Immune Reactions in Polysaccharide Media

INVESTIGATION ON COMPLEX-FORMATION BETWEEN SOME POLYSACCHARIDES, ALBUMIN AND IMMUNOGLOBULIN G

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Serum albumin and immunoglobulin G were chromatographed on columns of dextran, hyaluronate and chondroitin 4-sulphate. The partition of the two proteins between hyaluronate and buffer was also measured by equilibrium dialysis. The results accord with the view that there is no complex-formation between the polysaccharides and the proteins in 0.05M-phosphate buffer, pH 7.4, containing sodium chloride (0.1M). The observations support the hypothesis that the previously described polysaccharide enhancement of the precipitin reaction is due to exclusion and not to non-specific complex-formation.

Neutral polysaccharides such as dextran and Ficoll, and charged polysaccharides such as hyaluronate and chondroitin sulphate, increase the precipitation of antigen-antibody complexes (Hellsing, 1966, 1969). This effect has been explained as a steric exclusion of the complexes from the domains of the polysaccharides.

Complex-formation between serum proteins and polyanions, especially polysaccharides found in connective tissue, has been studied by many authors, and the results have been reviewed (Bettelheim-Jevons, 1958; Bernfeld, 1966; Scott, 1968). Proteins and polyanions form precipitates at low ionic strength and at pH values below the isoelectric points of the proteins. The existence of soluble protein-polysaccharide complexes has, however, been demonstrated even above the isoelectric point.

The present investigation was performed to determine whether non-specific complex-formation between the immunochemical reactants and the polysaccharides occurs at the pH and ionic strength used in the immunological experiments.

MATERIALS AND METHODS

All experiments were carried out at 4° in 0.05M-phosphate buffer, pH 7.4 (9.8mM-KH₂PO₄-40.2mM-Na₂HPO₄), containing NaCl (0.1M) (I/O-23), unless otherwise stated.

Proteins. Human serum albumin (RdO 54), human γ -globulin (RdO 38) and human transferrin (RfE 58) were kindly supplied by AB Kabi, Stockholm, Sweden. They were used without further purification for chromatography on Sephadex G-200 and gel diffusion and as carrier substances in equilibrium-dialysis experiments. Purified serum albumin, human IgG* and rabbit IgG, the last with anti-

albumin activity, were prepared and labelled with ¹²⁵I (Hellsing, 1966). Their radioactivity was measured as described in the preceding paper (Hellsing, 1969). Horse myoglobin was a gift from Professor A. Ehrenberg. Egg albumin was obtained from Baker and Adamson, Morristown, N.J., U.S.A. The concentrations of the last two proteins were determined by the Folin reaction (Eggstein & Kreutz, 1955).

Carbohydrates. Glucose, sucrose and raffinose were analytical-grade commercial preparations. Dextran preparations as used earlier were employed (Hellsing, 1968), and also a homogeneous dextran fraction with mol.wt. 14600 (fraction D-6 prepared by Laurent & Granath, 1967). An anthrone method was used for determinations of the concentrations of the neutral sugars (Scott & Melvin, 1953).

Hyaluronate was prepared from rooster comb by the method of Scott (1956), essentially as described by Laurent (1957). The two preparations used had the following characteristics. Preparation A had 43.1% hexuronic acid, 43.1% hexosamine and 3.80% total N; its mol.wt., from viscosity measurements, was 1.1×10^6 . Preparation B had 42.3% hexuronic acid, 41.4% hexosamine and 3.90% total N; its mol.wt. was 1.2×10^6 . The analytical techniques are referred to in the preceding paper (Hellsing, 1969). Hyaluronate concentrations were determined by the carbazole method (Galambos, 1967).

