The Influence of Sialic Acid Residues on the Ability of Glycoproteins to Interact Electrostatically with Chondromucoprotein

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1. Although glycoproteins with less than 1% of sialic acid (fibrinogen, lipoproteins, γ -globulins) interact electrostatically with chondromucoprotein to form insoluble complexes, interaction with glycoproteins containing larger amounts of sialic acid (orosomucoid, urine glycoprotein, seromucoid, fraction VI) was electrostatically impossible. Reasons for this are discussed. 2. The latter glycoproteins interacted with chondromucoprotein after mild acid hydrolysis or neuraminidase treatment, complex-formation being inversely related to their sialic acid content. 3. Complex-formation with sialic acid-deficient orosomucoid was maximum at pH 3.6 and negligible above its isoelectric point of pH 5, and was inhibited by Ca^{2+} ions and EDTA. 4. These results are discussed in relation to the carbohydrate composition and biological activities of euglobulin fractions, and of complexes formed by adding chondromucoprotein to abnormal plasmas which may contain sialic acid-deficient glycoproteins owing to faulty carbohydrate metabolism.

Chondromucoprotein, the naturally occurring form of chondroitin sulphate in which the mucopolysaccharide is covalently linked to non-collagenous protein (Muir, 1958; Anderson, 1962), is strongly anionic above pH 2.5 owing to dissociated carboxylic acid and sulphate groups. The addition of this polyelectrolyte to aqueous protein solutions, serum or plasma under mildly acid conditions results in the formation of precipitates (Anderson, 1963) the composition and biological properties of which are a function of the precipitating pH and the isoelectric points of the component proteins (Anderson & Lack, 1964; Anderson, 1965a). Although interactions between colloids of opposite charge have been studied by Bungenberg de Jong (1949), and the precipitation of various polyanions by long-chain aliphatic ammonium salts by Scott (1961), less is known of the effect of naturally occurring polyanions, such as chondromucoprotein, on the solubility of plasma proteins.

The latter interactions have been attributed to complex-formation as a result of electrostatic interaction between anionic chondromucoprotein and proteins functioning as cations. Similar complexes are present in the euglobulin fractions (Anderson, 1965b), which are precipitated spontaneously from acidified plasma, chondromucoprotein normally present *in vivo* functioning as the precipitating anion. The complexes dissolve at pH values above those at which they are precipitated, presumably owing to their dissociation into free components

after reversal of charge on the protein moiety, interactions between chondromucoprotein and any particular protein being electrostatically impossible above the isoelectric point of the latter.

During this work it was observed that glycoproteins containing more than 4% of sialic acid and isoelectric below pH 4.3 did not form insoluble complexes with chondromucoprotein. The acidic nature of glycoproteins is due to terminal sialic acid residues, the removal of which increases their cationic properties and should, if the formation of chondromucoprotein-protein complexes is due primarily to an electrostatic interaction, facilitate complex-formation with chondromucoprotein. The present study, a brief summary of which has been published (Anderson, 1965c), describes experiments designed to test this hypothesis, and relates the results to complex-formation between chondromucoprotein and other plasma components. Possible effects on the composition and biological properties of complexes precipitated by adding chondromucoprotein to plasmas containing sialic acid-deficient glycoproteins are discussed.

MATERIALS AND METHODS

Analytical methods. Protein, hexuronic acid and sialic acid were estimated as described by Anderson (1963). Turbidity measurements were carried out as described by Anderson & Lack (1964). Solutions of chondromucoprotein, isolated from human cartilage (Anderson, 1962), were

prepared in 0.9% NaCl as described by Anderson & Lack (1964), concentrations being expressed in terms of their chondroitin sulphate content.

