Interactions Between Glycosaminoglycans and Proteins, with Particular Reference to a New Technique for Isolating Serum Glycoproteins

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1. Chondroitin sulphate-serum protein complexes (A, B and C), successively precipitated by adding chondroitin sulphate to serum at three arbitrary descending pH values (5.2, 4.3 and 3.1), were dissociated at pH 6.7 and chromatographed on DEAE-Sephadex, when the liberated serum proteins were simultaneously freed of chondroitin sulphate and separated into five fractions. Evidence that serum proteins were precipitated as a result of electrostatic interactions with dissociated carboxylate groups on the glycosaminoglycan is presented. 2. Serum proteins (fraction G), unable to form complexes with chondroitin sulphate, contained 4.4% of sialic acid and accounted for 4 and 26% of the total protein and protein-bound sialic acid in serum respectively. This fraction interacted electrostatically with chondroitin sulphate only when rendered more basic by removal of sialic acid residues with neuraminidase. The heat stability, solubility properties and high carbohydrate content of fraction G classified it as a seromucoid fraction. 3. Fraction G contained several glycoprotein and hexuronic acid-containing fractions, including a hitherto undetected brown-pigmented high-molecular-weight serum component, which migrated in starch gel between the origin and $S\alpha_2$ -globulin and contained 3.1 and 4.1% of sialic acid and hexuronic acid respectively. 4. Glycosaminoglycan-protein interactions are discussed in relation to protein fractionation. By prior removal of less acidic proteins by these interactions, a new technique is available for isolating serum seromucoids in higher yields and under milder conditions than existing methods.

An important property of glycosaminoglycans (acid mucopolysaccharides), nucleic acids and other polyanions, which may be relevant to their role in cells, connective tissue and tissue fluids, is their strong anionic character over a wide pH range that enables them under certain conditions of pH and ionic strength to interact electrostatically with, and precipitate, cationic proteins. This mechanism has been implicated in the spontaneous precipitation of plasma and serum euglobulins (Anderson, 1965a), and in the more pronounced precipitation of proteins on addition of glycosaminoglycans to acidified plasma or serum (Anderson, 1963).

These interactions may have value in fractionating charged high-molecular-weight compounds in tissue fluids, and in isolating biologically active trace substances from serum. Chondromucoprotein-plasma protein complexes precipitated at pH 4.4, for instance, contain albumin-bound haematin (methaemalbumin) that has peroxidase activity, whereas those precipitated at pH 5.2 are rich in plasminogen, fibrinogen and lipoproteins,

the last two proteins being the principal carriers of plasminogen *in vivo* (Anderson & Lack, 1964; Anderson, 1965b).

Little is known, however, of the identity of serum proteins precipitated by glycosaminoglycans at different pH values and, in particular, of serum proteins unable under any conditions to interact electrostatically with these polyanions. The present study describes the successive precipitation at three arbitrary descending pH values of chondroitin sulphate-protein complexes from serum. After dissociation of the complexes, the liberated serum proteins were freed of chondroitin sulphate by chromatography on DEAE-Sephadex and their physical and chemical properties examined.

Theoretical considerations about the nature of these electrostatic interactions led to the observation that proteins with low isoelectric points, such as orosomucoid and other glycoproteins with high sialic acid contents, cannot form insoluble complexes with glycosaminoglycans until rendered more basic by the hydrolytic or enzymic removal of

terminal sialic acid residues (Anderson, 1965c). Thus serum proteins unable to interact electrostatically with glycosaminoglycans should possess low isoelectric points and therefore consist mainly of glycoproteins.

This prediction is confirmed in the present study, and a new technique for isolating serum glycoproteins in higher yield and under milder conditions than existing methods is described. Some properties of this serum glycoprotein-rich fraction are discussed.

METHODS AND MATERIALS

Materials

DEAE-Sephadex. The anion exchanger DEAE-Sephadex A-50 (capacity 3.5 ± 0.5 m-equiv./g.; particle size, 40–120 μ), bought from Pharmacia, Uppsala, Sweden, was allowed to swell in 0.02 M-phosphate buffer, pH 6.7, for 24 hr. After being washed several times with the same buffer, it was packed under gravity into a chromatographic tube (1.5 or 2.2 cm. diam.) to form bed heights from 14 to 18 cm.

