The Formation of Chondromucoprotein-Fibrinogen and Chondromucoprotein- β -Lipoprotein Complexes

CHEMICAL AND FIBRINOLYTIC PROPERTIES

By A. J. ANDERSON

Department of Pathology, Institute of Orthopaedics (University of London), Brockley Hill, Stanmore, Middlesex

(Received 10 December 1962)

The literature contains numerous references to the formation of insoluble complexes when polysulphate and poly(carboxylic acid) macromolecules are added to various plasma and serum colloids. Chondroitin sulphate, for instance, precipitates complexes containing protein and cholesterol (Badin & Schubert, 1955; Kerby, Taylor & Langley, 1961). Heparin forms insoluble complexes with fibrinogen (Smith & Korff, 1957; Godal, 1961) and when added to plasma precipitates a complex containing fibrinogen and plasminogen (Green, 1962). The addition of hyaluronic acid (Serafini-Cessi, 1959) to guinea-pig serum forms precipitates containing fibrinolytic enzymes. Dextran sulphate (Walton, 1954) and sulphated starches (Bernfeld & Nisselbaum, 1956) form insoluble complexes with fibrinogen. The presence of β -lipoprotein in precipitates formed between serum and heparin and between serum and various sulphate esters (including those of amylopectin, amylose, dextran and cellulose) has been reported (Bernfeld & Nisselbaum, 1956; Burstein, 1956; Bernfeld, Donahue & Berkowitz, 1957; Oncley, Walton & Cornwell, 1957). The formation of a complex also occurs between plasma β -lipoproteins and mucopolysaccharides isolated from both atherosclerotic (Gerö, Gergely, Jakab, Székely & Virag, 1961) and normal (Amenta & Waters, 1960) human aorta. Fibrin, formed by the addition of thrombin to plasma, contains β -lipoproteins (Morrison, 1947).

Many of these interactions, however, have been studied with substances either not normally present in the body or present in low concentrations. A large part of the chondroitin sulphate in cartilage, for instance, exists as chondromucoprotein in which chondroitin sulphate is bound covalently to non-collagenous protein (Shatton & Schubert, 1954; Partridge, Davis & Adair, 1961). Free chondroitin sulphate may not normally exist in the body, since even proteolytic attack by papain on chondromucoprotein preparations fails to remove all the bound amino acids (Muir, 1958). Also, viscometric studies on the digestion of chondromucoprotein by papain and by plasmin suggest that the enzymic products are smaller chondromucoprotein units (for

which the name 'chondromucopeptide' is proposed) rather than free chondroitin sulphate (Anderson, 1962a).

It was decided to investigate whether chondromucoprotein and chondromucopeptide possessed properties similar to those of chondroitin sulphate and other polysulphate and poly(carboxylic acid) polymers. The present study shows that the addition of chondromucoprotein to plasma, under specified conditions, forms insoluble complexes that contain α -, β - and γ -globulins, β -lipoproteins, cholesterol, mucopolysaccharides and several components of the fibrinolytic system (fibrinogen, plasminogen and streptokinase proactivator). A preliminary report of part of this work has been published (Anderson, 1962c).

METHODS AND MATERIALS

Analytical methods

Total nitrogen, total hexose and sulphate were determined as described by Anderson (1961). Hexosamine and hydroxyproline were determined as described by Anderson (1962a). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with reconstituted freeze-dried serum (Glaxo Laboratories Ltd., Greenford, Middlesex) of known protein content as the standard. Hexuronic acid was determined by the carbazole method of Dische (1955), as modified by Bitter & Ewins (1961), with glucuronic acid as the standard. Cholesterol was determined by the Liebermann-Burchard method as modified by Zlatkiss, Zak & Boyle (1953), with cholesterol as the standard. Sialic acid was determined by the method of Warren (1959), with *N*-acetylneuraminic acid as the standard. The complexes, which contained interfering chromogenic material in the lipid moiety, were first extracted with hot acetone-ethanol (1:1, v/v) mixture and the determination was performed on the lipid-free material. Paper electrophoresis (Durrum, 1950; Flynn & De Mayo, 1951) was performed in 0.05M-barbiturate buffer, pH 8.6. Spectrophotometric results were expressed as extinction values at 280 m μ determined in cells of 1 cm. light-path in a Unicam (SP. 500) spectrophotometer.

