Immune Reactions in Polysaccharide Media

POLYSACCHARIDE-ENHANCED PRECIPITIN REACTIONS WITH ANTIGENS OF VARIOUS SIZES

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The influence of the size of the antigen in the polysaccharide-enhanced precipitin reaction was investigated. The experiments were carried out by addition of homologous antigens of different molecular sizes (the monomer, dimer and trimer of serum albumin and the monomer and dimer of immunoglobulin G) to the same preparation of antibody in the absence and presence of dextran. Dextran decreased the solubility of the immune complexes to a larger extent when the antigen size increased. This is in accord with the view that the polysaccharide effect is due to steric exclusion.

The enhancement by polysaccharides of the precipitin reaction between serum albumin and anti-albumin IgG* has been demonstrated in preceding papers (Hellsing, 1966, 1969a,c). The effect was ascribed to a steric exclusion of soluble antigen-antibody complexes from the polysaccharide domain.

The magnitude of the steric exclusion of a protein from a polysaccharide solution increases with the molecular size of the protein (Laurent, 1968). The present study was carried out in an attempt to verify that the enhancement of the precipitin reaction also increases with increasing size of the immune complexes. This was achieved by varying the size of the antigen. Monomer, dimer and trimer preparations of serum albumin and monomer and dimer preparations of IgG were used as antigens.

MATERIALS AND METHODS

Unless otherwise stated all experiments were carried out at 4° in 0.05 M-phosphate buffer (9.8 mM-KH₂PO₄-40.2 mM-Na₂HPO₄), pH 7.4, containing 0.1 M-NaCl.

Proteins. Human serum albumin (RdO 23) and human γ -globulin (RdO 38) were kindly supplied by AB Kabi, Stockholm, Sweden. Another batch of human serum albumin (RHA 04) was obtained from Behringwerke A.-G., Marburg/Lahn, Germany.

Polysaccharides. The dextran preparations and Sephadex G-200 were kindly supplied by AB Pharmacia, Uppsala, Sweden. The following dextran fractions were used: Dextran 10 (weight-average molecular weight, $\bar{M}_w$ 9400); Dextran 35 ($\bar{M}_w$ 35 000); Dextran 80 ($\bar{M}_w$ 80 000); Dextran 500 ($\bar{M}_w$ 500 000); Dextran 12 000 ($\bar{M}_w$ 12 000 000).

Antisera. Rabbit anti-(human IgG), anti-(human IgA)

and anti-(human IgM) and rabbit antiserum against human serum proteins were obtained from Behringwerke A.-G. Rabbit anti-(human albumin) serum, rabbit anti-(human γ -globulin) serum, guinea-pig anti-(rabbit IgG) serum and sheep antiserum against rabbit serum proteins were prepared in this laboratory (Hellsing, 1966, 1969a).

Gel chromatography. Sephadex G-200 was used for the chromatography experiments. The handling of the gel columns, the determination of their void volume and total volume as well as the calculation of the K_{av} value for a compound were as described by Hellsing (1968).

Zone electrophoresis. Preparative zone electrophoresis with cellulose powder as supporting medium was performed as described by Hellsing (1966).

Immunochemical methods. Immuno-electrophoresis and double diffusion in gel analyses were performed as described by Hellsing (1966). The single radial immunodiffusion technique of Mancini, Carbonara & Heremans (1965) was used for IgA analysis with a myeloma IgA as standard.

Ultrafiltration. The protein solutions were concentrated by ultrafiltration in collodion bags (Mies, 1953).

Protein determination. Determination of protein concentrations and radioactivities were carried out as described by Hellsing (1966, 1969a).

Determination of molecular weights. Sedimentation coefficients were determined in a Spinco model E ultracentrifuge operated with 12 mm. single-sector cells and a schlieren optical system at 59 780 rev./min. The measurements were made at 20° with the same buffers as for gel chromatography. The sedimentation coefficients were obtained after extrapolation to zero concentration and applying corrections for the viscosity and density of the solutions (Svedberg & Pedersen, 1940).

Apparent diffusion coefficients were estimated from the Stokes radius derived from the gel-chromatographic data (K_{av} values) given in Table 1 as proposed by Siegel & Monty (1966). The K_{av} value obtained for the monomer of albumin was used for standardization of the column, i.e. the determination of the parameter L in the equation of Laurent & Killander (1964). The parameter r_r was assigned

* Abbreviations: IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M (macroglobulin).

a value of 6×10^{-8} cm. in this equation (Öbrink, Laurent & Rigler, 1967). Molecular weights were calculated by using the Svedberg equation. Values for the partial specific volume were taken from Schultze & Heremans (1966).