Neuraminidase. This was receptor-destroying enzyme from *Vibrio cholerae*, bought in portions (25 ml.) from Wellcome Research Laboratories, Beckenham, Kent, and used without dilution. Removal of sialic acid by neuraminidase was performed in 0.2 M-acetate buffer, pH 5.5, containing calcium acetate (10 mM), this solution being referred to below as 'neuraminidase buffer'.

Chondroitin sulphate. This was a preparation (batch no. 8340) bought from Koch-Light Laboratories Ltd., Colnbrook, Bucks. It contained 1.6% of protein.

Human chondromucoprotein. This was isolated from human cartilage (Anderson, 1962), solutions in 0.9% NaCl being prepared as described by Anderson (1965b).

Human serum. A non-haemolysed bulk sample from normal individuals was used.

Analytical methods

Protein, hexuronic acid, sialic acid, cholesterol, hexamine and hexose were estimated as described by Anderson (1963). Seromucoid, expressed as protein, was estimated as follows. The fraction under test was diluted to 5 ml. with 0.9% NaCl and 2.5 ml. of 1.8 N-HClO₄ added. After 10 min. precipitated proteins were removed by filtration through Whatman no. 50 filter paper and to 5 ml. of filtrate was added 1 ml. of 5% (w/v) phosphotungstic acid in 2 N-HCl. The seromucoid precipitate was centrifuged down after 10 min., washed with the phosphotungstic acid solution, dissolved in 1 ml. of 0.1 N-NaOH and estimated for protein.

Starch-gel electrophoresis. This was carried out by the method of Smithies (1955) in 0.3 M-borate buffer, pH 8.6, at 225 v for 17 hr. at 3.3 ma/gel. Proteins, detected by staining with 0.2% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid, were named by using the nomenclature suggested by Smithies (1955). A 40 μ l. sample of each fraction (4%, w/v) was applied to the gel.

Cellulose acetate electrophoresis. This was performed in barbiturate buffer, pH 8.6, at 200 v for 2 hr. at 1.8 ma/strip, proteins being detected with the above stain, and carbo-

hydrate-containing proteins with a periodic acid-Schiff stain (Keyser, 1964a). A 5 μ l. sample of each fraction (3%, w/v) was applied to the cellulose acetate.

Isolation, dissociation and chromatographic separation of chondroitin sulphate-serum protein complexes A, B and C

Isolation. Unless stated otherwise, operations were performed at 4°, and centrifugation at 16000g for 15 min. To 100 ml. of serum were added 850 ml. of water and 250 mg. of chondroitin sulphate dissolved in 50 ml. of water. The mixture was adjusted to pH 5.2 with 1 l. of 0.2 M-acetate buffer, pH 5.2. After 16 hr. the precipitate (complex A) was centrifuged down and washed with 0.1 M-acetate buffer, pH 5.2. Chondroitin sulphate (1.5 g., dissolved in 20 ml. of water) was added to the supernatant fluid and the pH lowered to 4.3 with acetic acid (13 ml.). After 16 hr. the precipitate (complex B) was centrifuged down and washed with 0.1 M-acetate buffer, pH 4.3. Chondroitin sulphate (200 mg. dissolved in 15 ml. of water) was added to the supernatant fluid and complex C was precipitated by adjusting the pH to 3.1 with acetic acid (210 ml.). After 16 hr. it was centrifuged down and washed with 0.1 M-acetate buffer, pH 3.1. The final supernatant fluid (S) was retained.

Dissociation. Complexes A, B and C, isolated as described above, were suspended in 26, 44 and 44 ml. respectively of 0.2 M-phosphate buffer, pH 8.5. After incubation at 37° they dissociated to form respectively orange-yellow, olive-green and white turbid solutions. These solutions were dialysed against 0.02 M-phosphate buffer, pH 6.7, the volumes after dialysis being 36, 96 and 60 ml. respectively. These solutions were estimated for protein, cholesterol, sialic acid and seromucoid (see the Results section) and stored deep-frozen.