Equilibrium dialysis. The experiments were performed in dialysis cells essentially as described by Ogston & Phelps (1960) and Laurent (1964), except that the depth of each cell compartment was only 4mm. and the volume 1.2ml. MF-Millipore filters, type VC (Millipore Corp., Bedford, Mass., U.S.A.), with mean pore size 100nm., were used. Isotope-labelled proteins with initial concentrations 0.15-0.30mg./ml. were equilibrated between a hyaluronate solution (preparation B) and buffer. Since at these low concentrations the proteins were adsorbed on the filter, the concentrations were increased to 10mg./ml. by addition of unlabelled albumin or γ -globulin. The concentration of hyaluronate was determined in both compartments after the termination of the experiments. Only those experiments

* Abbreviation: IgG, immunoglobulin G.

showing less than 5% of the hyaluronate in the buffer compartment were accepted.

Chromatography on Sephadex G-200. A column (2 cm. $\times$ 115 cm.) was packed and operated by reverse flow (Hellsing, 1968). The following samples, each dissolved in 1 ml., were applied separately or in mixtures to the column: 50 mg. of human serum albumin; 30 mg. of human γ -globulin; 10 mg. of the dextran fraction with mol.wt. 14600. The elution was performed with buffer alone or with a buffer to which Dextran 35 or Dextran 500 had been added to give concentrations of 0.25 or 1 mg./ml.

The void volume, the total volume of the column and the elution volumes of the proteins were recorded as described by Hellsing (1968).

Chromatography on a hyaluronate gel. Preparation A of hyaluronate (concentration 0.125 g./ml.) was cross-linked with 1,2:3,4-diepoxybutane (Fluka A.-G., Buchs SG, Switzerland) as described by Laurent, Hellsing & Gelotte (1964). The swollen gel was disintegrated in a gel press (Hjertén, 1962) and then transferred into 0.05 M-tris-HCl buffer, pH 8.5, containing NaCl (0.1 M). The polymer concentration of the gel grains suspended in the buffer was calculated from dry weight and ash determinations (Laurent, 1964), giving 36 mg./ml. The gel was packed into columns (1 cm. $\times$ 25 cm.) under a hydrostatic pressure of 15 cm. The same pressure was used during subsequent elutions of the columns and gave a flow rate of 2–3 ml./hr. Chromatographic experiments were performed in the tris-HCl buffer, in the standard phosphate buffer and in 19 mM-phosphate buffer, pH 7.4 (3.7 mM-KH₂PO₄–15.3 mM-Na₂HPO₄), containing NaCl (0.1 M) (I 0.15). The following materials were applied to the column in 0.15 ml. samples: 0.12 mg. of Dextran 12000 and 0.12 μ C of ³H₂O (for determination of the void volume and the total volume, respectively, of the columns); 0.19 mg. of ¹²⁵I-labelled rabbit IgG; 0.16 mg. of ¹²⁵I-labelled human serum albumin; 1 mg. of egg albumin; 1.8 mg. of myoglobin; 3.0 mg. of raffinose; 3.0 mg. of sucrose; 3.0 mg. of glucose. Fractions (0.5–1 ml.) were collected.

Chromatography on a chondroitin 4-sulphate gel. The experiments were performed with the gel used by Bettelheim, Laurent & Pertoft (1966). It was packed in a column (2 cm. $\times$ 25 cm.) equilibrated with 0.1 M-phosphate buffer, pH 5.0 (98.8 mM-KH₂PO₄–1.2 mM-Na₂HPO₄), and eluted

by reverse flow at a rate of 5 ml./hr. Rabbit serum with antibody activity against human transferrin (prepared as described for anti-albumin serum by Hellsing, 1966) was dialysed against the same buffer and 0.5 ml. was applied to the column. Fractions (2.5 ml.) were collected and analysed for pH and u.v. absorption at 280 nm. The protein fractions in the eluate were concentrated in collodion bags (Mies, 1953) to volumes of 0.5 ml. or less and were then analysed by gel electrophoresis, immunoelectrophoresis and gel diffusion (Hellsing, 1966). Anti-(rabbit serum) and anti-(rabbit IgG) prepared in sheep and guinea pig were used for the immunoelectrophoretic and gel-diffusion analyses (Hellsing, 1969). The anti-transferrin activity of the rabbit serum was tested by gel diffusion against serial dilutions of human transferrin.