Preparation of albumin monomer, dimer and trimer. Human serum albumin (Behringwerke A.-G.) was chromatographed in 1g. batches on a 5 cm. $\times$ 95 cm. column of Sephadex G-200 with 0.1 M-tris-HCl buffer, pH 8.0, containing 0.2 M- or 0.5 M-NaCl as eluent. The main peak containing the monomer gave on immunoelectrophoresis a single precipitin line with a rabbit antiserum to human serum.

Albumin (AB Kabi), which had been stored at 4° for about 3 years, was chromatographed in the same way and the material eluted before the monomer fraction was concentrated and stored at -30°. It was rechromatographed in approx. 500 mg. batches on the same column. Apart from material emerging close to the void volume, three larger peaks were obtained, the last two of which had K_{av} values corresponding to albumin dimer and monomer (Laurent & Killander, 1964). The dimer fractions were pooled, concentrated and rechromatographed on the same column. A single peak was obtained. After preparative zone electrophoresis no impurities were detected by immunoelectrophoresis.

The fractions from the Sephadex G-200 column preceding the dimer material were pooled, concentrated and rechromatographed on a 2 cm. $\times$ 113 cm. column of Sephadex G-200 in the phosphate buffer. Most of the material was eluted as one peak with a K_{av} value of 0.19. The material was concentrated to approx. 8 mg./ml. and analysed in the analytical ultracentrifuge. It showed a major component with a sedimentation coefficient, $S_{20,w}^0$, 8.1 s and a smaller slower-sedimenting contaminant (less than 5%). On immunoelectrophoresis the material showed a faint line in the region of β -globulin in addition to the dominating albumin line. From ultracentrifuge and gel-chromatographic data a molecular weight of 206 000 was derived, indicating that the isolated protein was a trimer of albumin. The content of dimer and trimer in the original albumin preparation from AB Kabi was estimated from u.v. data to be about 14% and 6% respectively.

Preparation of IgG monomer and dimer. Human γ -globulin (2g.) was chromatographed in two runs on the 5 cm. $\times$ 95 cm. column of Sephadex G-200. The chromatogram contained three peaks. The material in the second peak (14%) and the third peak (72%) was recovered and concentrated. After rechromatography on the same column each peak was analysed. On gel diffusion they showed no reaction against anti-IgM serum, but weak reactions against anti-IgA serum. The main precipitin line was produced by an anti-IgG serum. The content of IgA as determined by the single radial immunodiffusion technique of Mancini *et al.* (1965) corresponded to 6% and 2% respectively of the total protein contents in the second and third peaks.

Analytical ultracentrifugation of the second peak showed a main component having $S_{20,w}^0$ 9.7 s. A minor contaminant (9%) sedimented slower. The material in the third peak sedimented as a single component with $S_{20,w}^0$ of 6.2 s at a concentration of 9.9 mg./ml. The molecular weight, 301 000, estimated from ultracentrifuge and gel-chromatographic data suggested that the main component in the second peak was the dimer of IgG. It was concluded that the material in the third peak was the monomer of IgG.

Iodination of the compounds. The five different prepar-

Table 1. *Distribution of monomer, dimer and trimer in the iodinated antigen preparations*

The relative distributions of monomer, dimer and trimer were obtained by chromatography of the preparations on the 2 cm. $\times$ 113 cm. column of Sephadex G-200. The radioactivity of the eluate was plotted versus the elution volume and the elution diagram was resolved into symmetrical peaks. The distribution was estimated by measuring the area under each peak. The K_{av} value for each fraction is also tabulated.

Antigen preparation	Relative content (%)			K_{av} of main component
	Monomer	Dimer	Trimer	
Human serum albumin				
Monomer	97	3		0.45
Dimer	11	86	3	0.30
Trimer	14	12	74	0.19
Human IgG				
Monomer	99	1		0.29
Dimer	22	78		0.15

ations were labelled with ^{125}I by the method of McFarlane (1958), but with the preoxidation step omitted. The specific radioactivities of the iodinated compounds were 9, 19 and 20 $\mu\text{C}/\text{mg}$. for the monomer, dimer and trimer of serum albumin respectively and 15 and 17 $\mu\text{C}/\text{mg}$. for the monomer and dimer of IgG respectively. After dialysis against the phosphate buffer the preparations were stored at -30°. To establish the homogeneity of the samples they were subjected to gel chromatography in the phosphate buffer on the 2 cm. $\times$ 113 cm. column of Sephadex G-200 on the same day as they were used for the precipitin experiments. The approximate proportions of the monomer, dimer and trimer recorded by radioactivity measurements were estimated for each preparation from the chromatograms. The results (given in Table 1) indicate some conversion in the samples during the preparation procedure.

