The Quantitative Measurement of Alcian Blue–Glycosaminoglycan Complexes

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1. A simple new quantitative micro method was developed to study the interaction of the cationic dye Alcian Blue 8GX and acid glycosaminoglycans under different conditions. After washing with ethanol the precipitated Alcian Blue–glycosaminoglycan complex was dissociated in Manoxol IB solution and the amount of bound dye measured spectrophotometrically. 2. Reaction profiles of complex-formation were determined in the presence of different concentrations of MgCl_2 at pH 5.8, and could be used to study the critical electrolyte concentrations of glycosaminoglycans. At least 50 mM- MgCl_2 was required to produce maximum precipitation of, and maximum uptake of, Alcian Blue by standard glycosaminoglycans. Maximum uptake of Alcian Blue by glycosaminoglycans in the urine of a patient with Hurler's syndrome required the presence of 25–50 mM- MgCl_2 . 3. Under standard conditions of maximum interaction, calibration curves for the quantitative determination of a series of standard glycosaminoglycans in 20 μl volumes were nearly linear over the range 1–10 μg . 4. The technique was used to determine the molecular binding ratios of Alcian Blue to glycosaminoglycans under controlled conditions.

Most methods currently available for the measurement of glycosaminoglycans in biological fluids tend to be lengthy and complicated, and are subject to various limitations. For example, urinary glycosaminoglycans require 12 h equilibration at 4°C with cetylpyridinium chloride for maximum precipitation, after which time recoveries of keratan sulphate and hyaluronic acid may be incomplete (Di Ferrante, 1967). Complexes of low-molecular-weight keratan sulphate with cetylpyridinium chloride may redissolve in excess of complexing agent owing to micelle formation, although this occurs to a lesser extent at low temperatures (Scott, 1967, 1970a). Further, subsequent determination of the uronic acid content of the complexes of glycosaminoglycans with cetylpyridinium chloride by carbazole or orcinol reactions fails to measure keratan sulphate. Difficulties may also arise owing to structural complexities of proteoglycans and variations in the molecular weight and degree of sulphation of the glycosaminoglycan chains. Hybrid molecules consisting of chondroitin sulphate, keratan sulphate and peptides have been demonstrated in normal urine, and occur in excess in the urine of some patients with the Morquio syndrome (Kaplan *et al.*, 1968; Dean *et al.*, 1971).

Scott (1955, 1967, 1970a) has shown that the critical electrolyte concentration for a particular polyanion, i.e. the molarity of a salt at which there is an abrupt decrease in interaction between polyanion and precipitating organic cation (or polycation), is related to

the molecular weight of the polyanion and the nature of its ionic charges. Low-molecular-weight species of glycosaminoglycans have lower critical electrolyte concentrations than have higher-molecular-weight species of the same type (Laurent & Scott, 1964). The urine of some patients with mucopolysaccharidosis may contain two or more glycosaminoglycans with low average molecular weights, and in these circumstances overlapping of critical electrolyte concentrations may occur.

Ion-exchange chromatography has often been used for the fractionation of urinary glycosaminoglycans, but in view of the many factors involved it is not surprising that poor separations have been reported (Manley & Williams, 1969; Kaplan, 1969). Elution of the complexes formed between polyanions and polycationic exchange resins is governed by the characteristic critical electrolyte concentrations of these complexes, and to obtain reproducible conditions it is necessary to allow interactions to reach a state of equilibrium (Scott, 1970a). Such conditions are not usually attained by using continuous elution chromatographic procedures. Further, a series of complexes may be formed owing to competition for binding sites if the capacity of the ion-exchange resin or cellulose is not greatly in excess of that required for the applied polyanion. Diffusion and sieve effects are additional factors involved in the separation of glycosaminoglycans on ion-exchange resins (Scott, 1970a). Calatroni *et al.* (1969) measured plasma

glycosaminoglycans by using small ECTEOLA-cellulose columns, but the subsequent work of Neri *et al.* (1971) showed that the method considerably underestimated the true content.