Chromatographic separation. Complexes A, B and C, dissociated as described above, were thawed and incubated at 37° to redissolve material that may have come out of solution during storage. Appropriate portions were then applied to DEAE-Sephadex columns, excluded material (fraction 1) being washed through with 0.02 M-phosphate buffer, pH 6.7 (buffer 1), and retained. Adsorbed material was eluted successively with buffers 2–6, consisting of 0.02 M-phosphate buffer, pH 6.7, containing NaCl (0.1, 0.2, 0.4, 0.6 and 1.0 M respectively), thus providing fractions 2–6 respectively. Fractions 1–6, obtained from each complex, were dialysed against distilled water and freeze-dried.

Isolation and chromatographic separation of fraction G

Isolation. The final supernatant fluid (S), obtained as described above, contained serum proteins that were not precipitated by chondroitin sulphate. A portion (3 ml.) was retained for seromucoid estimation (see the Results section). The remainder (approx. 2.3 l.) was dialysed against distilled water until the pH had risen to 5.0, saturated with ammonium sulphate and stood at 18° for 16 hr. A white floating precipitate was skimmed off, and a light-brown suspended precipitate centrifuged down at 18°. The combined precipitates were suspended in 300 ml. of water and dialysed against distilled water. When the precipitates had dissolved and the solution was free of inorganic sulphate, non-

diffusible material was recovered by freeze-drying. The light-brown product (yield: 355 mg./100 ml. of serum) was termed fraction G.

Chromatographic separation. Fraction G (517 mg.) was suspended in 8 ml. of 0.02 M-phosphate buffer, pH 7.3, and the mixture incubated at 37° for 1 hr. The brown slightly turbid solution was dialysed against 0.02 M-phosphate buffer, pH 6.7, and chromatographed on a DEAE-Sephadex column under conditions identical with those used to fractionate the dissociated complexes A, B and C, as described above. The six fractions (G₁-G₆) were dialysed and freeze-dried.

Chromatography of chondroitin sulphate

Chondroitin sulphate (200 mg.) was dissolved in 7 ml. of 0.02 M-phosphate buffer, pH 6.7 (buffer A), dialysed against the same buffer and applied to a DEAE-Sephadex column. After excluded material (fraction 1) had been washed through with buffer A, adsorbed material was first eluted with 0.02 M-phosphate buffer, pH 6.7, containing NaCl (0.6 M) (buffer B) to yield fraction 2, and then with 0.05 M-phosphate buffer, pH 5.5, containing NaCl (1 M) (buffer C) to yield fraction 3. Effluents were examined for protein by measurement of *E*_{280 mμ}, and for chondroitin sulphate, by the method of Di Ferrante (1956), as follows. To 0.5 ml. of effluent were added 0.5 ml. of 0.2 M-acetate buffer, pH 4.0, and 1 ml. of cetyltrimethylammonium bromide solution (2.5%, w/v, in water). Precipitated chondroitin sulphate was measured turbidimetrically at 400 mμ, and the method made quantitative by reference to a standard curve obtained by treating known amounts of chondroitin sulphate with cetyltrimethylammonium bromide. Fractions 1, 2 and 3 were dialysed against distilled water and freeze-dried (yields: fraction 1, 0 mg.; fraction 2, 2.6 mg.; fraction 3, 152 mg.).

RESULTS

Chromatography of chondroitin sulphate on DEAE-Sephadex

The chromatogram is shown in Fig. 1. The excluded fraction (fraction 1) contained no measurable material, indicating the absence of basic components of high molecular weight in the chondroitin sulphate preparation. Fractions 2 and 3 contained 14.4 and 1.0% of protein respectively. Thus only 1% of the chondroitin sulphate preparation applied to the column was eluted with 0.02 M-phosphate buffer, pH 6.7, containing sodium chloride at concentrations of 0.6 M and below.

Complexes A, B and C

Chemical composition. Estimations were performed on portions from the solutions obtained after dialysis of the dissociated complexes A, B and C, the volumes after dialysis being 36, 96 and 60 ml. respectively (see Methods and Materials section). Protein, cholesterol, sialic acid and seromucoid

Fig. 1. Chromatogram obtained from the fractionation on DEAE-Sephadex of chondroitin sulphate (200 mg.). The effluent was collected in 5 ml. fractions. Compositions of the eluting buffers are described in the text. ----, Protein; —, chondroitin sulphate.