Preparation of antibody solutions. The IgG fractions from rabbit anti-albumin serum and rabbit anti- γ -globulin serum were prepared as described for anti-albumin IgG by Hellsing (1966). The same batches of anti-albumin IgG (10.7 mg./ml.) and anti- γ -globulin IgG (13.8 mg./ml.) were used throughout the precipitin experiments. The two batches gave on immunoelectrophoresis against a sheep anti-rabbit serum only one precipitin line, corresponding to IgG.

Precipitin reaction. The precipitin experiments were carried out in the phosphate buffer as described earlier (Hellsing, 1966, 1969a). Dextran was added to the following final concentrations: 40 mg. of Dextran 10/ml.; 40 mg. of Dextran 35/ml.; 10, 20 and 40 mg. of Dextran 80/ml.; 40 mg. of Dextran 500/ml.; 40 mg. of Dextran 12 000/ml. In control experiments where the antibody solution was replaced by human γ -globulin no precipitation occurred, even at the highest dextran concentration.

Free and antibody-bound albumin present in the supernatant fluids were separated and determined by two techniques, immunoelectrophoresis and $(\text{NH}_4)_2\text{SO}_4$ precipitation (Hellsing, 1969a). The results obtained by the two methods differed by 7-9% and by 21-24% when

albumin monomer and albumin trimer respectively were used as the antigen in the precipitin experiments. Controls showed, however, that the differences were due to salt precipitation of free albumin. Corrections were therefore made when the $(\text{NH}_4)_2\text{SO}_4$ precipitation technique was used.

RESULTS

The monomer, dimer and trimer preparations of human serum albumin and the monomer and dimer preparations of human IgG were used as antigens in the precipitin reactions. An increase in the molecular size of the antigen resulted, as expected, in an increased precipitation measured on a weight basis. The points of maximal precipitation also increased and corresponded to the addition of 21, 38 and 42 μg . of the monomer, dimer and trimer of albumin respectively and the addition of 20 and 27 μg . of the monomer and dimer of IgG respectively.

The experiments were also carried out in the presence of various dextran fractions (see above). In all experiments the addition of dextran increased the precipitation. The effect was most obvious in the region of antigen excess and increased with increasing molecular weight and concentration of the polysaccharide. A simultaneous shift of the point of maximal precipitation towards increased amounts of added antigen was also observed. The results agree with earlier observations by Helsing (1966, 1969a,c).

To compare the results obtained with antigens of different sizes, the data were recalculated on a molar basis. The molarities of the antigen solutions were calculated from the content of monomer, dimer and trimer (see Table 1) and the molecular

weights of albumin (69000) and IgG (156000) respectively (Schultze & Heremans, 1966).

The precipitin curves for albumin without added dextran were closely similar (Fig. 1). Maximal precipitation was obtained at 0.31 nmole for the monomer and dimer preparations, whereas the value for the trimer preparation was 0.29 nmole. The corresponding precipitin curves for IgG (Fig. 2) gave maximal precipitation at 0.135 and 0.115 nmole of added antigen in the monomer and dimer experiments respectively.

Fig. 2. Precipitin curves obtained with monomer (x) and dimer (o) preparations of IgG as antigens. Increasing amounts of ^{125}I -labelled IgG were mixed with 1.38 mg. of anti- γ -globulin IgG in a volume of 400 μl . The reactions were performed in the absence (—) or in the presence (---) of Dextran 500 (40 mg./ml.). The amount of IgG precipitated is shown on the ordinate. The values are expressed on a molar basis (see the text).

Fig. 1. Precipitin curves obtained with monomer (x), dimer (o) and trimer (Δ) preparations of serum albumin as antigens. Increasing amounts of ^{125}I -labelled albumin were mixed with 1.07 mg. of anti-albumin IgG in a volume of 400 μl . The reactions were performed in the absence (—) or in the presence (---) of Dextran 500 (40 mg./ml.). The amount of albumin precipitated is shown on the ordinate. The values are expressed on a molar basis (see the text).