Protein and glycoprotein fractions used for experiments on the formation of complexes. Bovine plasma fibrinogen (fraction I; 0.6% of sialic acid) and crystallized bovine plasma albumin were bought from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex). Bovine plasma γ -globulin (fraction II; containing less than 0.5% of sialic acid) was bought from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.). Serum fraction VI (8.0% of sialic acid; containing equal amounts of albumin and of glycoproteins from the α_1 - and α_2 -globulin fractions) was bought from Merck, Sharp and Dohme Research Laboratories (Rahway, N.J., U.S.A.). Human serum orosomucoid (10.2% of sialic acid) was a gift from Dr R. J. Winzler. Urine glycoprotein (4.3% of sialic acid) was isolated from human urine by the method of Anderson & MacLagan (1955). Seromucoid (4.1% of sialic acid) was isolated from serum as follows: to a mixture of 30 ml. of human serum and 270 ml. of 0.9% NaCl was added 150 ml. of 0.6M-sulphosalicylic acid solution in 0.9% NaCl. After 10 min. at room temperature, the precipitate was centrifuged down and to the supernatant fluid was added 84 ml. of a solution (5%, w/v) of phosphotungstic acid in 2N-HCl. After 10 min. the seromucoid fraction was centrifuged down, washed successively with 5 ml. of phosphotungstic acid solution, twice with 5 ml. of absolute ethanol and twice with 5 ml. of diethyl ether. It was then dried *in vacuo*. Yield: 243 mg./100 ml. This fraction contained predominantly orosomucoid, haptoglobins and 3.5S α_1 -glycoprotein (Doležalová, Brada & Kočent, 1964). Human plasma was an unhaemolysed bulk sample from normal individuals. It was stored 'deep-frozen' and immediately before use was thawed, incubated at 37° for 30 min. and centrifuged. Samples frozen and thawed more than once were not used.

Neuraminidase. Two samples of neuraminidase (*N*-acetyl-neuraminate glycohydrolase, EC 3.2.1.18) were used: (1) receptor-destroying enzyme from *Vibrio cholerae*, bought in 25 ml. portions from Burroughs Wellcome and Co. (Beckenham, Kent). This enzyme solution was used without dilution; (2) partially purified neuraminidase (20–40% purity) was a gift from Dr G. L. Ada. One ampoule contained 30000 units of biological activity, as defined by French & Ada (1959) and Ada, French & Lind (1961). Removal of sialic acid by neuraminidase was performed in acetate buffer (0.2M, pH 6.0) containing calcium acetate at a concentration of 10 mM, this solution being referred to below as 'neuraminidase buffer'.

EXPERIMENTAL AND RESULTS

Interaction between chondromucoprotein and plasma fractions. The experiment described in the legend to Fig. 1 shows the inability of native orosomucoid to form an insoluble complex with chondromucoprotein, compared with the complex-forming abilities of plasma proteins with higher isoelectric points.

Interaction between chondromucoprotein and sialic acid-deficient glycoproteins. Sialic acid-deficient orosomucoid. When sialic acid residues were removed from orosomucoid by mild acid hydrolysis

Fig. 1. Effect of pH on the formation of insoluble complexes between chondromucoprotein and plasma proteins. To the protein fraction (○, 20 mg. of γ -globulin; ●, 10 mg. of albumin; □, 5 mg. of fibrinogen; ■, 5 mg. of orosomucoid), dissolved in 0.5 ml. of 0.9% NaCl, was added 0.01 ml. of chondromucoprotein solution (31.2 μ g. of chondroitin sulphate), followed by 3 ml. of an acetate buffer (0.2M) of pH between 2.8 and 6.3.

Fig. 2. Effect of pH on complex-formation between chondromucoprotein and orosomucoid, before (○) and after (Δ) the hydrolytic removal of sialic acid. Orosomucoid (10 mg.) was placed in each of two tubes (B and T). To tube B was added 10 ml. of 0.9% NaCl. To tube T was added 5 ml. of 0.9% NaCl, followed by 5 ml. of 0.2N-H₂SO₄. Each tube was then heated at 80° for 1 hr. To 1 ml. portions from each mixture were added 0.1 ml. of chondromucoprotein solution (36.4 μ g. of chondroitin sulphate) and 0.9 ml. of 0.9% NaCl. The complexes were then precipitated by adding to each tube 3 ml. of a 0.2M-acetate buffer of pH between 3.0 and 6.3, and the pH values of the final mixtures determined.

