (N) and after (T) treatment with endoglycosidase, using DNP–BSA as antigen. Antibody concentration 1·5 mg/ml. The antigen concentration is indicated in μg/ml.

Table 2. Agglutination of DNP-sensitized human and sheep red cells by precipitating and non-precipitating antibodies and their $F(ab')_2$ fragments, before and after treatment with endoglycosidase

	Haemagglutination							
	DNP-sheep red cells, antibody in well (μg)				DNP-human red cells, antibody in well (μg)			
	1·6	0·8	0·4	0·2	1·6	0·8	0·4	0·2
Whole pp. Ab	+	+	+	+	+	+	+	+
Whole pp. Ab enzyme-treated	+	+	+	+	+	+	+	+
Whole non-pp. Ab	+	+	+	−	−	−	−	−
Whole non-pp. Ab enzyme-treated	+	+	+	−	+	+	+	−
$F(ab')_2$ pp. Ab	+	+	+	+	+	+	+	+
$F(ab')_2$ pp. Ab enzyme-treated	+	+	+	+	+	+	+	+
$F(ab')_2$ non-pp. Ab	−	−	−	−	−	−	−	−
$F(ab')_2$ non-pp. Ab enzyme-treated	+	+	+	−	+	+	+	−

Table 3. Complement fixation, with DNP–BSA as antigen, of precipitating and non-precipitating purified anti-DNP sheep antibodies, before and after treatment with endoclycosidase

	Precipitating antibody		Non-precipitating antibody			
					DNP–BSA	
Tube	Untreated	Treated	Untreated	Treated	(μg)	% lysis
1	20	—	—	—	1	0·2
2	20	—	—	—	—	45·0
3	—	20	—	—	1	0·1
4	—	20	—	—	—	46·2
5	—	—	20	—	1	49·3
6	—	—	20	—	—	50·0
7	—	—	—	80	4	0·6
8	—	—	—	80	—	40·9
9	—	—	—	—	4	48·8

$0{\cdot}72 \times 10^4\ M^{-1}$ for the fraction retained. That means that the Fab fraction containing the carbohydrate residue has an affinity 50 times less than that of the fraction without carbohydrate.

DISCUSSION

The results of the various experiments reported in this paper indicate that the functional univalence of the molecule of non-precipitating antibody is due to a structural asymmetry of the molecule and, more precisely, to the presence of a carbohydrate residue in only one of the Fab regions. It can be assumed that this residue produces a steric hindrance that affects the combination of the corresponding antibody site with the antigen.

It was found that the reaction between antibodies of the non-precipitating type and their specific antigenic determinant is very much dependent of the position of the determinant in the antigenic molecule. The fact of interposing a short aliphatic chain spacer between the haptenic DNP group and the bulk of

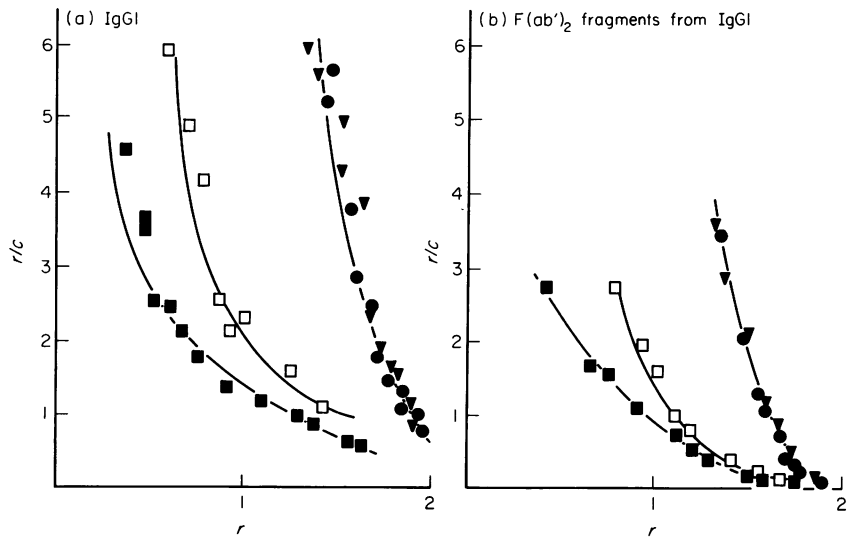

Figure 4. Interaction between DNP–GABA and sheep anti–DNP antibody and its $F(ab')_2$ fragment. Data obtained by fluorescence quenching. Precipitating antibody (▼); precipitating antibody treated with endoglycosidase (●); non-precipitating antibody (■); non-precipitating antibody treated with endoglycosidase (□).

the BSA molecule modified the accessibility of the hapten to the antibody site and allowed the formation of complexes able to precipitate and to fix complement. The DNP groups coupled directly to the lysine residues of the BSA may be unable to react with the antibody-combining site of low affinity because the BSA molecule cannot get close enough. On the other hand, when the DNP groups are further apart, as in the case of DNP–GABA–BSA, the access to the combining site may be possible. BSA may not approach the antibody site because of electrical repulsion or steric hindrance. A particular position of the hapten in one of the antigens cannot be invoked, since both antigens were highly substituted.