Fibrinolytic and caseinolytic methods

These procedures were based on the fibrinolytic and caseinolytic methods of Anderson (1962b, d) and Norman

(1957) respectively. The following were used: borate-saline buffer (0.2M; pH 7.4), glycine buffer (0.1M; pH 9.5), urea solution (40%, w/v, in glycine buffer), bovine fibrinogen solution (10%, w/v, in borate-saline buffer), trichloroacetic acid (20%, w/v, in water), all prepared as described by Anderson (1962*d*).

Plasminogen. Human plasminogen was prepared as described by Anderson (1962*d*). Rabbit plasminogen was prepared from rabbit serum by an acetone-precipitation method (S. Y. Ali, personal communication).

Urokinase. A glycoprotein fraction isolated from normal human urine by a benzoic acid-adsorption method (Anderson & MacLagan, 1955) was used as a source of urokinase.

Streptokinase. Varidase (Lederle Laboratories Division, American Cyanamid Co., New York, U.S.A.) was dissolved in borate-saline buffer. This stock solution (2000 units/ml.) was stored at 4° and diluted to the desired concentration with borate-saline buffer before use.

Bovine thrombin. Bovine thrombin for topical use (Parke, Davis and Co., Detroit, Mich., U.S.A.) was dissolved in aq. 50% (v/v) glycerol. This stock solution (500 units/ml.) was stored at -20° and diluted to the desired concentration with borate-saline buffer before use.

Casein solution. A solution of light white soluble casein (British Drug Houses Ltd., Poole, Dorset) was used, at a concentration of 4% (w/v) in borate-saline buffer.

Perchloric acid. This was 10% (w/v) in water.

Papain. Papain powder (East African) was bought from Chas. Zimmermann and Co. Ltd. (Walmgate Road, Perivale, Middlesex).

Immunochemical methods

Antisera. Rabbits (800-900 g.) were injected intramuscularly with 20 mg. of complex A or of complex B (see the Results section) in sterile 0.9% NaCl (2 ml.). The injections were repeated after 7 days. After 16 days good antibody response had occurred and the rabbits were bled to death by cardiac puncture. The sera were separated and stored deep-frozen.

Agar diffusion. This was as described by Ouchterlony (1953).

Mucopolysaccharide and chondromucoprotein preparations used for experiments on the formation of complexes

Fractions A and 1A. The fractions were crude chondromucoprotein preparations from human cartilage isolated at pH 10.6 and 10.1 with glycine buffers (0.1M) at pH 11.5 and pH 11.0 respectively.

Subfraction 1. This fraction was the highly viscous chondromucoprotein constituent of fraction 1A which was not adsorbed when fraction 1A was chromatographed on diethylaminoethylcellulose columns.

Subfractions 2-6. These were the constituents of fraction 1A which were adsorbed on diethylaminoethylcellulose columns and could be progressively eluted by buffers of decreasing pH and increasing ionic strength. The detailed preparation and properties of these cartilage preparations were described by Anderson (1961, 1962*a*).

Rabbit chondromucoprotein. Pieces of rabbit-ear cartilage (approx. 3 mm.²) were homogenized in 15 g. lots with 0.1M-glycine buffer, pH 10.9 (50 ml.), at 4°. The homogenates (at pH 10.2) were pooled, dialysed exhaustively against water

and centrifuged, and 3 vol. of ethanol solution [95% (v/v) ethanol containing 1% (w/v) of potassium acetate and 1% (v/v) of acetic acid] was added to the supernatant fluid. The chondromucoprotein precipitate was centrifuged down, washed twice with ethanol and once with ether, and dried *in vacuo*. The yield was 2.2 g./100 g. wet wt. of cartilage (Found: protein, 71.0; hexuronide, 5.1%).

Chondroitin sulphate C. This was a gift from Dr A. Dorfman.

