Biochem. J. (1969) **112**, 475
Printed in Great Britain

Immune Reactions in Polysaccharide Media

THE EFFECT OF HYALURONATE, CHONDROITIN SULPHATE AND CHONDROITIN SULPHATE–PROTEIN COMPLEX ON THE PRECIPITIN REACTION

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(*Received* 23 *September* 1968)

The influence of the connective-tissue polysaccharides hyaluronate, chondroitin 4-sulphate and a chondroitin 4-sulphate–protein complex (PP-L) from cartilage on the precipitin reaction was investigated. In a system consisting of ^{125}I-labelled human serum albumin and the immunoglobulin G fraction from rabbit anti-albumin sera, the precipitation is greatly increased in the region of antigen excess. This effect depends on the concentration, molecular weight and configuration of the polysaccharide. The increase parallels a decrease in the amount of soluble immune complexes in the supernatant. It is suggested that the effect is due to steric exclusion of the complexes from the domains of the polysaccharides. The possibility that such a mechanism might enhance precipitation of antigen–antibody complexes in certain pathological conditions is discussed.

The polysaccharide dextran has been found to increase the precipitation of soluble albumin–anti-albumin complexes (Hellsing, 1966). The increased precipitation depended on the concentration and the molecular weight of the dextran. It was suggested that the effect was due to steric exclusion of the immune complexes from the domain of the polysaccharide. Since polysaccharides may play a role in immune reactions *in vivo*, the effect of some connective-tissue polysaccharides, namely hyaluronate, chondroitin 4-sulphate and a chondroitin 4-sulphate–protein complex, was investigated. It is shown below that these polysaccharides all have a pronounced effect on the precipitation of immune complexes.

MATERIALS AND METHODS

Antigen. Human serum albumin (RdO 56) was kindly supplied by AB Kabi, Stockholm, Sweden. It was further purified and labelled with ^{125}I (AB Atomenergi, Studsvik, Sweden) as described by Hellsing (1966) to yield a product of specific radioactivity about 12 μc/mg.

Antibody fractions. The IgG* fraction from rabbit anti-(human albumin) sera was prepared as described by Hellsing (1966). The same batch was used in most of the experiments. Immunoelectrophoresis of this material against guinea-pig anti-(rabbit IgG) showed three parallel bands (Hellsing, 1966) whereas only one could be seen after electrophoresis against sheep anti-(rabbit serum).

A batch of anti-albumin was prepared by an immunosorbent technique (K. Hellsing, unpublished work) and was labelled with ^{125}I. The final solution contained 0·92 mg./ml. and approx. 3 μc/mg. A precipitin curve was determined by the addition of 0·5–39 μg. of unlabelled human serum albumin to 100 μl. of this solution in a final volume of 400 μl.; maximal precipitation, corresponding to 52% of the labelled antibody, was obtained on the addition of 8 μg. of albumin. The antibody was, however, more than 95% reactive in gel-electrophoresis experiments (K. Hellsing, unpublished work).

Human γ-globulin. Human γ-globulin (RdO 38) was kindly supplied by AB Kabi, Stockholm, Sweden. It contained polymers of IgG and IgA, but was used without further purification.

Testicular hyaluronidase. Testicular hyaluronidase (14000 i.u./mg.) was kindly supplied by AB Leo, Hälsingborg, Sweden.

Polysaccharides. Hyaluronate was isolated from extracts of rooster comb mainly by the technique described for umbilical cord by Blix & Snellman (1945). Two samples were used with weight-average mol.wt. $3{\cdot}6 \times 10^6$ and 4×10^5 respectively, determined from viscosity measurements (Laurent, Ryan & Pietruszkiewicz, 1960). The latter preparation, which was used in most of the experiments, contained 42·1% hexuronic acid, 40·4% hexosamine and 3·39% total N.

A polysaccharide–protein complex (PP-L) from bovine nasal cartilage was prepared as described by Schubert and co-workers (Malavista & Schubert, 1958; Gerber, Franklin & Schubert, 1960). The preparation contained 24·3% hexuronic acid, 23·0% hexosamine, 4·36% total N and 4·19% S.