(0.1N-H₂SO₄ at 80° for 1 hr.), the modified glycoprotein formed an insoluble complex with chondromucoprotein, maximum amounts being precipitated at pH 3.4–3.8 (Fig. 2). When the sialic acid residues were gradually removed from orosomucoid, whether by acid hydrolysis or neuraminidase action, the ability of the modified glycoprotein to form complexes with chondromucoprotein at pH 3.8 was progressively increased (Figs. 3 and 4 respectively). In Fig. 5 is shown the formation of insoluble complexes between sialic acid-deficient orosomucoid and chondromucoprotein, as a function of chondromucoprotein concentration.

Sialic acid-deficient seromucoid. When sialic acid residues were progressively removed from seromucoid by neuraminidase action, the ability of the modified seromucoid fraction to form complexes with chondromucoprotein was increased (Fig. 6).

Sialic acid-deficient urine glycoprotein and fraction VI. In Table 1 are compared the complex-forming abilities of sialic acid-deficient orosomucoid, urine glycoprotein and fraction VI at pH 3.5 and 4.7.

Inhibitory effect of calcium ions and EDTA on the formation of chondromucoprotein-protein complexes. The inhibitory effect of Ca²⁺ ions on the formation of complexes between chondromucoprotein and sialic acid-deficient orosomucoid is shown in Fig. 7. This Figure also contains, for comparison, the inhibitory effect of Ca²⁺ ions on the formation of complexes between chondromucoprotein and serum proteins. When a similar experiment was carried out with EDTA in place of Ca²⁺ ions, the formation of insoluble complexes was inhibited at a concentration of EDTA of 0.07M.

The inhibitory effect of 0.2M-Ca²⁺ ions on the formation of complexes between chondromucoprotein and other sialic acid-deficient glycoprotein fractions (urine glycoprotein and fraction VI) is shown in Table 1.

DISCUSSION

The pH range in which a protein interacts electrostatically with anionic chondromucoprotein is related to its isoelectric point, a net positive charge

Fig. 3. Complex-formation at pH 3.8 between chondromucoprotein and orosomucoid after the progressive hydrolytic removal of sialic acid. Orosomucoid (8mg.) was dissolved in 2ml. of 0.9% NaCl, and 2ml. of 0.2N-H₂SO₄ added. A portion (0.05ml.) was immediately removed and free sialic acid estimated. A further portion (0.5ml.) was also removed and 0.1ml. of chondromucoprotein solution (36.4μg. of chondroitin sulphate) added, followed by 4ml. of acetate buffer (0.2M, pH 4.1). The complexes, precipitated at the final pH of 3.8, were measured turbidimetrically. The remainder of the orosomucoid solution in 0.1N-H₂SO₄ was then heated at 80°, 0.05ml. portions were removed at intervals and free sialic acid was determined. Portions (0.5ml.) were also removed at intervals and the extent of complex-formation with chondromucoprotein was determined as described above. O, Complex-formation between chondromucoprotein and orosomucoid; Δ, hydrolytic release of sialic acid from orosomucoid.

Fig. 4. Complex-formation at pH 3.8 between chondromucoprotein and orosomucoid after the progressive enzymic removal of sialic acid. Orosomucoid (10mg.) was dissolved in 5ml. of neuraminidase buffer. Neuraminidase (7500 units), dissolved in 0.5ml. of 0.9% NaCl, was added. A portion (0.05ml.) was immediately removed and free sialic acid estimated. A further portion (1.0ml.) was removed and 0.2ml. of chondromucoprotein solution (72.5μg. of chondroitin sulphate) added, followed by 4ml. of acetate buffer (0.2M, pH 3.0). The complexes, precipitated at pH 3.8, were measured turbidimetrically. The remainder of the orosomucoid solution was incubated at 37°, portions (0.05ml.) were removed at intervals and free sialic acid was estimated. Portions (1ml.) were also removed at intervals and the extent of complex-formation with chondromucoprotein determined as described above. O, Complex-formation between chondromucoprotein and orosomucoid; Δ, enzymic removal of sialic acid from orosomucoid.