RESULTS

Formation of complexes between human chondromucoprotein and plasma proteins

The amount and composition of the insoluble complexes that were precipitated on adding mucopolysaccharides to diluted plasma depended on the pH (Fig. 1) and the amount of mucopolysaccharide (Figs. 2 and 3). Fig. 4 illustrates the formation of insoluble complexes on adding chondromucoprotein to bovine plasma fibrinogen. In view of these results the following method was developed for preparing the complexes.

Preparation of complexes. Complex A (chondroitin sulphate-human plasma protein complex) and complex B (subfraction 1-human plasma protein complex) were prepared at pH 4.4 as follows. All operations were carried out at 4°. To citrated blood-bank plasma (158 ml.) was added 7.5 mN-hydrochloric acid (1.422 l.). The resulting mixture (at pH 4.4) was centrifuged to remove the euglobulin precipitate, and the supernatant fluid was divided into two equal portions. To one portion was added 80 mg. of chondroitin sulphate (to form complex A) and to the other 80 mg. of subfraction 1

Fig. 1. Effect of pH on the formation of complexes between mucopolysaccharides and human plasma proteins. The contents of two series of tubes, containing human plasma (2 ml.) and water (18 ml.), were adjusted to various pH values (between 2.3 and 6.8) with 0.1N-HCl and the euglobulin precipitates were removed by centrifuging. To the resulting supernatant fluids from each series were added, dissolved in 0.9% NaCl (1 ml.): O, chondroitin sulphate (2 mg.); Δ, subfraction 1 (2 mg.). The resulting insoluble complexes were centrifuged down, washed with acetate buffer of the same pH, dissolved in 0.1N-NaOH (10 ml.) and the protein contents determined.

(to form complex B), both mucopolysaccharides being added as solutions in 0.9% sodium chloride (40 ml.). After standing for 2 hr. the insoluble complexes were centrifuged down and washed with 0.1M-acetate buffer, pH 4.4. They were suspended in water (100 ml.), dissolved by addition of 0.1N-sodium hydroxide to give pH 8, dialysed exhaustively against water and the light-yellow solutions freeze-dried. The yields of complexes A and B were 9.8 and 7.7 mg./ml. of plasma respectively.

Chemical properties. Analytical results on complexes A and B are presented in Table 1. If it is assumed that chondroitin sulphate contains 33% of hexuronic acid, complexes A and B contained 5 and 3% of mucopolysaccharide respectively. The lipid contents were determined as follows. The light-yellow complexes (25 mg.) were extracted three times with boiling acetone-ethanol (1:1, v/v) mixture, washed twice with ether and dried *in vacuo*. The yields of the resulting cholesterol-free white materials were 19.8 and 17.9 mg., showing that the untreated complexes A and B contained 21 and 28% of lipid respectively. Calculations based on results in Table 1 show that the lipid components of the complexes contained 51 and 47% of cholesterol

respectively. Application of the thiobarbituric acid reaction of Warren (1959) to the acetone-ethanol extracts, after evaporation to dryness and suspension in water, produced a chromophore ($E_{\max}$ at 532 m μ) due either to 2-deoxy-D-ribose or unsaturated lipids (Bernheim, Bernheim & Wilbur,

Fig. 3. Effect of chondromucoprotein (subfraction 1) concentration on the formation of complexes with human plasma proteins. Experimental details are given in the legend to Fig. 2, subfraction 1 being used in place of chondroitin sulphate. The resulting alkali solutions were assayed for: ○, protein; △, cholesterol; □, sialic acid. Sialic acid in the alkali solution (1 ml.) was determined after hydrolysis at 80° for 1 hr. with $N-H_2SO_4$ (0.3 ml.).

Fig. 2. Effect of chondroitin sulphate concentration on the formation of complexes with human plasma proteins. Human plasma (1 ml.) and water (9 ml.) were measured into a series of tubes and adjusted to pH 5.6 with $N-HCl$; the euglobulin precipitates were removed by centrifuging. Increasing amounts of chondroitin sulphate (0.2-2.5 mg.), dissolved in 0.9% NaCl (0.5 ml.), were added to the supernatant fluids. After incubation at 37° for 2 hr., the insoluble complexes were centrifuged down, washed once with 0.1M-acetate buffer, pH 5.6, dissolved in 0.1N-NaOH (5 ml.) and the solutions assayed for: ○, protein; △, cholesterol.