Chondroitin 4-sulphate was prepared from bovine nasal cartilage by the method described by Scott (1960) by using papain digestion and cetylpyridinium chloride precipitation. The preparation contained 31·7% hexuronic acid,

* Abbreviations: IgG, immunoglobulin G; IgA, immunoglobulin A; PP-L, 'polysaccharide–protein complex, light fraction' (chondroitin 4-sulphate–protein complex).

32·3% hexosamine, 3·2% total N and 5·4% S. It had average mol.wt. 25×10^3, determined by gel chromatography and analytical ultracentrifugation (Wasteson, 1969).

Dextran preparations were kindly supplied by Pharmacia AB, Uppsala, Sweden. They had the following weight-average mol.wt.: 35000 (Dextran 35), 420000 (Dextran 500) and 12000000 (Dextran 12000).

Immunological techniques. Gel diffusion (Ouchterlony, 1948) and immunoelectrophoresis (Scheidegger, 1955) were performed as described by Hellsing (1966). The following antisera were used: rabbit antiserum against human serum proteins, obtained from Behringwerke A.-G., Marburg/Lahn, Germany; guinea-pig antiserum against rabbit IgG (Hellsing, 1966); sheep antiserum against rabbit serum proteins. The antiserum to rabbit serum was prepared by injecting a sheep intramuscularly in the forelegs with 0·5 ml. of normal rabbit serum emulsified in 2 ml. of complete Freund's adjuvant (Freund & McDermott, 1942), followed 2 weeks later by equal-sized injections in the hindlegs; antiserum was collected 2 months after the last injection by puncture of the jugular vein.

Before use all solutions were dialysed against two changes of 0·05 M-phosphate buffer, pH 7·4 (9·8 mM-KH_2PO_4–40·2 mM-Na_2HPO_4), containing NaCl (0·1 M), and were then centrifuged at 80000*g* for 20 min. All reactions were performed in this buffer unless otherwise stated. The polysaccharides were dissolved in the buffer by stirring for 12 hr.

The precipitin reaction was carried out in the following manner: 100 μl. of antibody solution (10·7 mg./ml.) and 100 μl. of a solution of labelled albumin (0·1–3·17 mg./ml.) were added to 200 μl. of a solution containing polysaccharide or other materials or both. The test tubes were incubated at 37° for 60 min., and at 4° for 5–7 days before the radioactivity in the supernatant and precipitate was measured. Other details of the technique were identical with those described by Hellsing (1966).

After complete precipitation the supernatants in the region of antigen excess were analysed for their content of free albumin and antibody-bound albumin by two methods.

(*a*) Radioactivity measurements after separation of the components by immunoelectrophoresis as described by Hellsing (1966). This technique was not applicable in the presence of hyaluronate unless the polysaccharide had been enzymically degraded. (*b*) Precipitation of the soluble complexes by addition of an equal volume of saturated $(NH_4)_2SO_4$ at 4° (Farr, 1958). The samples were kept for 30 min. at 4° before the radioactivity in the supernatant and precipitate was measured. This technique could also be used when hyaluronate was present. Both methods gave reproducible results, but with the immunoelectrophoretic method values 6–8% higher for free albumin were obtained. The discrepancy was probably due to the precipitation of a small amount of albumin by $(NH_4)_2SO_4$, in accordance with the results of Farr (1958). Addition of polysaccharide did not affect this discrepancy.