RESULTS

Chromatography on Sephadex G-200. Serum albumin and γ -globulin (mainly IgG) were chromatographed on Sephadex G-200 separately or mixed with a low-molecular-weight fraction of dextran (D-6) (Expts. 1–3 in Table 1). There was no indication of complex-formation between the proteins and the dextran fraction, as each substance was eluted at the same place (K_{av} value) in the chromatogram, both when run alone and when mixed.

In a different type of experiment the two proteins were chromatographed with eluents containing low concentrations of dextran (Expts. 4–7 in Table 1). The K_{av} values of the proteins did not decrease in the presence of dextrans with molecular weights of 35 000 (Dextran 35) and 420 000 (Dextran 500), in support of the view that a complex had not been formed between the components.

Equilibrium dialysis. The partition of ¹²⁵I-labelled serum albumin and IgG between a hyaluronate phase and a pure-buffer phase is shown in Fig. 1. The concentrations of the proteins were always higher on the buffer side, indicating that

Table 1. *Chromatography of human serum albumin and human γ -globulin, separately or mixed, with a homogeneous dextran fraction (D-6) on Sephadex G-200 in 0.05 M-phosphate buffer, pH 7.4, containing sodium chloride (0.1 M)*

Dextran (35 or 500) was added to the eluent in Expts. 4–7. The coefficient for the partition of the various compounds between the gel and liquid phases on the column, K_{av} , was calculated as described by Hellsing (1968).

Expt. no.	Dextran added to eluent	Concn. of dextran (mg./ml.)	K_{av} (serum albumin)	K_{av} (IgG)	K_{av} (Dextran D-6)
1	—	—	0.47	0.29	
2	—	—			0.60
3	—	—	0.48	0.30	0.60
4	Dextran 35	1	0.48		
5	Dextran 35	0.25	0.47		
6	Dextran 500	1	0.48	0.30	
7	Dextran 500	0.25	0.48	0.29

Fig. 1. Equilibration of ^{125}I -labelled proteins between a hyaluronate solution and buffer. The ratio C'/C'' , where C' is the protein concentration in the hyaluronate compartment and C'' is the protein concentration in the buffer compartment of the dialysis cell is plotted against the hyaluronate concentration inside the dialysis bag. $\times$, Human serum albumin; $\circ$, human IgG; $\blacktriangle$, rabbit IgG; all in 0.05M-phosphate buffer, pH 7.4, containing NaCl (0.1M) (I0-23). $\blacksquare$, Human serum albumin in veronal buffer, pH 8.6 (I0-1). For details see the text.

the main interaction is an exclusion of proteins by hyaluronate. The results are qualitatively similar to those obtained by Laurent (1964) at higher pH or ionic strength, but quantitatively the effect exerted by hyaluronate is larger in the present experiments. Although the behaviour of rabbit IgG deviated from that of human IgG, the deviation was not very large.

Veronal buffer, pH 8.6 (50mM-sodium diethylbarbiturate-5mM-diethylbarbituric acid-50mM-sodium acetate), was used in one series of experiments instead of phosphate buffer without influencing the results (Fig. 1).

Chromatography on the hyaluronate gel. The ^{125}I -labelled proteins, when chromatographed in 0.05M-tris-hydrochloric acid buffer, pH 8.5, containing sodium chloride (0.1M), or in the standard phosphate buffer, were eluted in almost symmetrical peaks near the void volume of the column with a yield of 92–106%. Human albumin and rabbit IgG therefore seem to be almost completely excluded from the hyaluronate gel and cannot have been adsorbed.