Fig. 4. Effect of chondromucoprotein (fraction A) concentration on the formation of complexes with bovine plasma fibrinogen. To a series of tubes containing bovine plasma fibrinogen (10 mg.) in 0.1M-phosphate buffer, pH 5.6 (10 ml.), were added increasing amounts of fraction A dissolved in 0.9% NaCl (0.5 ml.). After incubation at 37° for 2 hr., the insoluble complexes were centrifuged down, washed with phosphate buffer, dissolved in 0.1N-NaOH (5 ml.) and the protein contents determined.

1948). There was no absorption maximum at 549 m μ , indicating the absence of sialic acid.

Physical properties. Freeze-drying of the complexes caused considerable loss of solubility. Only 28% by weight of complex B dissolved in 0.9% sodium chloride, this extract containing 4.3% of the total cholesterol originally present. Both complexes, however, formed opalescent solutions in 0.1N-sodium hydroxide, and in *m*- ϵ -aminocaproic acid (6-aminohexanoic acid) at pH 7.5. Since this amino acid inhibits the activation of plasminogen (Alkjaersig, Fletcher & Sherry, 1958, 1959) and is important in the fibrinolytic system, its inhibitory effect on the formation of insoluble complexes was investigated (Fig. 5).

Protein and cholesterol contents of the precipitated complexes were expressed as percentages of the total amounts that were precipitated in the absence of this amino acid.

Electrophoretic properties. The only components detected in the complexes migrated with the mobilities of normal human serum globulins (α and β) and β -lipoproteins. Smaller amounts of γ -globulins, but no albumin or α -lipoproteins, were detected.

Immunochemical properties. A diagram of a typical agar-diffusion pattern, resulting from the reaction of antiserum to complex A with complex A, complex B and human fibrinogen, is shown in Fig. 6. Similar results were obtained with antiserum to complex B. Further evidence for the presence of fibrinogen in complex B was obtained from a quantitative precipitin reaction between human fibrinogen and antiserum to complex B (Fig. 7).

Table 1. *Percentage composition of chondroitin sulphate-human plasma protein (A) and subfraction 1-human plasma protein (B) complexes isolated at pH 4.4*

Experimental details are given in the text.

Constituent	Complex A	Complex B
Nitrogen	12.0	11.3
Protein*	58.4	57.6
Protein†	73.0	69.0
Sialic acid‡	0.7	0.9
Hexuronic acid	1.8	1.0
Hexosamine	3.3	3.1
Sulphur	3.5	1.5
Hexose	2.3	3.1
Collagen§	0.3	0.4
Cholesterol	10.7	13.1

* By the method of Lowry *et al.* (1951).

† Calculated by multiplying the total nitrogen, after subtracting hexosamine N (7.8%) and sialic acid N (4.5%), by 6.25.

‡ After removal of interfering lipid material with hot acetone-ethanol (1:1, v/v) mixture.

§ Calculated by multiplying the percentage concentration of hydroxyproline by 7.15 (on the assumption that collagen contains 14% of hydroxyproline).

Specificity of formation of complexes

Formation of complexes between human chondromucoprotein and various colloid fractions. The abilities of various colloid fractions to form a complex with chondromucoprotein (fraction A) are shown in Table 2. The results indicated that the principal

Fig. 5. Effect of ϵ -aminocaproic acid on the formation of insoluble complexes between chondromucoprotein (subfraction 1) and human plasma proteins. Human plasma (30 ml.) was diluted with water (270 ml.) and the mixture adjusted to pH 5.6 with N-HCl. The euglobulin precipitate was centrifuged down. Portions (4 ml.) of the supernatant fluid were measured into a series of tubes and solutions of ϵ -aminocaproic acid in water (0.5 ml.) added to successive tubes to give final amino acid concentrations between 0.1 and 1000 mM. To a control tube no amino acid was added. To each tube was added subfraction 1 (0.5 mg.), dissolved in 0.9% NaCl (0.5 ml.). After incubation at 37° for 2 hr., the insoluble complexes were centrifuged down, washed with 0.1M-acetate buffer, pH 5.6, dissolved in 0.1N-NaOH (5 ml.) and the solutions assayed for: O, protein; Δ, cholesterol.