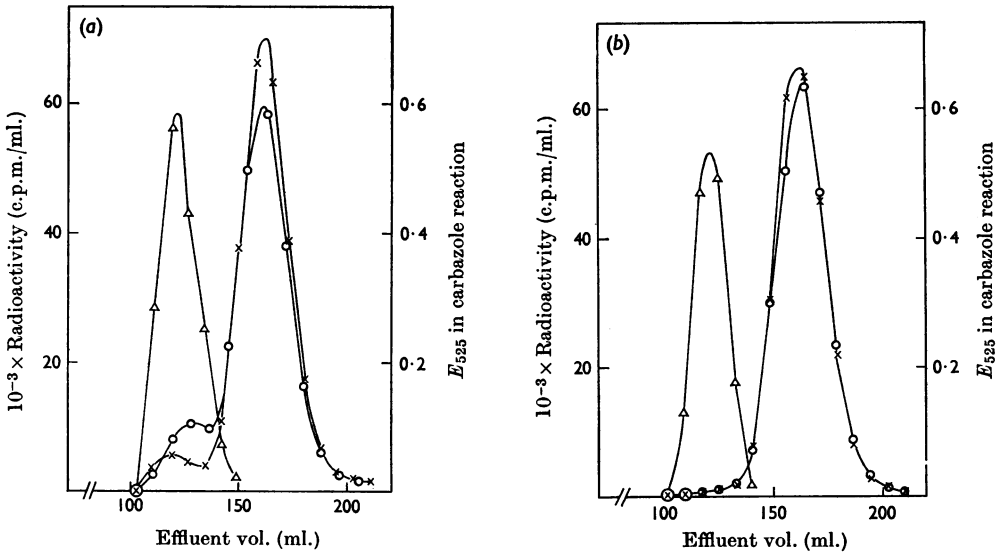

Fig. 1. Interaction of ^{125}I-labelled IgG with PP-L studied by gel chromatography on Sephadex G-200. A column (2 cm. × 108 cm.) was operated as described by Hellsing (1968). Samples (0·5–0·8 ml.) containing either (*a*) 0·5 mg. of labelled rabbit anti-albumin IgG or (*b*) 0·5 mg. of labelled human IgG, alone or together with 5 mg. of PP-L, were applied to the column, followed by a small volume of 2 M-NaCl to avoid convection disturbances. The reversed-flow elution was then performed with the phosphate buffer (9·8 mM-KH_2PO_4–40·2 mM-Na_2HPO_4–0·1 M-NaCl, pH 7·4) at a flow rate of 10 ml./hr. Fractions (7 ml.) were collected. The radioactivity of IgG chromatographed with PP-L (○) and alone (×) was measured in 1 ml. samples and plotted versus the elution volume. PP-L (△) was assayed by the carbazole reaction.

Immunochemical characterization of the polysaccharides. Since it was essential to eliminate the possibility of a reaction between the anti-albumin preparation and the polysaccharides, this possibility was tested by gel diffusion. A faint band was seen between the anti-albumin and the PP-L wells containing more than 7mg./ml., indicating the presence of small amounts of albumin in the PP-L preparation. No precipitin bands were observed between anti-albumin and the other polysaccharide preparations over a wide range of concentrations.

Different approaches were used to characterize and quantitatively determine the reacting component in PP-L. A 200mg. sample of PP-L was subjected to gel chromatography on a column (5cm.×94·5cm.) of Sephadex G-200, which was eluted with 0·1M-tris–HCl buffer, pH8·0, containing NaCl (0·5M). Fractions (160ml.) were analysed against the anti-albumin preparation in gel-diffusion experiments after concentration to 5ml. by ultrafiltration (Mies, 1953). The component reacting as albumin emerged at the tail end of the broad asymmetric polysaccharide–protein peak, at $K_{av.}$ 0·32–0·44 (Hellsing, 1968). Human serum albumin was eluted with $K_{av.}$ 0·38 on this column.

When ^{125}I-labelled anti-albumin was chromatographed on Sephadex G-200 alone and mixed with PP-L about 3% of the anti-albumin fraction was eluted earlier (Fig. 1*a*), indicating that it had been bound to the reacting component. When the same experiments were performed with isotope-labelled purified human IgG (K. Hellsing, unpublished work) instead of anti-albumin, no such effect could be observed (Fig. 1*b*).

The effect of the albumin-like component in precipitin experiments was investigated by adding 92μg. of labelled anti-albumin to undegraded and hyaluronidase-degraded PP-L in a final volume of 400μl. No precipitation could be detected over the range 9–2100μg. of PP-L.

These experiments showed that the PP-L preparation was contaminated with bovine serum albumin, which cross-reacted with anti-(human albumin). In the main precipitin series a maximum of 5·6mg. of PP-L was used, which was calculated to bind, at the most, 5% of the anti-albumin molecules. Since it is known that the reactivity of an antibody towards a cross-reacting antigen should be less than that towards the specific one, it may be concluded that the binding of anti-albumin molecules to the PP-L preparation should not influence the results to any great extent. The presence of bovine serum proteins in PP-L preparations has been observed previously (Di Ferrante, 1968).