Table 1. Composition of chondroitin sulphate-serum protein complexes A, B and C precipitated successively from 100 ml. of serum at pH values 5.2, 4.3 and 3.1

Component	Complex A	Complex B	Complex C
Protein (g.)	0.53	5.2	0.53
Sialic acid (mg.)	2.0	11.6	12.0
Cholesterol (mg.)	120.0	144.0	29.0
Seromucoid (mg.)	34.0	4.0	8.0
Sialic acid (μg./mg. of protein)	3.8	2.2	23.0
Cholesterol (μg./mg. of protein)	226.0	28.0	55.0

contents of complexes A, B and C, precipitated from 100 ml. of serum, are shown in Table 1.

In the thiobarbituric acid reaction for estimating sialic acid in the lipid-rich complex A, correction was made (Warren, 1959) for interfering chromophores formed by unsaturated lipids. Complexes B and C formed chromophores with adsorption spectra (*E*_{max} 549 mμ) identical with that formed by authentic *N*-acetylneuraminic acid.

Chromatography of dissociated complexes. The chromatograms obtained when the dissociated complexes A, B and C were chromatographed on DEAE-Sephadex were qualitatively similar. The chromatogram obtained from complex A is shown in Fig. 2. Chemical compositions of the six fractions (A₁-A₆, B₁-B₆, C₁-C₆), obtained from the chromatography of each complex, are shown in Table 2. During the chromatography of complex B

red and yellow bands were observed, which were eluted in fractions B₂ and B₃ respectively. Their absorption spectra are shown in Fig. 3.

Electrophoretic separation of the fractions obtained from the chromatography of complexes A, B and C on DEAE-Sephadex. Protein fractions (1-6), obtained from the chromatography of dissociated complexes A, B and C on DEAE-Sephadex, were subjected to electrophoresis in starch gel. The proteins detected are listed in Table 3.

Fig. 2. Chromatogram obtained from the fractionation on DEAE-Sephadex of the dissociated chondroitin sulphate-serum protein complex A. The effluent was collected in 5 ml. fractions. Compositions of the eluting buffers are described in the text.

Fig. 3. Absorption spectra of coloured components eluted during the chromatography of the dissociated chondroitin sulphate-serum protein complex B, and of fraction G, on DEAE-Sephadex. —, Red component in fraction B₂, - · -, yellow component in fraction B₃; —, brown component in fraction G₁.

Fraction G

The chemical composition of fraction G is given in Table 4. The supernatant fluid (S), from which fraction G was isolated (see the Methods and Materials section), contained 232 mg. of seromucoid. Protein and carbohydrate-containing components of fraction G, detected after electrophoresis on starch gel and cellulose acetate, are listed in Table 5.

Solubility properties. Fraction G (0.5%, w/v, in 0.9% sodium chloride) remained soluble on heating at 100° for 10 min., and in the presence of dilute acids (0.6 M-perchloric acid, 0.2 M-sulphosalicylic acid), N-hydrochloric acid, M-phosphoric acid and 8 M-urea. It was partially precipitated by 5% (w/v) trichloroacetic acid and 0.2 M-lead acetate, and completely precipitated by 5% (w/v) phosphotungstic acid in 2 N-hydrochloric acid and saturated ammonium sulphate solution.

Chromatography of fraction G on DEAE-Sephadex. Table 4 contains the yields and chemical compositions of fractions G₁-G₆ obtained from the chromatography of fraction G on DEAE-Sephadex. Recovery from the column, by weight, was 72%. The absorption spectrum of fraction G₁, which was light-brown in colour, is shown in Fig. 3. The protein and protein-bound carbohydrate components detected after electrophoresis of fractions G₁, G₃ and G₄ in starch gel and on cellulose acetate are listed in Table 5.