Fig. 6. Diagram of agar-diffusion pattern of antiserum to complex A (4) reacting against: (1), complex A; (2), complex B; (3), human fibrinogen. A control, consisting of normal rabbit serum in (4), gave no precipitin lines.

plasma proteins forming insoluble complexes with chondromucoprotein were fibrinogen and β -lipoprotein.

Formation of complexes between human plasma proteins and colloids isolated from human cartilage. The complexing abilities of cartilage subfractions 2-6 (Anderson, 1962a), some of which contain

Fig. 7. Quantitative precipitin reaction between human fibrinogen and antiserum to complex B. Two series of tubes were set up containing increasing amounts of human fibrinogen (0-6.5 mg.) dissolved in 0.9% NaCl (0.5 ml.). Antiserum to complex B (0.1 ml.) was added to one series and to the other normal rabbit serum (0.1 ml.) was added. Both series were incubated at 37° for 2 hr. and their contents centrifuged. The precipitates were washed three times with 0.9% NaCl (5 ml.), dissolved in 0.1N-NaOH (0.5 ml.) and the protein contents determined. The Figure shows the protein contents of precipitates formed after addition of: O, antiserum to complex B; Δ , normal rabbit serum. The difference between the two curves ($\square$) represents the amount of antigen-antibody complex precipitated from antiserum to complex B by human fibrinogen.

proteins and sialoproteins in the absence of chondromucoprotein, were examined. The relationship (Table 3) between chondromucoprotein content (i.e. hexuronide, hexosamine, sulphate) and ability to form complexes indicates that the principal cartilage component responsible for reaction with plasma proteins was chondromucoprotein (subfraction 4), since the other subfractions, deficient in chondromucoprotein, showed in general less ability to react, particularly at pH 5.5.

Formation of complexes between rabbit chondromucoprotein and normal rabbit plasma proteins. Rabbit chondromucoprotein formed complexes with rabbit plasma proteins under conditions similar to those used to prepare the human complexes A and B. The rabbit complex was precipitated at pH 5.6, by using 5 mg. of chondromucoprotein for every 1 ml. of rabbit plasma. The yield was 6.7 mg./ml. of plasma (Found: protein, 60.4; hexuronide, 1.2; cholesterol, 16.4; sialic acid, 0.12 %).

Presence of components of the fibrinolytic system in the complexes

The immunochemical evidence for fibrinogen in complex B prompted a search for components of the fibrinolytic system.

Fibrinolytic enzymes and plasminogen activators. Fibrinolytic enzymes were absent since no visible fibrinolysis resulted when complex B (5 mg.) in borate-saline buffer (0.5 ml.) was incubated at 37° for 2 hr. with bovine fibrinogen solution (0.5 ml.) and bovine thrombin (0.05 ml.; 1 unit). Similar results were obtained when human plasminogen

Table 2. *Formation of complexes between human chondromucoprotein and various colloid fractions*

A suspension of the colloid (1 mg.) in 0.1M-phosphate buffer, pH 5.6 (1 ml.), was incubated at 37° for 3 hr. and centrifuged, and the supernatant fluid was added to phosphate buffer (9 ml.). A solution of fraction A (0.5 mg.) in 0.9% NaCl (0.05 ml.) was added, and any insoluble complex was centrifuged down, washed with phosphate buffer, dissolved in 0.1N-NaOH (3 ml.) and the protein content determined.