The inhibition test that we have performed showed that competition between the two antigens and the labelled tracer was not the same. The reason for this difference is probably not related to the slight difference in the substitution ratios of DNP, which are rather high in both antigens, but to the fact that DNP–BSA reacts with only one site while DNP–GAGA–BSA reacts with both sites. Consequently, more DNP–GABA–BSA than DNP–BSA should be necessary to displace the tracer, as was found.

When all this tests (immunodiffusion, complement fixation and radiobinding) were performed with precipitating antibodies, no differences were observed between DNP–BSA and DNP–GABA–BSA. It must be noted that in these experiments we have employed antibodies from two different animal species (rabbit and sheep) with the same results. We can, then, reasonably assume that we are dealing with a characteristic inherent to the non-precipitating antibodies in general.

It has previously been shown (Margni *et al.*, 1977; Ronco *et al.*, 1984) that the two sites of the non-precipitating antibody molecule have association constants the values of which differ by about two logs when measured with a small hapten. It is clear that, if the two Fab regions of the same molecule have a different configuration, there must be differences in their molecular configuration. One explanation of an asymmetrical molecule could be found in the presence and configuration of their carbohydrate residues, and it seemed interesting to study the behaviour of the asymmetric antibody after treatment with endoglycosidase. The results obtained demonstrate that the enzymatic treatment modifies the non-precipitating molecule in such a way that it becomes precipitant and behaves as such in the various tests performed. The haemagglutination test showed that precipitating antibody agglutinated both human and sheep sensitized red cells, while non-precipitating antibody agglutinated sheep but not human cells. This result confirms previous observations (Hajos *et al.*, 1978) demonstrating that univalent antibodies can agglutinate sensitized red cells of various animal species because their red cells have an Fc receptor. In that case, the antibody can attach to one red cell by its antibody-combining site, and to another red cell via the Fc receptor. Human red cells do not have the Fc receptor normally expressed, and therefore univalent non-precipitating antibodies do not agglutinate them. Obviously, no agglutination can be obtained in any case with the $F(ab')_2$ fragment of the univalent antibody. After treatment with endoglycosidase, it was found that non-precipitating antibody, as well as its $F(ab')_2$ fragment, agglutinated both human and sheep red cells, indicating the bivalence of the treated molecule.

The essential change induced by the enzyme was an increase in the affinity of the low affinity antibody site. This was clearly shown by studying the interaction of the treated antibody with the monovalent hapten DNP–GABA by fluorescence quenching. The Scatchard plots indicated a net increase in affinity (Fig. 4). Even if the enzyme produced a substantial modification, the treated non-precipitating antibody is not equal to the precipitating one. This may be due to incomplete digestion or to some structural feature produced by the acetylglucosamine remaining after digestion.

The results obtained by chromatography on Con A–Sepharose showed that half of the Fab fragments of the non-precipitating antibody were retained. This carbohydrate-containing Fab was shown to have an affinity much lower than that of the fragments not retained. The fact that all the $F(ab')_2$ fragments are retained by the Con A whereas only half of the Fab are, indicate that carbohydrate is present in only one of the Fab regions of each molecule. These results strongly support the hypothesis that the steric hindrance affecting the accessibility to the antibody site is exerted by the carbohydrate residue.

The existence of an asymmetrical attached oligosaccharide has been demonstrated in rabbit immunoglobulin G (Hinrichs & Smyth, 1970a, b). Altered glycosylation of the Fab region has also been reported in a dextran-binding mouse plasmocytoma (Matsuuchi, Wims & Morrison, 1981), in which case the carbohydrate moïety affected the reactivity with the antigen.

The present observations reveal some structural aspects of the molecule of non-precipitating antibody. The exact composition of the carbohydrate residue and its precise location on the antibody chains remain to be determined. It is also very important to explain the modalities of the synthesis of that asymmetric molecule and to understand its physiological role, since these non-precipitating antibodies, on account of their peculiar structure, behave as functionally univalent and are, therefore, unable to activate most effector mechanisms.

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