The demonstration by Scott *et al.* (1964) that the cationic dye Alcian Blue 8GX formed insoluble complexes with acid glycosaminoglycans under controlled conditions was extended to a study of the critical electrolyte concentrations for the interaction of the dye with polyanions (Scott & Dorling, 1965; Scott & Willett, 1966). Magnesium chloride was found to be the most effective salt for differentiating critical electrolyte concentrations of polycarboxylates, polysulphates and polyanions containing both carboxylate and ester sulphate groups.

The present report describes the effect of $MgCl_2$ on the interaction of Alcian Blue 8GX with glycosaminoglycans, a new quantitative technique being used for the measurement of the Alcian Blue–glycosaminoglycan complex. The method is simple and problems encountered with conventional methods of analysis of glycosaminoglycans are largely overcome by careful control of the initial precipitation procedure. The method has been used to determine the glycosaminoglycan content of urine, a preliminary report of which has appeared (Whiteman, 1972).

Experimental

Materials

Alcian Blue 8GX was a gift from I.C.I. Ltd., Blackley, Manchester, U.K. Manoxol IB (dibutyl ester of sodium sulphosuccinic acid) was obtained from B.D.H. Chemicals Ltd., Poole, Dorset, U.K. D-Glucuronic acid and crude chondroitin sulphate (mixed isomers) were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Highly purified sodium salts of glycosaminoglycans (International Reference Standards) were kindly given by Dr. M. B. Mathews, Dr. J. A. Cifonelli and Dr. L. Rodén of the Department of Paediatrics, University of Chicago. All other reagents were A.R. grade.

Methods

Formation and analyses of Alcian Blue–glycosaminoglycan complexes. The complexing reagent, freshly prepared, consisted of Alcian Blue (0.05%, w/v) in 50mM-sodium acetate buffer (pH 5.8) containing 50mM- $MgCl_2$ (except where otherwise stated). This reagent was centrifuged at 2000g for 15min in a MSE Mistral 6L centrifuge and any deposit discarded.

The reagent for dissociating the Alcian Blue–glycosaminoglycan complex in the standard procedure was a 40% (w/v) solution of Manoxol IB in

50mM-sodium acetate buffer (pH 5.8), although similar results were also obtained in water in the absence of the buffer. Centrifugation of the Manoxol IB reagent produced a clear colourless solution.

In the standard procedure, glycosaminoglycan solutions (20 μ l, containing 1–10 μ g of glycosaminoglycan) were equilibrated for 2h at room temperature with Alcian Blue reagent (1ml) in small disposable plastic tubes. The complete recovery of Alcian Blue–chondroitin 4-sulphate complexes required centrifugation at 2000g for 10min. A 15min period of centrifugation at 2000g was therefore adopted in the standard procedure. After separation of the Alcian Blue–glycosaminoglycan complex, the supernatant solution was carefully drained by decantation and the complex washed once with ethanol (2ml) and again isolated by centrifugation. After removal of the ethanolic supernatant as completely as possible by decantation, the complex was dissociated by mixing briefly with Manoxol IB reagent (1ml). The extinction of the resulting clear blue solution was read at 620nm in 1cm microcuvettes in a Unicam SP.500 spectrophotometer. Variations in this standard procedure are indicated in the appropriate sections.

Determination of the uronic acid content of complexes of glycosaminoglycans with Alcian Blue. Alcian Blue reagent (4ml) containing different concentrations of $MgCl_2$ was added to urine (50 μ l) of a patient with Hurler's syndrome or to a solution of chondroitin 4-sulphate (50 μ g/50 μ l of water). Equilibration was allowed to proceed for 2h at room temperature, the complexes were then centrifuged and the supernatants discarded. The uronic acid contents of the complexes were determined by the procedure of Scott *et al.* (1964). The complex was dissociated by vigorous shaking with a mixture of 4M-NaCl (0.4ml) and methanol (0.2ml). Alcian Blue was then precipitated by the addition of 0.1M- Na_2CO_3 (0.2ml) (J. E. Scott, personal communication) and water (0.4ml). The mixture was left at room temperature for 30min followed by centrifugation. Portions (0.5ml) of the supernatant solution were analysed for their uronic acid contents by the method of Bitter & Muir (1962). Low blanks and linear glucuronic acid standard curves were obtained when analysis was carried out under the same conditions in the presence of NaCl, Na_2CO_3 and methanol. For convenience, the uronic acid content of the complexes analysed has been expressed as E_{530} , since it was intended to compare the relative glycosaminoglycan content of the complexes formed under different conditions.