Colloid	Reference	Protein content of the precipitated complex (μ g.)
Bovine fibrinogen	—	216
Urine colloid fractions 3 and 4* (containing γ - and β -globulins)	Anderson, Lepper & Winzler (1960)	36
Urine colloid fraction 7* (containing α - and β -globulins)	Anderson <i>et al.</i> (1960)	7
Total normal urine colloids	Anderson <i>et al.</i> (1960)	0
Urine colloid fractions (RS1, UF-0 and R-1)	Boyce, King, Little & Artom (1958)	0
Normal urine glycoprotein	Anderson & MacLagan (1955)	0
Collagen (from compact bone)	—	0
Crystallized bovine plasma albumin	—	0
Deoxyribonucleic acid (from herring sperm)	—	0
Human serum orosomucoid	—	0
Cohn's plasma fraction VI (containing 50% of albumin and 50% of glycoprotein)	—	0
Ovomucoid	—	0

* These were separated from total normal urine colloids by chromatography on diethylaminoethylcellulose (Anderson *et al.* 1960).

Table 3. *Formation of complexes between human plasma proteins and colloid fractions isolated from human cartilage*

Details of the method of isolation of the cartilage fractions have been given by Anderson (1962*a*). Three lots of human plasma (20 ml.) were diluted with water (180 ml.) and adjusted to pH 3.5, 5.5 or 6.5 with *N*-HCl. The resulting euglobulin precipitates were centrifuged down and the supernatant fluids retained. To a portion (10 ml.) of each supernatant fluid was added the subfraction under investigation (1 mg.), dissolved in 0.9% NaCl (0.5 ml.). After incubation at 37° for 2 hr. the insoluble complexes were centrifuged down, washed with 0.1 *M*-acetate buffer (5 ml.) of the appropriate pH value, dissolved in 0.1 *N*-NaOH (5 ml.) and the protein contents determined.

Subfraction	Constituent of subfraction (%)			Amount of complex precipitated (as mg. of protein)		
	Hexuronide	Hexosamine	Sulphate	pH 3.5	pH 5.5	pH 6.5
2	0	2.6	3.2	0.09	0.15	0.15
3	0.5	3.8	5.0	0.03	0.15	0.13
4	12.6	17.6	10.8	1.60	4.58	0.38
5	3.2	6.6	4.7	0.30	3.12	0.45
6	2.4	4.0	4.5	0.33	1.62	0.62

(0.2 ml.) was added to the mixture before the addition of thrombin, indicating the absence of plasminogen activators.

Plasminogen. Plasminogen was detected in complex B after incubation with streptokinase, the plasmin formed being caseinolytic and fibrinolytic (Fig. 8).

Complex as plasmin substrate. The incubation of complex B with plasminogen activator (streptokinase or urokinase) resulted in proteolysis (fibrinogenolysis) owing to the presence in the complex of plasminogen and fibrinogen (Fig. 8).

Formation of complexes between rabbit plasma proteins and the chondromucopeptides produced by papain and plasmin attack on rabbit cartilage

The results presented above showed that rabbit chondromucoprotein formed insoluble complexes with normal rabbit plasma. Since injection of papain (Thomas, 1956; Bryant, Leder & Stetten, 1958) and plasmin (Lack, Anderson & Ali, 1961; Lack, 1961, 1962) into rabbits leads to partial proteolysis of the protein moiety of chondromucoprotein and release of chondromucopeptides into the blood and urine, it was decided to determine whether these products had similar properties.

Formation of complexes by chondromucopeptides released in vivo by papain. Chondromucopeptides, released into rabbit plasma after the injection of papain (Table 4), combined with plasma proteins to form a large euglobulin precipitate. After centrifuging and the addition of chondromucoprotein to the supernatant fluid, only traces of complexes formed, since most of the complex-forming plasma proteins had been precipitated in the euglobulin fraction. Traces of plasma chondromucoprotein normally present resulted in a small euglobulin precipitate, most of the proteins available appearing in complexes formed on the addition of chondromucoprotein.