Table 2. Percentage composition of fractions obtained from the chromatography of dissociated chondroitin sulphate-serum protein complexes A, B and C on DEAE-Sephadex

		Composition (%)		
	Fraction	Sialic acid	Hexuronic acid	Cholesterol
Complex A	1	0.3	0.5	13.7
	2	0.4	0.5	5.9
	3	0.3	0.5	2.0
	4	0.3	0.4	0.6
	5	—	—	—
	6	0.2	29.3	0
Complex B	1	0.1	0.2	0.1
	2	0.3	0.2	0.8
	3	0.1	0.2	0.2
	4	0.1	0.3	0.4
	5	0.1	1.0	0.1
	6	0.4	27.5	0
Complex C	1	0.3	0.6	0.1
	2	0.4	1.1	0.3
	3	0.2	0.5	0
	4	1.1	0.9	0.6
	5	1.4	—	—
	6	0.3	29.4	0

Table 3. *Composition of fractions obtained from the chromatography of dissociated chondroitin sulphate-serum protein complexes A, B and C on DEAE-Sephadex*

Components were identified by electrophoresis on starch gel; the nomenclature used is that of Smithies (1955).

	Fraction	Protein component
Complex A	1	γ -Globulin
	2	$\alpha\beta$ -Globulin
	3	β -Globulin
	4	$\alpha\beta$ -Globulin
	5	Nil
Complex B	1	γ -Globulin
	2	β -Lipoprotein, $\alpha\beta$ -globulin
	3	S α_2 -globulin, post-albumins, albumin
	4	S α_2 -globulin, β -globulin, post-albumins, albumin
	5	S α_2 -globulin, β -globulin, post-albumins, albumin
Complex C	1	X*
	2	$\alpha\beta$ -Globulin
	3	$\alpha\beta$ -Globulin, F α_2 -globulin, post-albumins, albumin
	4	$\alpha\beta$ -Globulin, β -globulin, post-albumins, albumin, pre-albumins
	5	Post-albumins (trace), albumin (trace)

* This component migrated as a broad diffuse band midway between the origin and S α_2 -globulin.Table 4. *Percentage composition of fraction G, and the yields and chemical compositions of fractions G₁-G₆ obtained from the chromatography of fraction G on DEAE-Sephadex*

Fraction	Yield		Composition (%)					
	(mg.)	(% of total fraction placed on column)	Protein	Sialic acid	Hexuronic acid	Cholesterol	Hexose	Hexosamine
1	135.0	26	69.3	3.1	4.1	0.2	7.4	10.8
2	21.7	4	48.9	2.3	1.0	0.6	10.2	—*
3	129.4	25	71.3	0.5	1.0	0.1	13.8	10.9
4	34.1	7	65.0	0.5	5.5	0.1	10.2	—*
5	18.7	4	40.9	2.2	9.7	0.2	7.8	—*
6	28.8	6	18.9	0.5	16.0	0	9.9	—*
Fraction G	—	—	57.3	4.4	3.8	0.4	7.9	11.5

* Not determined.

Table 5. *Electrophoretic composition of fraction G, and of fractions obtained from the chromatography of fraction G on DEAE-Sephadex*

	Electrophoretic method		
	Starch gel (stained for protein)	Cellulose acetate	
		(stained for protein)	(stained for carbohydrate)
Chromatographic fraction 1	X*	Immobile material	Immobile material
Chromatographic fraction 3	Post-albumins, albumin	Albumin, β -globulins	Nil
Chromatographic fraction 4	$\alpha\beta$ -Globulins, post-albumins	γ -Globulins (trace) α_1 -, α_2 - and β -globulins, albumin (trace)	α_1 - and α_2 -Globulins, β -globulins (trace)
Fraction G	X*, $\alpha\beta$ -globulins, post-albumins, albumin (trace), pre-albumins	Immobile material, α_1 -, α_2 - and β -globulins, albumin (trace)	Immobile material, α_1 -, α_2 - and β -globulins

* This component migrated as a broad diffuse band midway between the origin and S α_2 -globulin.

Fig. 4. Complex-formation between chondroitin sulphate and fraction G before (O) and after (Δ) the removal of terminal sialic acid residues by neuraminidase. Into two tubes (A and B) were placed 20 mg. of fraction G (containing 880 μg. of protein-bound sialic acid), 3 ml. of 0.9% NaCl and 5 ml. of neuraminidase buffer. To tube A was added 2 ml. of neuraminidase solution and to tube B was added 2 ml. of neuraminidase solution previously inactivated by heating at 100° for 10 min. Each tube was incubated at 37° for 17 hr., after which time estimation of free sialic acid in tube A indicated that 98% of the protein-bound sialic acid in fraction G had been liberated. To 0.5 ml. of each mixture (containing 1 mg. of fraction G) was added 1 ml. of an 0.2 M-acetate buffer (of pH value 5.8, 5.0, 4.5, 3.9 or 3.5) followed by 100 μg. of chondroitin sulphate (dissolved in 0.2 ml. of water). The complexes precipitated were measured turbidimetrically at 400 mμ.