Determination of bound Alcian Blue by copper analysis. Each molecule of Alcian Blue 8GX contains one atom of copper. Determination of the copper content of the complex therefore gave a measure of binding of Alcian Blue to glycosaminoglycan. Complexes were formed between sodium hyaluronate (40 μ g/40 μ l) or sodium chondroitin 4-sulphate (40 μ g/

40 μ l) and Alcian Blue reagent (4ml) containing 50mM-MgCl₂. In one series of experiments, excess of reagent was drained as completely as possible from the isolated complex, but the complex was not washed, and in another series the complex was washed with ethanol (2ml) as in the standard procedure. After the complex was dissociated in Manoxol IB reagent (2ml), 0.2ml of the resulting solution was diluted with water (2ml) and its copper content measured by using a Perkin-Elmer 303 atomic absorption spectrophotometer at 324.7nm with an air-acetylene flame. Blanks and standard copper solutions were made up in a similar concentration of Manoxol IB reagent. The copper contents of reagent blanks, both washed and unwashed, were relatively low.

Results and Discussion

Effect of Alcian Blue concentration on complex-formation

The effect of varying the concentration of Alcian Blue on complex-formation with chondroitin 4-sulphate (10 μ g) in the presence of 50mM-MgCl₂ is shown in Table 1. A tenfold increase in concentration of Alcian Blue in the complexing reagent from 0.01 to 0.10% (w/v) resulted in a 5% increase in the Alcian Blue content of the complex. Free dye occluded by the precipitate could account for some of the increase, since this factor would not be offset by the reagent blank. Blank values over the range specified were low but became unacceptably high when higher concentrations of Alcian Blue were used. Thus 0.05% (w/v) appeared to be a useful working concentration for

Alcian Blue in the complexing reagent; small variations in this concentration cause negligible changes in the dye content of the complexes. Complex-formation with the recommended quantities of glycosaminoglycan causes only small changes in the Alcian Blue concentration of the reagent with the standard procedure described under 'Methods'. Scott & Dorling (1965) showed that moderate changes in Alcian Blue concentration caused only slight changes in critical electrolyte concentrations of glycosaminoglycans, provided that a large excess of the dye was present.

An interesting observation was the sharp decrease in complex-formation that occurred at high Alcian Blue concentrations (0.4–0.8%, w/v). This phenomenon was not further investigated, but may have been due to some other constituent, e.g. boric acid, of the commercial Alcian Blue 8GX rather than to the dye itself.

Dissociation of the Alcian Blue-glycosaminoglycan complex

To assess quantitatively the amount of glycosaminoglycan in the complex by measurement of bound Alcian Blue it was necessary to find a reagent that would rapidly and completely dissociate the complex and also provide a suitable medium in which to measure the concentration of the dye spectrophotometrically. A 40% (w/v) solution of the anionic surfactant Manoxol IB fulfilled both these criteria. Less-concentrated solutions dissociated the complexes incompletely. The final solutions were optically clear and showed an absorption maximum at 620nm. The precipitation of glycosaminoglycans from these solutions by the addition of 6vol. of butan-1-ol indicated that dissociation had taken place.

Other methods tested included the use of concentrated salt solutions, e.g. 4M-MgCl₂ and 5M-NaCl, but were unsuitable because dissociation of the complex was either slow or incomplete. Also, broadening of the absorption curve occurred with strong salt solutions. Dimethyl sulphoxide would theoretically provide an ideal medium in which to measure the extinction of Alcian Blue, since in this reagent the dye assumes its non-metachromatic state and exhibits a marked increase in light-absorption at 678nm (Scott, 1970b). In practice, however, this solvent was not an efficient complex-dissociating reagent.