To define the exclusion properties of the hyaluronate gel, a number of lower-molecular-weight compounds were also chromatographed in the phosphate buffer (3.7mM- KH_2PO_4 -15.3mM- Na_2HPO_4 -0.1M-NaCl) of I0-15. The partition coefficients, K_{av} , calculated for the various substances were plotted versus their Stokes radii

Fig. 2. Various substances were chromatographed on a column (1 cm. $\times$ 25 cm.) of hyaluronate gel (36 mg./ml.), and eluted with 19mM-phosphate buffer, pH 7.4, containing NaCl (0.1M). The partition coefficient, K_{av} , for each substance was plotted against its Stokes radius (taken from Laurent, 1964). 1, Glucose; 2, sucrose; 3, raffinose; 4, myoglobulin; 5, ovalbumin; 6, human serum albumin; 7, rabbit IgG. The line, drawn by the relationship established by Laurent (1964), gives the exclusion from a three-dimensional random network of hyaluronate chains.

(Fig. 2). Laurent (1964), assuming the partition to be due to steric exclusion, calculated the theoretical relationship between the partition coefficient and the Stokes radius. There is good agreement between the experimental values obtained in this investigation and values calculated by Laurent's (1964) method for the gel having a concentration of 36 mg./ml.

Chromatography on the chondroitin 4-sulphate gel. The following experiment was originally designed to define the ionic conditions under which different plasma proteins are bound to chondroitin sulphate.

Anti-(human transferrin) serum from rabbits was applied to the chondroitin sulphate column in 0.1M-phosphate buffer, pH 5.0, and most of the proteins (92%) were adsorbed on the column. They were recovered by stepwise elution with 100–230ml. portions of 0.1M-phosphate buffer, pH 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. Each time the protein elution occurred at the point of pH rise. About 70% of the proteins were eluted at pH 5.5 and most of the remainder at pH 6.0 and 6.5. These fractions contained all the serum albumin, γ -globulin and anti-transferrin activity that could be recovered. Even at pH 7.0 (where the buffer had I0-22) a small u.v.-absorbing fraction was eluted. Gel electrophoresis, immunoelectrophoresis and gel diffusion revealed the presence of α - and β -globulin but no γ -globulin or anti-transferrin activity. At pH 7.5

further traces of u.v.-absorbing material were obtained. This could not be identified by gel electrophoresis, immunoelectrophoresis or gel diffusion. No material was eluted at pH 8.0.

The experiment suggests that at pH 7.4 and I₀23 complexes are not formed between chondroitin 4-sulphate and serum albumin or IgG.

DISCUSSION

Most earlier investigations support the assumption that no complexes are formed between the polysaccharides and the immunochemical reactants used in the preceding precipitin experiments (Hellsing, 1966, 1969). However, claims have been made for complex-formation under conditions similar to those used in these experiments.

Dextran. Dextran decreases the plasma concentration of several serum proteins *in vivo*, e.g. fibrinogen and lipoproteins (Abildgaard & Skjörten, 1968; Morrison, 1956). This was ascribed by some authors to complex-formation between different proteins and dextran (Ricketts, 1952; Fletcher, Martin & Ratcliffe, 1952; Melik-Sarkisyan & Rosenfeld, 1957; Stroër, 1967). However, Laurent (1963), Iverius & Laurent (1967) and Iverius (1968) have, on the basis of experiments *in vitro*, discussed the lowering in plasma concentration of the proteins in terms of a steric exclusion phenomenon. Ponder & Ponder (1960) studied the interaction between albumin and dextran during free electrophoresis at physiological ionic strength and pH 6.6–9.6; they claimed that a complex was formed between one albumin molecule and four dextran molecules. This was not observed by others (Melik-Sarkisyan & Rosenfeld, 1957; Noguchi, 1961). A frictional interaction, proposed earlier from experiments on a hyaluronate–albumin system (Davies, Nichol & Ogston, 1963), could also explain the results obtained by Ponder & Ponder (1960).

The chromatographic experiments reported in this paper were carried out on a dextran gel, Sephadex G-200. The fact that serum proteins are eluted according to their molecular size when chromatographed on dextran gels does not favour the idea that they form complexes with dextran (Laurent, Öbrink, Hellsing & Wasteson, 1969). It does not, however, exclude the possibility of binding between soluble dextran molecules and the proteins.