Complex-formation between fraction G and chondroitin sulphate. Fraction G was only able to form an insoluble complex with chondroitin sulphate when sialic acid residues were first removed by neuraminidase (Fig. 4).

Complex-formation between fraction G and serum proteins. Compared with the complex-forming properties of chondroitin sulphate and chondromucoprotein between pH 3.5 and 6.0, fraction G did not form insoluble complexes with serum proteins. A comparison of the complex-forming properties of chondroitin sulphate, chondromucoprotein and fraction G with serum proteins at pH 5.2 is shown in Fig. 5.

Hexuronic acid-containing components in seromucoid precipitated from fraction G. To two tubes (A and B), containing 2 ml. of serum, were added: (A) 7.5 mg. of fraction G, containing 285 μg. of bound hexuronic acid; (B) 2 mg. of chondroitin sulphate, containing 660 μg. of bound hexuronic acid. A control tube (C) contained 2 ml. of serum only. After diluting the contents of each tube to 10 ml. with 0.9% sodium chloride, the seromucoid fractions were precipitated (see the Methods and Materials section) and estimated for hexuronic

Fig. 5. Complex-formation at pH 5.2 between serum proteins and chondroitin sulphate (O), chondromucoprotein (Δ) and fraction G (□). Euglobulin-free serum was prepared as follows. Serum (6 ml.) was diluted to 30 ml. with water and the euglobulin fraction precipitated by adding 30 ml. of 0.2 M-acetate buffer, pH 5.2. After centrifugation, the supernatant fluid was used as a source of serum proteins. To 0.6 ml. of the fraction under investigation (containing 0–100 μg.) was added 1 ml. of euglobulin-free serum. The complexes precipitated were measured turbidimetrically at 400 mμ.

acid. Seromucoids precipitated in tubes A, B and C contained 188, 120 and 60 μg. of hexuronic acid respectively. Subtraction of bound hexuronic acid in the serum control (tube C) showed that recoveries of bound hexuronic acid from fraction G (tube A) and chondroitin sulphate (tube B) were 128 and 60 μg. respectively. Thus recovery of chondroitin sulphate added to serum was 9%, whereas recovery of glycosaminoglycan material in fraction G was 45%. A subsidiary experiment under identical conditions showed that chondroitin sulphate in aqueous solution (0.02%, w/v) was not precipitated by either 0.6 M-perchloric acid or phosphotungstic acid.

DISCUSSION

Chemical and electrophoretic differences, in complexes A, B and C, precipitated successively from serum by chondroitin sulphate at the arbitrary descending pH values of 5.2, 4.3 and 3.1 (Tables 1–3), indicate that their formation involves electrostatic interactions between the anionic glycosaminoglycan and serum proteins positively charged at the precipitation pH. The complexes dissolve at pH 6.7 as a result of dissociation caused by charge reversal on the protein moiety. When the dissociated complexes were chromatographed on

DEAE-Sephadex the free highly anionic chondroitin sulphate, with molecular weight under 50000 (Mathews & Dorfman, 1952), was strongly retained at pH 6.7 and only eluted with sodium chloride concentrations above 0.6M (Fig. 1). Serum proteins, being less anionic and of generally higher molecular weight, were eluted at lower salt concentrations. The five serum protein fractions obtained from each complex (fractions 1-5; Fig. 2) were thus virtually free of chondroitin sulphate, the latter only eluting with M-sodium chloride (fraction 6; Table 2).

Since a protein requires a sufficiently strong positive charge for it to interact electrostatically with a glycosaminoglycan, precipitation of glycosaminoglycan-protein complexes is only maximal at pH values at least 0.5 unit below the isoelectric point of the protein. For instance, when chondromucoprotein was added to solutions of γ -globulins (isoelectric above pH 5.8) and albumin (isoelectric at pH 4.9), maximum precipitation of chondroitin sulphate-protein complexes occurred at pH 5.2 and 4.4 respectively (Anderson, 1965c). Similar pH relationships apply to the precipitation of nucleic acid-protein complexes (Hofstee, 1964).