Measurement of radioactivity. This was carried out in a Packard model 3003 Tri-Carb liquid-scintillation system with a well-type crystal detector. A standard curve for the ^{125}I-labelled antigen solution was linear to about 500000 c.p.m. When $(NH_4)_2SO_4$ was included to a final concentration of half-saturation, a 3% correction for decreased efficiency was made. It has been reported that solutions of some electrolytes can absorb the low-energy radiation from ^{125}I (Bakhle, Prusoff & McCrea, 1964).

Chemical analyses. Protein concentrations were determined as described by Hellsing (1966). The following $E^{1\%}_{1cm.}$ values at 280nm. were used: human serum albumin, 5·2 (Hellsing, 1966); human IgG, 13·8 (Schultze & Heremans, 1966); rabbit IgG, 13·5 (Crumpton & Wilkinson, 1963). Hexuronic acid was determined by the carbazole method (Dische, 1947) as modified by Galambos (1967). A modification of the Elson & Morgan (1933) method was used for hexosamine determinations. N values were obtained by micro-Kjeldahl analyses (Ma & Zuazaga, 1942). Sulphate analyses were performed by a benzidine method (Antonopoulos, 1962).

RESULTS

The precipitation of albumin by anti-albumin was greatly increased in the region of antigen excess by addition of the connective-tissue polysaccharides hyaluronate, chondroitin 4-sulphate and chondroitin 4-sulphate–protein complex. Parallel to the increased precipitation, a decrease in the concentration of soluble immune complexes was observed in the supernatants, whereas the concentration of free albumin remained unaltered within the limits of detection. When human γ-globulin was substituted for the anti-albumin solution in control experiments, no precipitates were formed either with or without added polysaccharide.

Effect of hyaluronate. The effect of hyaluronate on the precipitin reaction is shown in Fig. 2 and Table 1. In the region of antigen excess the precipitation was markedly increased by hyaluronate (mol.wt. 4×10^5). The effect of the polysaccharide was presumably greater than was indicated by these results, since the sedimentation of the precipitates by centrifugation was apparently not complete under the conditions used. This was suggested by the fact that at the highest polysaccharide concentration 5–11% of the radioactivity was found in the supernatant fluid in the region of antibody excess, compared with 2–3% in samples without polysaccharide. Attempts to perform experiments with hyaluronate of higher mol.wt. ($3·6\times10^6$) were

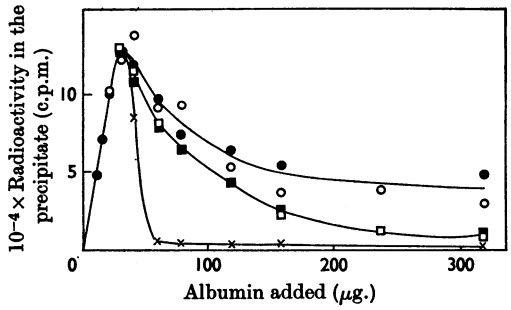

Fig. 2. Effect of hyaluronate on the precipitin reaction. Increasing amounts of ^{125}I-labelled human serum albumin were added to constant amounts of anti-albumin IgG in the presence of the polysaccharide (mol.wt. 4×10^5). The radioactivity in the precipitate was plotted versus the amount of albumin added. Final concentration of hyaluronate: ×, none; ○ and ●, 3·8mg./ml.; □ and ■, 1·9mg./ml. The open and filled symbols represent two different experiments.