Effect of washing on the Alcian Blue-glycosaminoglycan complex

Methods of washing the Alcian Blue-glycosaminoglycan complex free of unbound dye may cause partial dissociation of the complex. To investigate this problem Alcian Blue-glycosaminoglycan complexes were washed once with different fluids (2ml) and the

Table 1. *Effect of Alcian Blue concentration on complex-formation*

Chondroitin 4-sulphate (10 μ g) complexes were formed by the standard procedure with Alcian Blue reagents (1 ml) containing 50mM-MgCl₂. A commercial preparation of Alcian Blue 8GX was used for the reagent. Reagent blanks were taken through the entire procedure with the omission of glycosaminoglycan.

Concn. of Alcian Blue in reagent (%, w/v)	Reagent blank (E ₆₂₀)	Alcian Blue in complex (E ₆₂₀ -blank)
0.01	0.022	0.878
0.02	0.033	0.887
0.05	0.033	0.907
0.10	0.041	0.919
0.20	0.050	0.980
0.40	0.087	0.783
0.80	0.161	0.469

Table 2. *Methods of washing Alcian Blue-glycosaminoglycan complexes*

Complexes were formed from glycosaminoglycans (10 μ g) under standard conditions (i.e. with 0.05% Alcian Blue) and their Alcian Blue contents measured (E_{620} -blank) after one washing with 2ml of washing fluid. Reagent blanks were taken through the whole procedure, with omission of glycosaminoglycans.

Glycosaminoglycan in complex	Washing fluid ...	Alcian Blue content (E_{620} -blank)			
		Water	Salt* solution	Methanol	Ethanol
Hyaluronate		0.558	0.957	0.232†	0.796
Chondroitin 4-sulphate		0.598	0.980	0.570	0.930
Dermatan sulphate		0.718	0.980	0.575	0.940
Heparan sulphate		0.708	0.990	0.555	0.915
Heparin		0.863	1.14	0.760	1.105
Keratan sulphate		0.346	0.682	0.354	0.665
Reagent blank		0.097	0.143	0.070	0.055

* Salt solution contained 50mM-MgCl₂ in 50mM-sodium acetate buffer, pH5.8.

† After washing in methanol the hyaluronate-Alcian Blue complex would not dissociate fully in the Manoxol IB reagent.

amount of dye remaining in the complex was measured by using the standard procedure (Table 2). The results indicated that water and especially methanol caused considerable dissociation of the complexes compared with the use of ethanol or an electrolyte solution containing MgCl₂ and buffer at the same concentrations as those used in the complexing reagent. Although washing with ethanol gave values slightly lower than those obtained by using the electrolyte solution, the method showed some distinct advantages when applied as an analytical technique. Only one wash with ethanol was required to produce a constant and very low concentration of dye in the supernatant washing fluid, whereas aqueous solutions required two washes to produce the same effect. Ethanol was more easily and completely decanted and left the complex firmly fixed to the bottom of the test tube. A further advantage in the use of ethanol was the low blank value obtained. The addition of water (5%, v/v) to ethanol or the presence of 50mM-MgCl₂ had no advantage over the use of ethanol alone.

Interaction of Alcian Blue and hyaluronate

The interaction of sodium hyaluronate and Alcian Blue reagent containing different concentrations of MgCl₂ is shown in Fig. 1. Maximum complex-formation occurred in the presence of 50mM-MgCl₂; at higher concentrations of MgCl₂ there was an abrupt decrease in complex-formation consistent with the known low critical electrolyte concentration for hyaluronic acid (Scott *et al.*, 1964; Scott & Dorling, 1965). There was no significant change in the point of maximum interaction or the critical electrolyte concentration when the amount of hyaluronate present was increased, but the amount of complex formed

Fig. 1. *Effect of MgCl₂ concentration on hyaluronic acid-Alcian Blue interaction*

The ordinate shows the extinction of Alcian Blue released from the Alcian Blue-hyaluronate complexes, which were formed in different concentrations of MgCl₂. Conditions were as for the standard procedure (see under 'Methods'). Amount of hyaluronic acid: □, 4 μ g; ○, 8 μ g; ●, 12 μ g.

was directly proportional to the concentration of hyaluronate. This observation formed the basis of subsequent quantitative investigations.