Complex A, precipitated at pH 5.2, lacked albumin but was rich in 7S γ -globulins and lipoproteins, the fraction excluded from DEAE-Sephadex (fraction A₁) containing 13.7% of cholesterol. Complex B contained several protein fractions absent from complex A, including post-albumins, albumin and a yellow pigment (Fig. 3) in fraction B₃ (Table 3), which may be complexes of albumin and small coloured molecules previously observed in albumin-rich fractions separated on DEAE-cellulose (Sober, Gutter, Wyckhoff & Peterson, 1956). The absorption properties of the red component in fraction B₂ (Table 3) indicate the presence of transferrin, the iron-binding β -globulin described by Holmberg & Laurell (1945). S α_2 -globulin, the high-molecular-weight glycoprotein described by Wallenius, Trautman, Kunkel & Franklin (1957), also known as α_2 -macroglobulin and 19S α_2 -glycoprotein, was only present in complex B. All the serum 7S γ -globulins formed complexes with chondroitin sulphate at pH 4.3 and above, since they were absent from complex C. Complex C contained pre-albumin, although orosomucoid, which occupies the same position as pre-albumin on starch-gel electrophoresis (Poulik & Smithies, 1958), was absent since it cannot interact electrostatically with chondroitin sulphate owing to its low isoelectric point of pH 2.7 (Anderson, 1965c). The F α_2 -globulin was only detected in complex C (fraction 3). Complexes A, B and C contained 17% of the total perchloric acid-soluble proteins (seromucoid) in serum.

Methods for protein fractionation employing

electrostatic interaction with polyanions have advantages over those using organic solvents and salt fractionation. The necessity of maintaining low temperatures, for instance, is avoided. Also, since these interactions depend principally on the electric charge and not molecular weight of the protein, they are readily abolished by raising the pH above the isoelectric point of the protein and thus reversing its charge. The liberated protein, freed of the polyanion by chromatographic methods that take advantage of its high acidity and, with chondroitin sulphate, its low molecular weight, is recovered with no apparent change in solubility or electrophoretic behaviour. When proteins in a mixture have widely differing isoelectric points, their separation by electrostatic interaction with a polyanion is particularly effective. Chondromucoprotein added to a mixture of albumin and γ -globulins at pH 5.2, for instance, precipitates only the latter (Anderson, 1965c). Interactions between plasma proteins and other polyanions, including polyphosphates (Rane & Newhouser, 1954; Nitschmann, Rickli & Kistler, 1958) and nucleic acids (Hofstee, 1964; Pusztai, 1966), have also been studied, whereas Mathews (1965) has investigated interactions between glycosaminoglycans and solubilized collagen.

Decreased electrostatic interaction between glycosaminoglycans and cationic proteins at pH 3.5, and the absence of these interactions at pH 2.5 (Anderson, 1965c), are probably due to glycosaminoglycan carboxylate groups' becoming progressively less dissociated, causing changes in the tertiary structure of the molecule that sterically hinder the approach of cationic proteins to dissociated sulphate groups. Thus serum proteins isoelectric below about pH 4.5, most of which are glycoproteins, cannot theoretically form insoluble complexes with glycosaminoglycans since, although some may function as cations below pH 3.5, anionic sites on glycosaminoglycans are inaccessible.

It was confirmed in the present study that the serum protein fraction, unable to interact electrostatically with chondroitin sulphate, was rich in glycoproteins. This fraction, termed fraction G, comprised 4, 26 and 83% of the total protein, protein-bound sialic acid and perchloric acid-soluble proteins (seromucoid) in serum respectively. It contained protein-bound carbohydrates migrating at pH 8.6 with α_1 -, α_2 - and β -globulins; the small amount of protein migrating with the mobility of albumin (Table 5) probably contained glycoproteins that on paper electrophoresis at pH 8.6 migrate as α_1 -globulins (Poulik & Smithies, 1958). In common with orosomucoid and other glycoprotein fractions (Anderson, 1965c), fraction G interacted electrostatically with chondroitin sulphate only when rendered more basic by removal

of terminal sialic acid residues with neuraminidase (Fig. 4).