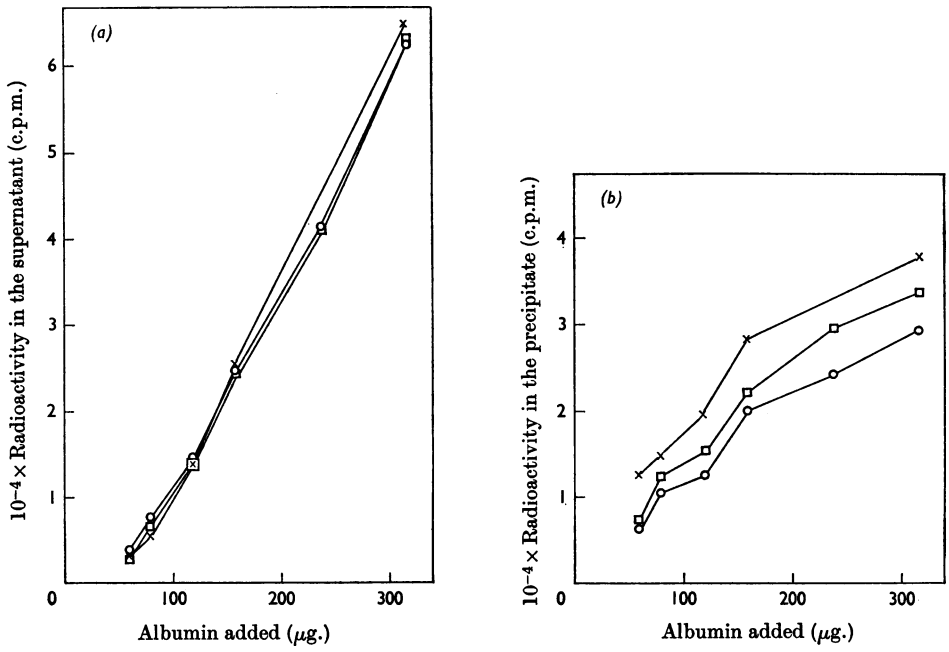

Fig. 3. Free albumin (*a*) and soluble albumin–anti-albumin complexes (*b*) in the supernatants from the precipitin reactions described in Fig. 2. The separation was carried out by $(NH_4)_2SO_4$ precipitation. The same symbols are used as in Fig. 2.

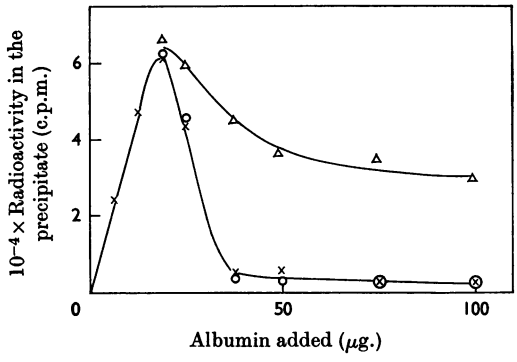

Fig. 4. Abolition by hyaluronidase of the effect of hyaluronate on the precipitin reaction. To a series of test tubes were added in volumes of 200 μl.: △, 0·7 mg. of hyaluronate; ×, 0·2 mg. of hyaluronidase; ○, 0·7 mg. of hyaluronate and 0·2 mg. of hyaluronidase. After incubation at 37° for 12 hr., 100 μl. of an anti-albumin solution and 100 μl. of labelled albumin were added. A different batch of anti-albumin to that in earlier experiments was used. The results were plotted as in Fig. 2.

not successful, since the recoveries of the immune precipitates were too low.

Separation of the free-albumin and soluble antibody-bound-albumin fractions that remained in the supernatants showed that the polysaccharide significantly decreased the concentration of the soluble complexes. The results illustrated in Figs. 3(*a*) and 3(*b*) were obtained with the ammonium sulphate precipitation technique, but the immuno-electrophoretic technique gave similar results.

When hyaluronate was degraded by testicular hyaluronidase before the addition of the immunochemical reactants, no effect on the precipitate formation was detected (Fig. 4).

A comparison between hyaluronate and Dextran 500, which have approximately the same molecular weight, is shown in Fig. 5. In the region of large antigen excess the effect of hyaluronate was more pronounced, although its concentration was only one-tenth of that of dextran.

Effect of PP-L and chondroitin sulphate. PP-L exerted an effect similar to that of hyaluronate, although higher concentrations were required to obtain the same degree of increase of the precipitation (Table 1). The effect of PP-L was greater than that of chondroitin sulphate, a finding that illustrates the importance of the molecular weight of the added polysaccharide. On the other hand, the effect of chondroitin sulphate was greater than that of Dextran 35, although these two compounds have almost the same molecular weight. This result, which may be related to the structural differences

between the two polysaccharides, is discussed below.