Fig. 5. Complex-formation at pH 3.8 between chondromucoprotein and sialic acid-deficient orosomucoid as a function of chondromucoprotein concentration. To a solution of orosomucoid (8mg.) in 4ml. of neuraminidase buffer was added 0.4ml. of neuraminidase solution (6000 units). The mixture was incubated at 37° for 17hr., after which time 96% of the sialic acid had been enzymically removed. To 0.5ml. portions were added varying amounts of chondromucoprotein solution (0–125 μg. of chondroitin sulphate), dissolved in 1 ml. of 0.9% NaCl, followed by 2 ml. of acetate buffer (0.2M, pH 3.0). The complexes, precipitated at the final pH of 3.8, were measured turbidimetrically (○). The complexes were then centrifuged down, washed with acetate buffer (0.1M, pH 3.8) and dissolved in 1 ml. of 0.1N-NaOH, and hexuronide was estimated (□).

Fig. 6. Complex-formation at pH 3.8 between chondromucoprotein and seromucoid after the progressive enzymic removal of sialic acid. Seromucoid (10mg.) was incubated at 37° with 2ml. of neuraminidase buffer. The fraction dissolved to give a slightly turbid solution. Receptor-destroying enzyme (0.5ml.) was then added. A portion (0.05ml.) was immediately removed and free sialic acid estimated. A further portion (0.3ml.) was also removed and 0.5ml. of chondromucoprotein solution (31.2 μg. of chondroitin sulphate) added, followed by 1.5 ml. of acetate buffer (0.2M, pH 3.0). The complex, precipitated at the final pH of 3.8, was measured turbidimetrically. The remainder of the seromucoid solution was incubated at 37°, portions (0.05ml.) were removed at intervals and free sialic acid was estimated. Portions (0.3ml.) were also removed at intervals and the extent of complex-formation with chondromucoprotein was determined as described above. Δ, Removal of sialic acid from seromucoid; ○, increase in complex-formation between sialic acid-deficient seromucoid and chondromucoprotein.

Table 1. *Complex-formation between chondromucoprotein and glycoprotein fractions after the enzymic removal of sialic acid*

A solution of the glycoprotein fraction (5mg.) in neuraminidase buffer (1ml.) was divided into two halves. To one-half was added receptor-destroying enzyme (0.125 ml.) which had previously been inactivated by heat (at 100° for 10 min.). To the other half was added 0.125 ml. of the active enzyme. Each mixture was incubated at 37° for 17hr. and free sialic acid estimated in 0.05 ml. portions (column 2). To the remaining incubation mixtures was added 0.4 ml. of chondromucoprotein solution (125 μg. of chondroitin sulphate), and the complexes were precipitated at pH 3.5 by the addition of 2 ml. of acetate buffer (0.2M, pH 3.0). These were measured turbidimetrically, the amounts formed from the native and sialic acid-deficient glycoprotein being shown in columns 3 and 4 respectively. In column 5 are shown the calculated percentage increases in complex-formation after the removal of sialic acid. Calcium chloride was then added to these mixtures at a final concentration of 0.2M, and the percentage decreases in complex-formation between chondromucoprotein and the sialic acid-deficient glycoproteins are shown in column 6. When complex-formation was done by the same method at pH 4.7 instead of pH 3.5, the amounts of complexes formed between chondromucoprotein and sialic acid-deficient glycoproteins were decreased by the percentages shown in column 7.

Complex-formation with chondromucoprotein

Glycoprotein fraction	Sialic acid removed (μg.)	At pH 3.5				At pH 4.7
		From native glycoprotein	From sialic acid-deficient glycoprotein	Increase after removal of sialic acid	Decrease in presence of 0.2M-Ca ²⁺	Decrease at pH 4.7 compared with pH 3.5 (%)
		(E)	(E)	(%)	(%)	
Orosomucoid	14.6	0.048	0.248	416	82	87
Urine glycoprotein	13.2	0.182	0.386	112	74	63
Fraction VI	13.9	0.584	0.870	49	84	85

Fig. 7. Inhibitory effect of Ca^{2+} ions on the formation of insoluble complexes between chondromucoprotein and sialic-deficient orosomucoid at pH 3.6 (O), and between chondromucoprotein and serum proteins at pH 5.2 (Δ).