The heat stability, solubility properties and high carbohydrate content of fraction G classify it as a seromucoid fraction (Rimington, 1940; Winzler, 1955). The classical method for isolating serum seromucoids is first to precipitate serum proteins at pH 1.1 with perchloric acid or sulphosalicylic acid (Winzler, Devor, Mehl & Smyth, 1948), seromucoid being recovered from solution by freeze-drying or by precipitation with ammonium sulphate or phosphotungstic acid. This fraction, which contains at least 20 components including orosomucoid, haptoglobins, β_1 -seromucoid, β_2 -globulin and glycosaminoglycans, has been extensively studied (Winzler, 1960; Miettinen, 1961; Doležalová & Brada, 1964; Doležalová, Brada & Kočent, 1964) and its diagnostic significance in various diseases investigated (Lockey, Anderson & MacLagan, 1956; Keyser, 1964b; Anderson, 1965d). Isolated in this way, however, seromucoid is partially denatured and degraded since it is completely soluble only in alkali. Moreover, since at least 30% of serum seromucoids, mainly those in the α_2 -globulin fraction (Markham, 1955), are lost by co-precipitation with serum proteins during the initial precipitation with dilute acids (Winzler *et al.* 1948), the resulting seromucoid accounts for only 1 and 17% of the total protein and protein-bound sialic acid in serum respectively. Fraction G, on the other hand, was isolated under milder conditions at pH 3.1 and in higher yield. It may contain serum glycoproteins so far undetected, either because they are absent from the seromucoid fraction isolated by existing methods or, if present, they have suffered degradative changes and become biologically inactive owing to the drastic conditions used.

It is unlikely that the relatively large amounts of bound hexuronic acid in fractions G₁, G₄ and G₅, which were eluted with sodium chloride at a concentration of 0.6 M or less (Table 4), were present in the form of free chondroitin sulphate, since this glycosaminoglycan was strongly retained by DEAE-Sephadex and eluted only at salt concentrations above 0.6 M (Fig. 1 and Table 2). Since only 9% of free chondroitin sulphate added to serum was recovered in the seromucoid fraction, whereas recovery of glycosaminoglycan in fraction G under identical conditions was 46%, fraction G may contain a glycosaminoglycan-protein complex similar to cartilage chondromucoprotein (Muir, 1958; Anderson, 1962). Presumably, however, it was less anionic owing to a lower glycosaminoglycan-protein ratio, since it was unable to interact electrostatically with cationic proteins (Fig. 5).

The exclusion from DEAE-Sephadex of fraction G₁ suggests the presence in serum not only of protein-bound glycosaminoglycan but of glyco-

proteins with low isoelectric point and high molecular weight. Largely immobile on cellulose acetate electrophoresis, this brown-pigmented fraction migrated in starch gel as a broad diffuse zone midway between the origin and S α_2 -globulin. Owing to its heat stability, solubility properties and negligible cholesterol content it cannot contain the high-molecular-weight β -lipoprotein described by Silberman (1957), the only serum component so far identified in this region of starch-gel electrophoretograms. Obtained from serum in a yield of 93 mg./100 ml., fraction G₁ appears to represent a previously undetected serum protein fraction that is absent from serum seromucoids precipitated by classical methods (Schultze, Heide & Haupt, 1962).

The method of isolating the glycoprotein-rich serum seromucoid fraction by first precipitating less acidic serum proteins with a polyanion differs fundamentally from the classical method, since the latter does not rely on the anionic properties of glycoproteins but on their solubility in dilute acids due to the hydrophilic properties of hexose, hexosamine, fucose and sialic acid residues. This conclusion appears justified since, by increasing the isoelectric point of orosomucoid from 2.8 to 5.0 by removal of terminal sialic acid residues with neuraminidase, its solubility in perchloric acid was unaffected (Anderson, 1965d), although electrostatic interaction with chondroitin sulphate became possible (Anderson, 1965c).

A more detailed investigation of interactions between serum proteins and various polyanions would appear profitable, particularly in the isolation of serum glycoproteins where it is important that extremes of pH be avoided owing to the notorious lability of terminal sialic acid residues, which impart to glycoproteins their acidic properties and, in many instances, their biological activities.

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