In the presence of Dextran 500 at 1–4% (w/v) concentration, albumin was eluted later from a Sephadex G-200 gel than when the experiment was conducted in the absence of the polysaccharide (Hellsing, 1968). This phenomenon is due to an exclusion effect and may make it impossible to detect complex-formation, which should decrease the elution volume. The present experi-

ments were therefore carried out at much lower dextran concentrations (0.025–0.1%), where the exclusion effect is small. There were, however, no indications of earlier elution (lower K_{av} values), and thus of complex-formation, at these concentrations (Table 1). A range of molecular weights of dextran between 14600 and 420000 was used. This excludes the possibility that complex-formation depends on the degree of polymerization of the polysaccharide.

The results obtained support the conclusion, based on experiments with [¹⁴C]dextran (Hellsing, 1966), that the polysaccharide is not bound to the immunochemical reactants in the precipitin reaction between serum albumin and anti-albumin.

Hyaluronate. The interaction between hyaluronate and various proteins is probably of steric nature at the ionic strength and pH at which the present investigations were carried out (Ogston & Phelps, 1960; Laurent, 1964; Laurent & Pietruszkiewicz, 1961; Laurent, Björk, Pietruszkiewicz & Persson, 1963), although contrary evidence was produced by Pigman, Gramling & Holley (1961) and Gramling, Niedermeier, Holley & Pigman (1963). In free-electrophoretic runs of bovine serum albumin and hyaluronate in veronal buffer, pH 8.6 and I₀1 Gramling *et al.* (1963) found an extra boundary and claimed that this consisted of a stable complex between hyaluronate and albumin. The complex was difficult to demonstrate in phosphate buffer (Pigman *et al.* 1961) and veronal buffer was presumably required for its formation (Niedermeier, Gramling & Pigman, 1966). Davies *et al.* (1963) suggested, however, that the extra boundary could arise from a frictional effect rather than from complex-formation. Strong objections to this supposition were raised by Niedermeier *et al.* (1966).

The equilibrium-dialysis experiments performed here confirm the conclusion made by Ogston & Phelps (1960) and Laurent (1964) that the main interaction between hyaluronate and serum albumin or IgG is a steric exclusion. This is also the case in veronal buffer, in contradiction of the hypothesis by Pigman and co-workers of complex-formation. The present partition values (C'/C'') are lower than those found by Laurent (1964), and no explanation was found for this fact, but it cannot invalidate the main conclusion. Further strong evidence against complex-formation is provided by the results of chromatography of serum albumin and IgG on the hyaluronate column.

As the hyaluronate gel used in the present study was more concentrated than that used by Laurent (1964), an opportunity was available to test the validity of the steric-exclusion hypothesis at a hyaluronate concentration not used earlier. Fig. 2 shows that the present results are in good agree-

ment with the view that solutes are excluded from a three-dimensional random network of linear hyaluronate chains. These experiments were carried out at physiological pH and ionic strength, unlike earlier work.

Chondroitin 4-sulphate. Gerber & Schubert (1964) demonstrated that the partition of bovine serum albumin between solutions of chondroitin sulphate-protein complex and buffer above a salt concentration of 0.15M and within a pH range of 5–10 was due essentially to exclusion. Bettelheim *et al.* (1966), using electrophoretic, ultracentrifugal and chromatographic techniques, concluded that under physiological conditions no complex-formation took place between the polysaccharide and serum albumin. An indication that the chondroitin sulphate-protein complex and human IgG do not form a complex in the standard phosphate buffer was obtained in the preceding paper (Hellsing, 1969), in which the two compounds were chromatographed together on Sephadex G-200.

The present experiments substantiate the conclusion that chondroitin 4-sulphate and the two immunochemical reactants do not form complexes in the precipitin reaction.

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