Complex-formation with sialic acid-deficient orosomucoid. Orosomucoid (8mg.) was dissolved in 2ml. of 0.9% NaCl, 2ml. of 0.2N- H_2SO_4 added and the solution heated at 80° for 45 min. to hydrolyse sialic acid. Portions (0.5 ml.) were then taken and 0.1 ml. of chondromucoprotein solution (36.4 μg . of chondroitin sulphate) was added. Into each tube was placed 0, 0.25, 0.5 or 1.0 ml. of 1M- CaCl_2 and the volumes in each tube were made up to 1.6 ml. with 0.9% NaCl. Complexes were precipitated at pH 3.6 by the addition of 4 ml. of 0.2M-acetate buffer (pH 4.1) and measured turbidimetrically.

Complex-formation with serum proteins. Into a series of tubes were added 0.5 ml. of human serum and 0.1 ml. of chondromucoprotein solution (36.4 μg . of chondroitin sulphate). Into each tube was placed 0, 0.125, 0.25, 0.5 or 1.0 ml. of 1M- CaCl_2 and the volume in each tube was made up to 3.6 ml. with 0.9% NaCl. Complexes were precipitated at pH 5.2 by the addition of 4 ml. of 0.2M-acetate buffer (pH 5.2) and measured turbidimetrically.

facilitating electrostatic interaction (Fig. 1). Complex-formation with albumin was decreased above its isoelectric point of pH 4.8, whereas more basic proteins, such as fibrinogen and γ -globulins isoelectric at pH 5.8 (Phelps & Putnam, 1960) and above pH 6.5 (Pennell, 1960) respectively, interacted with chondromucoprotein under more alkaline conditions and formed complexes at pH 5.2 where electrostatic interaction with albumin was impossible.

Complex-formation below pH 2.5 was much decreased, indicating that carboxylic acid groups on the chondroitin sulphate moiety of chondromucoprotein were largely responsible for the formation of complexes, since at this pH and below, these

groups were undissociated. It would not appear that sulphate groups are primarily involved in complex-formation, since at pH 2.5 they remained dissociated. For a protein to interact electrostatically with chondromucoprotein it must therefore possess a sufficiently strong net positive charge above pH 2.5, and it is evident that orosomucoid, isoelectric at pH 2.7 (Schmid, 1953), does not fulfil these conditions and cannot take part in complex-formation (Fig. 1).

If complex-formation is due primarily to an electrostatic interaction, it should be possible by rendering a protein more basic to facilitate complex-formation with chondromucoprotein at higher pH values. Glycoproteins provide convenient models since their low isoelectric points, mainly owing to dissociated carboxylic acid groups on terminal sialic acid residues, are inversely related to their sialic acid contents, selective removal of the latter decreasing their acidity (Winzler, 1960).

Sialic acid-deficient orosomucoid, isoelectric at pH 5 (Popenoe & Drew, 1957), should therefore possess above pH 2.5 the positive charge necessary for electrostatic interaction with chondromucoprotein, and the experiment shown in Fig. 2 indicates that this was so. Complex-formation above pH 5, when sialic acid-deficient orosomucoid functioned as an anion, was negligible. Similar results were obtained for albumin (Fig. 1) and methaemalbumin (Anderson, 1965a), which are also isoelectric near pH 5.

The gradual removal of sialic acid from orosomucoid, by mild acid hydrolysis (Fig. 3) or neuraminidase (Fig. 4), progressively increased its complex-forming ability with chondromucoprotein at pH 3.8, similar results being obtained with serumucoid (Fig. 6), urine glycoprotein and fraction VI (Table 1). An inverse relationship therefore exists between the sialic acid content of glycoproteins and their ability to form insoluble complexes with chondromucoprotein, agreeing with the hypothesis that these interactions are primarily electrostatic. Partial removal of sialic acid was sufficient to initiate electrostatic interaction, and the turbidities formed on adding chondromucoprotein to the modified glycoprotein at pH 3.8 provided a reliable estimate of its sialic acid content.