Fig. 3. Decrease in the amount of soluble complexes in the precipitin reaction after addition of dextran. The supernatant fluids from the precipitin series with monomer (x) and trimer (Δ) of albumin (Fig. 1) were analysed for content of antibody-bound albumin. The ratio of the content found in the presence to that in the absence of dextran was plotted versus the amount of antigen added.

In the presence of Dextran 500 the enhancement increased with the size of the antigen even when calculated on a molar basis (Figs. 1 and 2). This effect was also demonstrated by analysing the supernatant solutions from the albumin monomer and trimer precipitin series for free and antibody-bound antigen. Dextran caused a decrease in the amount of soluble antigen-antibody complexes, and the relative decrease was larger in the trimer than in the monomer experiments (Fig. 3). In these, as in the previously reported experiments (Helsing, 1969c), there was a small increase in the amount of free antigen when dextran was added.

DISCUSSION

From observations of the effect of polysaccharides of different molecular weight, configuration and concentration (Helsing, 1966, 1969a) it was concluded that the enhancement of the precipitin reaction by polysaccharides is due to steric exclusion. Complex-formation between the polysaccharides and the immune reactants could be excluded (Helsing, 1969b).

The size of the antigen-antibody complexes should be an important parameter if the polysaccharide enhancement is a steric phenomenon: the larger the complex the greater the exclusion effect. However, it is difficult to investigate the influence of size of the immune complex on the antigen-antibody reaction without disturbing other parameters in the system.

Fundamentally the molecular size of the immune complexes will depend on (a) the size of the antigen, (b) the size of the antibody or (c) the ratio of antigen to antibody in the complex. Alternative (b) is of biological interest in view of the different molecular sizes of the various immunoglobulin classes, but it is difficult to investigate experimentally since every antibody preparation has its own intrinsic affinity for the antigen.

Alternative (c) was investigated indirectly in the preceding paper (Helsing, 1969c). It was shown that when dextran was added to a solution of soluble immune complexes the largest ones (i.e. those with a high ratio of antibody to antigen) were removed first.

The present experiments are based on alternative (a). To permit a comparison the experiments must be carried out with the same batch of antibody and the antigens must have identical antigenic sites. Although masking of some of the antigenic sites through polymerization of proteins cannot be excluded, it is reasonable to assume that most of the sites still are available and unchanged.

The control curves for the albumin monomer, dimer and trimer experiments, when recalculated on a molar basis, were nearly identical (Fig. 1),

facilitating a comparison of the effects of dextran in the separate albumin systems. Although the difference between the control curves for the experiments with the monomer and dimer of IgG was large (Fig. 2), it was possible to demonstrate clearly the difference in enhancement caused by dextran.

The enhancement by polysaccharides increased with antigen size (Figs. 1 and 2). As the antibody content of the soluble immune complex fraction was not determined, one cannot, however, definitely state that an increase in the size of the antigen was followed by an increase in the size of the soluble complexes and that this had caused the increase of the enhancement.

It is well known that immune precipitates of large antigens contain, on a molar basis, more antibodies than do those of small ones (Kabat & Mayer, 1961). As a result, one should obtain a larger withdrawal of antibodies from the supernatants in the experiments with albumin polymer than those with monomer, since the amounts of antigen in the precipitates are almost identical when calculated on a molar basis. The supernatant fluids in the dimer and trimer experiments should therefore have lower antibody/antigen ratios than in the monomer experiments. A decrease in this ratio is paralleled by a decrease in the size of the soluble immune complexes (Helsing, 1969c). Nevertheless there is a larger enhancement by polysaccharides in the albumin polymer experiments than in the monomer experiments. Had it been feasible to maintain identical antibody concentrations in the supernatant solutions in the different antigen systems, the difference in enhancement might have been even larger.

Although it has not been possible to determine the size of the soluble immune complexes themselves it is striking that the enhancement by polysaccharides increases with increasing antigen size. It is reasonable to interpret this phenomenon in terms of a steric exclusion of immune complexes of increasing size.

These findings are of interest from a pathological point of view since it is known that the deposition of soluble antigen-antibody complexes (prepared *in vitro*) in vessel walls is related to the size of the immune complexes rather than to their net charge or any specific affinity between complexes and structures of vessel walls (Cochrane & Hawkins, 1968).

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