In the experiments with PP-L the removal of the precipitates by centrifugation was not complete, and consequently the values given in Table 1 are minimal ones. At the highest concentration of PP-L 30% of the radioactivity was found in the supernatant in the region of antibody excess, whereas the corresponding values at lower concentrations were about 5%.

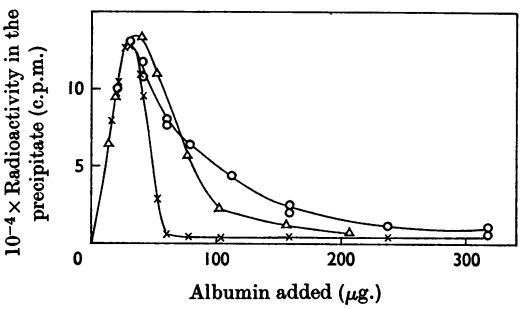

Fig. 5. Comparison of the effects on the precipitin reaction of hyaluronate and dextran of almost the same molecular weight. The result was plotted as in Fig. 2. The concentrations used were: ×, none; ○, hyaluronate (1·9mg./ml.); Dextran 500 (20mg./ml.).

DISCUSSION

The steric interaction between proteins and connective-tissue polysaccharides, especially hyaluronate, has been studied extensively recently (Laurent, 1966, 1968). Since hyaluronate has a random-coil structure that is extended over a large domain (Balazs, 1958) the chains will entangle and form a continuous network even at low polysaccharide concentrations. The sulphated polysaccharides have a shorter chain length than has hyaluronate, but in the tissue they are covalently linked to protein and occur as large complexes that, like hyaluronate, take part in the formation of an extracellular network. Characteristic properties of such a continuous network are its steric exclusion of other macromolecules and its sieving effect.

The present investigation examined the steric exclusion effects exerted by connective-tissue polysaccharides on the precipitation of antigen–antibody complexes. The polysaccharides used were hyaluronate, chondroitin 4-sulphate and a chondroitin 4-sulphate–protein complex (PP-L). All three compounds caused an increased immune precipitation that was correlated with the concentration of the polysaccharide. The increase was

Table 1. *Comparison of the effect of different polysaccharides on the precipitation of albumin–anti-albumin complexes*

The amount of albumin precipitated (μg.) was measured (or calculated by interpolation) at different albumin concentrations in the precipitin reaction. Different batches of albumin were used, but the same batch of anti-albumin was used in all the experiments. Maximal precipitation in the absence of polysaccharide occurred with the addition of 30μg. of albumin.

Polysaccharide	Concn. of polysaccharide (mg./ml.)	Concn. of albumin (μg./400μl.)	Albumin precipitated (μg.)					
			60	90	120	180	240	300
None			1·2	0·8	0·6	0·6	0·6	0·4
Hyaluronate	3·8		17·8	13·5	10·9	8·1	7·1	7·1
	1·9		14·6	10·4	7·6	3·6	2·1	1·7
PP-L	14·0		19·9	15·9	13·3	10·0	7·8	5·9
	7·0		16·8	13·3	10·7	7·3	5·0	4·3
	3·5		12·1	5·7	3·3	1·9	1·2	1·2
Chondroitin 4-sulphate	11·2		16·1	8·5	3·8	2·1	1·4	1·4
	5·6		13·5	3·8	0·9	0·6	0·6	0·6
Dextran 35	20		12·1	3·6	1·7	1·2		
	10		8·5	0·9	0·9	0·9		
Dextran 500	20		17·3	6·9	3·6	1·7		
	10		11·4	1·7	1·2	1·2		
Dextran 12000	20		19·6	15·4	12·1	7·6		
	10		11·4	2·1	1·9	1·9		

accompanied by a decrease of the concentration of soluble antigen–antibody complexes in the supernatants.

In addition to the concentration the molecular weight and the configuration of the polysaccharides were of particular importance for the size of the increase.

The influence of molecular weight is indicated by the finding that PP-L had a greater effect than chondroitin sulphate. Further, when hyaluronate was degraded to oligosaccharides by hyaluronidase (Weissman, Meyer, Sampson & Linker, 1954) the effect was abolished. These results are in keeping with the idea that shorter polysaccharide or oligosaccharide molecules cannot take part in network formation.