Interaction of Alcian Blue and standard sulphated glycosaminoglycans

Complex-formation resulting from the interaction of a variety of standard sulphated glycosaminoglycans

Fig. 2. Effect of MgCl_2 concentration on interaction of Alcian Blue with sulphated glycosaminoglycans

The ordinates indicate the extinction of Alcian Blue released from Alcian Blue-glycosaminoglycan complexes, which were formed in different concentrations of MgCl_2 . Conditions were as for the standard procedure (see under 'Methods'), $6\mu\text{g}$ of individual sulphated glycosaminoglycans being used. (a) ●, Chondroitin 4-sulphate; ▲, chondroitin 6-sulphate; ■, keratan sulphate. (b) ○, Dermatan sulphate; △, heparan sulphate.

and Alcian Blue reagent containing different concentrations of MgCl_2 is shown in Fig. 2.

For all glycosaminoglycans tested at least 50mM- MgCl_2 was required for maximum complex-formation. The critical electrolyte concentrations for the sulphated glycosaminoglycans were higher than that for hyaluronate, and with the exception of keratan sulphate all glycosaminoglycans showed a relatively sharp decrease in complex-formation at their individual critical electrolyte concentrations. The anomalous behaviour of keratan sulphate would be consistent with considerable polydispersity of its molecular weight.

Maximum precipitation of Alcian Blue complexes of chondroitin 4-sulphate and urinary glycosaminoglycans

The effect of MgCl_2 on the precipitation of Alcian Blue-glycosaminoglycan complexes from standard solutions of chondroitin 4-sulphate and from urine

Fig. 3. Effect of MgCl_2 concentration on the uronic acid and Alcian Blue contents of complexes

Complexes were formed by using the standard procedure (see under 'Methods'). Uronic acid content determined by the modified carbazole reaction is expressed as E_{530} : ○, chondroitin 4-sulphate standard; □, glycosaminoglycans in the urine of patients with Hurler's syndrome. Alcian Blue content is expressed as E_{620} : ●, chondroitin 4-sulphate standard; ■, glycosaminoglycans in the urine ($10\mu\text{l}$) of patients with Hurler's syndrome; ▲, normal adult urine ($50\mu\text{l}$).

of patients with Hurler's syndrome was assessed by determination of the uronic acid and Alcian Blue contents of the resultant complexes. The uronic acid content of the chondroitin 4-sulphate complexes again indicated that maximum precipitation had occurred in the presence of 50mM- MgCl_2 (Fig. 3). These results also show that with increasing concentration of MgCl_2 the binding of Alcian Blue to chondroitin 4-sulphate increased disproportionately relative to the uronic acid content of the complexes, but attained a maximum value coincident with complete precipitation of glycosaminoglycan. Similar observations were made by Scott *et al.* (1964) with heparin and NaCl. They postulated that Alcian Blue, which may have up to four cationic groups, required associated anions in solution to maintain electrical neutrality in the neighbourhood of the molecule. Thus, at low MgCl_2 concentrations, more cationic sites on the Alcian Blue molecule are effectively available for binding with the polyanion and the number of dye molecules bound to polyanion will be fewer than at higher Cl^- concentrations.