At high concentrations, chondromucoprotein inhibits chondromucoprotein-plasma protein complex-formation (Anderson, 1963, 1965a; Anderson & Lack, 1964), and a similar inhibitory effect was observed in the formation of complexes between chondromucoprotein and sialic acid-deficient orosomucoid (Fig. 5). For maximum precipitation of complexes it is therefore necessary that chondromucoprotein concentration, as well as pH, should be at optimum values. In experiments in which the concentration of chondromucoprotein was kept

constant, care was therefore taken that this concentration did not exceed the optimum value needed for maximum interaction with the protein under investigation.

Calcium ions inhibited complex-formation between chondromucoprotein and sialic acid-deficient glycoproteins and, as had been observed with ϵ -aminohexanoic acid (Anderson, 1963), complex-formation between chondromucoprotein and serum proteins (Fig. 7), providing additional evidence for the electrostatic nature of these interactions. Calcium ions probably compete more strongly than cationic proteins for negatively charged carboxylic acid groups on the chondromucoprotein molecule. The inhibitory effect of EDTA may be related to the competition of this negatively charged molecule with chondromucoprotein for reactive sites on the protein, probably the imidazolium, ϵ -ammonium and guanidinium groups, which are positively charged in acid solutions. At higher pH values these groups become less charged, accounting for the decreased ability of proteins to form a complex with chondromucoprotein above pH 6.5 (Fig. 1). ϵ -Aminohexanoic acid, Ca^{2+} ions and EDTA inhibit the actual formation of complexes; also their addition in appropriate amounts to complexes, suspended in buffers of pH values at which they were precipitated, causes their dissociation into soluble components. The ease of this dissociation indicates the relative weakness of the ionic bonds present in chondromucoprotein-protein complexes.

Small amounts of sialic acid in chondromucoprotein-plasma protein complexes (Anderson, 1963) partly derive from chondromucoprotein itself, the protein moiety of which may be a sialoprotein (Anderson, 1962). However, plasma proteins with less than 1% of sialic acid are present which, although classed as glycoproteins (cf. the nomenclature devised by Winzler, 1960), can form complexes with chondromucoprotein owing to their relatively high isoelectric points. The complex-forming properties of this group, which includes fibrinogen, lipoproteins and γ -globulins, were discussed by Anderson (1963) and Anderson & Lack (1964). Glycoproteins with sialic acid contents high enough to depress their isoelectric points below about pH 4 cannot form complexes with chondromucoprotein and are absent from complexes formed on adding chondromucoprotein to normal acidified plasma. These include the haptoglobins (Anderson, 1965a) and, as the present study shows, orosomucoid and glycoproteins in serumucoid and fraction VI.

Complexes formed between chondromucoprotein and sialic acid-deficient glycoproteins may have certain physiological implications. In plasma of patients with certain chronic diseases the sialic acid content of orosomucoid is sometimes only half the normal value (Schmid, Burke, Debray-Sachs &

Tokita, 1964). Chondromucoprotein added to such a plasma would be expected to interact with this glycoprotein, as with orosomucoid rendered sialic acid-deficient *in vitro* by acid hydrolysis or neuraminidase, the precipitated complexes containing abnormally high amounts of fucose, mannose and other glycoprotein components. Similar arguments apply to the composition of euglobin fractions (Anderson, 1965b).

If other sialic acid-deficient plasma glycoproteins are present, then complexes precipitated by chondromucoprotein may contain biologically active substances not normally present. Haptoglobins, for instance, cannot form complexes with chondromucoprotein (Anderson, 1965a), since they contain about 5.5% of sialic acid and have isoelectric points below pH 4.3. As sialic acid residues are not essential for haptoglobin activity (Rafelson, Cloarec, Moretti & Jayle, 1961), complexes precipitated by adding chondromucoprotein to abnormal plasma containing sialic acid-deficient haptoglobins may have haemoglobin-binding properties. Similar arguments can be advanced for the presence in these complexes of thyroxine-binding protein, whose activity is also independent of sialic acid residues (Blumberg & Warren, 1961).

Labile, negatively charged sialic acid residues evidently maintain glycoproteins in solution under conditions of pH and mucopolysaccharide concentration that precipitate other plasma proteins. Whether a disturbance in carbohydrate metabolism, producing sialic acid-deficient glycoproteins, causes a breakdown of this protective mechanism *in vivo* must await further knowledge of the composition of these abnormal mucosubstances.

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