The influence of the configuration of the polysaccharide on its ability to increase precipitation is shown by a comparison between hyaluronate and dextran of almost equal molecular weight (Fig. 5). Although hyaluronate was used in a concentration only one-tenth of that of dextran, its effect was greater in the region of large antigen excess. These results are consistent with previous studies (Laurent, 1964), demonstrating that hyaluronate has steric exclusion effects four to five times as great as those of dextran. Hyaluronate is a linear molecule and may therefore be expected to have a greater effect than dextran, which has a more branched and coiled structure. The dependence on the configuration was further substantiated by a comparison between PP-L and hyaluronate (Table 1). In spite of its lower molecular weight, hyaluronate exerted more than twice the effect of PP-L. The 'branched' structure of PP-L (a protein core to which a number of chondroitin sulphate chains are attached) may be the reason for this difference. Further evidence for the importance of the configuration of the polysaccharide was the fact that the linear chondroitin 4-sulphate alone exhibited greater precipitating effects than the branched dextran of almost the same molecular weight (Dextran 35).

Thus the enhanced immune precipitation depends on the concentration, the molecular weight and the configuration of the added polysaccharide. This agrees with the hypothesis that the fundamental mechanism is a steric exclusion of the immune complexes from the domains of the polysaccharides. Similar observations have been made with neutral polysaccharides (Hellsing, 1966). Other studies seem to exclude the interpretation that the effect is due to complex-formation between the polysaccharides and the proteins (Hellsing, 1969).

Some of the experimental observations suggest that the sieving property of the polysaccharides also influenced the results, but in an opposite direction to that of exclusion. Thus, at high concentrations of hyaluronate and PP-L, a considerable amount of radioactivity was found in the supernatant in the region of antibody excess. Owing probably to restricted transport (sieving) in the solution, the immune complexes were not completely recovered in the precipitate. The most pronounced sieving effect would be expected in the region of antibody excess where the largest immune complexes are found.

Hyaluronate was shown to have more pronounced sieving properties than dextran (Laurent & Persson, 1964). This fact may explain the intersection of the two curves in Fig. 5, since the radioactivity values found in the precipitate at a moderate excess of antigen when hyaluronate had been added were apparently too low.

When the immunoelectrophoretic technique was used to separate free and antibody-bound albumin in the supernatant containing hyaluronate, no separation was possible, nor did the fractions have their normal mobilities, unless the hyaluronate had previously been enzymically degraded. It is possible that hyaluronate moved towards the anodic side of the holes in the agarose gels, became packed and formed a sieve for the protein molecules.

The question arises whether the exclusion properties of polysaccharides described here play a role in human disease. During the second week of experimental serum sickness, soluble antigen–antibody complexes are found in the circulation of the experimental animals (Dixon, 1965). At the same time, inflammatory lesions appear in the connective tissue, especially in that of the heart, arteries, joints and kidneys. Immunohistochemical studies have demonstrated the existence of antigen as well as of host complement factors and γ-globulin at the site of the lesions. The inflammatory reactions observed have been ascribed to the precipitation of immune complexes originating from the blood. It is tempting to speculate that, especially in areas containing high concentrations of connective-tissue polysaccharides, this may be due to a steric exclusion effect. Such a mechanism might participate in the pathogenesis of several immunological diseases.

This investigation was supported by grants from the Swedish Medical Research Council (B69-13X-4-05A), the Swedish Cancer Society (53-B68-03XC), Konung Gustaf V:s 80-årsfond, Svenska Sällskapet för Medicinsk Forskning, AB Pharmacia and the University of Uppsala. I thank Professor T. C. Laurent for his interest in this work, for his criticism and for his helpful suggestions. Thanks are also extended to Dr L. Rodén, who revised the manuscript, and to Mr E. Lindberg, who prepared some of the polysaccharide material. AB Kabi, AB Leo, and Pharmacia AB kindly supplied material. This paper is part 2 in the series 'Immune reactions in polysaccharide media' (part 1: Hellsing, 1966).

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