Constant maximum precipitation of glycosaminoglycans from the urine of patients with Hurler's syndrome occurred at concentrations of MgCl_2 up to 50mM but decreased significantly at higher concentrations. Uptake of Alcian Blue by polyanions in the urine of these patients was at a maximum in the presence of 25–50mM- MgCl_2 and decreased at higher MgCl_2 concentrations. The decrease in both components of the Alcian Blue–glycosaminoglycan complex at 100mM- MgCl_2 indicates the presence in the urine of these patients of polyanions with low critical electrolyte concentrations and is consistent with the behaviour of glycosaminoglycans of low molecular weight. Slightly less than maximum complex-formation was obtained with normal adult human urine at 50mM- MgCl_2 and a further decrease occurred at 100mM- MgCl_2 .

Rate of formation of Alcian Blue–glycosaminoglycan complexes with standard glycosaminoglycans

An Alcian Blue reagent containing 50mM- MgCl_2 appeared to be generally useful for the determination of total acid glycosaminoglycans in solution. Accordingly this reagent (1 ml) was used to study the rate of complex-formation with standard glycosaminoglycans ($10\mu\text{g}$).

The amounts of complex formed after different periods of equilibration at room temperature were

Fig. 4. Rate of formation of Alcian Blue–glycosaminoglycan complexes

The ordinate shows the extinction of Alcian Blue released from Alcian Blue–glycosaminoglycan complexes. Standard glycosaminoglycans ($10\mu\text{g}$) formed complexes by the standard procedure in the presence of 50mM- MgCl_2 and measurements were made after different periods of equilibration at room temperature. ●, Chondroitin 4-sulphate; ○, dermatan sulphate; △, heparan sulphate; ■, keratan sulphate; □, hyaluronic acid.

measured by the standard procedure described under 'Methods'. For all glycosaminoglycans tested the initial rate of reaction was extremely rapid and, with the exception of dermatan sulphate, which required 2h for complete precipitation, complex-formation was maximum after 1h (Fig. 4). A 2h period of equilibration was therefore judged to be adequate as a general procedure.

Calibration curves for standard glycosaminoglycans

Standard glycosaminoglycans were equilibrated for 2h at room temperature with Alcian Blue reagent (1 ml) containing 50mM- MgCl_2 , and the amount of dye in complex was measured by using the standard procedure. Chondroitin 4-sulphate, chondroitin 6-sulphate, heparan sulphate and dermatan sulphate, which contained close to two anionic groups per disaccharide repeating unit, gave similar and almost linear calibration curves over the range 1– $10\mu\text{g}$ (Fig. 5).

Fig. 5. Quantitative interaction of standard reference glycosaminoglycans and Alcian Blue

The ordinate shows the extinction of Alcian Blue released from Alcian Blue–glycosaminoglycan complexes, which were formed in the presence of 50mM- MgCl_2 . The abscissa shows the amount of glycosaminoglycan present in each sample ($20\mu\text{l}$). Conditions were as for the standard procedure (see under 'Methods'). ●, Chondroitin 4-sulphate, chondroitin 6-sulphate and heparan sulphate; ○, dermatan sulphate; ■, keratan sulphate; □, hyaluronic acid; △, heparin.

Hyaluronic acid and keratan sulphate, which have lower charge densities, gave slightly lower values, and heparin, which has a relatively high charge density, gave higher values than chondroitin 4-sulphate.

In view of its commercial availability, sodium chondroitin 4-sulphate was always used as a reference standard for the determination of glycosaminoglycans in unknown solutions. Crude chondroitin sulphate (Sigma Chemical Co.) containing mixed isomers gave an almost identical response.

Molar ratio of Alcian Blue to glycosaminoglycan in the complex

Complexes formed from the interactions of known weights of hyaluronic acid or chondroitin 4-sulphate with Alcian Blue reagent containing 50mm-MgCl₂ were either washed with ethanol or drained free of reagent as far as possible. The amount of Alcian Blue in each complex was estimated from the extinction of the dye after dissociation of the complex in Manoxol IB reagent and also by determination of the copper content of the dissociated complexes (each molecule of Alcian Blue contains one atom of copper). The amount of dye present in the complex was calculated by comparing its extinction with that of the commercial dye dissolved in the Manoxol IB reagent. It was necessary to allow for the presence of approx. 40% (w/w) of boric acid in the commercial preparation of Alcian Blue 8GX (Scott *et al.*, 1972; Scott, 1972a). The molarity of Alcian Blue 8GX was then calculated on the assumption that the dye is a copper phthalocyanin with four *S*-methylene tetramethylisothiuronium chloride side chains per molecule (Scott *et al.*, 1972; Scott, 1972b). Results were expressed in terms of the quantity of Alcian Blue (nmol) and repeating units (nmol) of glycosaminoglycans in the complexes. Hyaluronic acid and chondroitin 4-sulphate were reference standards bearing approximately one and two charges respectively per disaccharide repeating unit. Unwashed complexes were examined to determine an upper limit for binding of Alcian Blue to glycosaminoglycan, although these results would be falsely high, owing to the presence of free reagent absorbed in the precipitate (reagent blanks would not allow for this factor). After the complexes had been washed with ethanol there appeared to be approximately one molecule of Alcian Blue for each disaccharide repeating unit of chondroitin 4-sulphate. The Alcian Blue 8GX molecule has up to four cationic isothiuronium groups, and it appeared that on average two of the groups were linked with chondroitin 4-sulphate when complexes were formed in the presence of 50mm-MgCl₂.

In view of the reasonable agreement between the independent methods of determining Alcian Blue in the complexes, further measurements of molecular binding ratios were made by measurement of the

extinction of the bound dye only. Complexes washed in ethanol or 50mm-sodium acetate buffer (pH 5.8) containing 50mm-MgCl₂ were compared. Slightly lower values were obtained when the complexes were washed in ethanol, which may have been due either to slight dissociation of the complex or to more efficient washing. Hyaluronate appeared to bind more Alcian Blue per anionic charge than did chondroitin 4-sulphate, and heparin, with its high charge density, showed more binding of Alcian Blue to one repeating unit than that observed for chondroitin 4-sulphate. Chondroitin sulphate, dermatan sulphate and heparan sulphate gave similar molecular binding ratios.

General Discussion

The interaction of organic cations or polycations with polyanions is related to the pH and electrolyte content of the medium. Some proteins present in biological fluids may form salt links with glycosaminoglycans in acidic solutions (pH 2.6) and block interaction with Alcian Blue (Scott & Dorling, 1965). This effect is diminished at pH 5.8, but in this condition some proteins and glycoproteins may become weakly ionized polyanions capable of forming complexes with Alcian Blue. These undesirable situations are largely abolished and maximum reaction between glycosaminoglycans and Alcian Blue is achieved when complex-formation takes place at pH 5.8 in the presence of low concentrations of salt, for example 50mm-MgCl₂ (Scott & Dorling, 1965). However, the Tamm-Horsfall urinary glycoprotein appears to be an exception, and is discussed in the next paper (Whiteman, 1973).

The method described here provides a measure of polyanions in solution and is not restricted to glycosaminoglycans containing uronic acids. It provides a new parameter in quantitative glycosaminoglycan analysis, since it is dependent on the charge on the polyanion rather than the constitution of the backbone chain of the glycosaminoglycan. Further, profiles of the interaction of glycosaminoglycan with Alcian Blue at different concentrations of MgCl₂ provide qualitative information on the type of glycosaminoglycan that is forming the complex, and this might be applicable to the analysis of biological fluids. The critical electrolyte concentrations of glycosaminoglycans are better differentiated at pH 5.8 than under more acidic conditions (Scott & Dorling, 1965).

Fractionation of glycosaminoglycans may be achieved by forming complexes with Alcian Blue 8GX in different concentrations of MgCl₂. The method described here allows interactions to reach the equilibrium conditions required for reproducible critical electrolyte concentration effects and overcomes some of the disadvantages of ion-exchange chromatography. In effect the glycosaminoglycan

remaining in complex with Alcian Blue in a given concentration of $MgCl_2$ represents glycosaminoglycan that would be retained on an ion-exchange column when an equivalent chromatographic procedure was used. This method therefore provides a profile essentially complementary to that of ion-exchange chromatography.

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