Formation of complexes by chondromucopeptides released in vitro by plasmin. The complex-forming ability of chondromucopeptides, released when cartilage is incubated *in vitro* with plasmin (Lack & Rogers, 1958), was investigated as follows. Rabbit ears were pierced with sterile cork borers (diam. 1.2 cm.), the skin was removed and the resulting disks were washed four times with sterile 0.9% sodium chloride. After brief drying on filter paper they were weighed while still moist. Six flasks were set up (Table 5). After incubation, a sample of each digest was removed and centrifuged. Chondromucopeptide contents (as hexuronic acid), and the abilities of the chondromucopeptides in each sample to form complexes with rabbit plasma proteins, were then determined (Fig. 9). The results show a linear relationship between the amount of chondromucopeptides liberated and their ability to combine with plasma proteins.

DISCUSSION

The amount and composition of the complexes precipitated from plasma by chondroitin sulphate and chondromucoprotein depended on pH and mucopolysaccharide concentration. By adjustments of these two variables complexes consisting principally of β -lipoproteins or proteins can be isolated, the use of serum leading to fibrinogen-free complexes. Lipoprotein-free complexes were obtained by using fibrinogen solution in place of plasma or serum. Complexes A and B contained α -, β - and γ -globulins, lipid and mucopolysaccharide materials, the percentage compositions being respectively: 73.0, 69.0; 21.0, 28.0; and 5.0, 3.0. Sialic acid, none of which was bound to lipid material, originated from fibrinogen, plasminogen and β -lipoproteins, which are classed as glycoproteins (cf. the nomenclature adopted by Winzler, 1960). Part of the sialic acid in complex B was derived from chondro-

Fig. 8. Quantitative investigation of plasminogen in complex B.

Caseinolytic method. Increasing amounts of complex B (0–6 mg.) in borate-saline buffer (0.5 ml.) were added to a series of tubes, followed by streptokinase (0.5 ml.; 5 units) and casein solution (1 ml.). After incubation at 37° for 3 hr., perchloric acid (3 ml.) was added to precipitate undigested casein, the mixtures were filtered and the acid-soluble caseinolytic products released by plasmin were measured spectrophotometrically.

Fibrinolytic method. Increasing amounts of complex B (0–8.3 mg.) in borate-saline buffer (0.5 ml.) were added to a series of tubes, followed by bovine fibrinogen solution (0.5 ml.), streptokinase (0.5 ml.; 5 units) and bovine thrombin (0.05 ml.; 1 unit). After incubation at 37° for 3 hr., the tubes were placed in ice-water, and chilled urea solution (3 ml.) was added to dissolve undigested fibrin (about 2 hr.). Trichloroacetic acid (2 ml.) was added, the mixtures were filtered and acid-soluble peptides released by plasmin were measured spectrophotometrically. The extinction values O and Δ are proportional to the amount of plasminogen in complex B when fibrin and casein respectively were used as the plasmin substrate.

Complex B as plasmin substrate. Increasing amounts of complex B (0–20 mg.) in borate-saline buffer (2 ml.) were measured into two series of tubes (X and Y). Streptokinase (0.05 ml.; 100 units) was added to series X and borate-saline buffer (0.05 ml.) to series Y. After incubation at 37° for 3 hr., perchloric acid (3 ml.) was added, the mixtures were filtered and acid-soluble products of plasmin digestion were measured spectrophotometrically. The differences in extinction values (X – Y) (i.e. □) represent proteolysis (fibrinogenolysis) of complex B resulting from plasminogen activation by streptokinase.

mucoprotein (Anderson, 1961, 1962a). Although freeze-drying of the complexes in the preparative method caused some loss of solubility in 0.9% sodium chloride, probably owing to lability of the β-lipoprotein moiety (Oncley *et al.* 1957), they dissolved in dilute alkali and *m*-ε-aminocaproic acid, pH 7.5. At molar concentration this amino acid completely inhibited the formation of insoluble complexes, whereas at lower concentrations the complexes containing protein were more soluble than those containing β-lipoprotein.

Chondromucoprotein and chondromucopeptides, released from cartilage by papain and plasmin, were the main cartilage components responsible for the formation of complexes, the principal plasma proteins involved being fibrinogen and β-lipoproteins. This conclusion conformed with the finding that normal urine colloids, which contain most of the plasma proteins except fibrinogen and β-lipoproteins, formed insignificant amounts of insoluble complexes with mucopolysaccharides.

It was calculated that the addition of 1 mg. of chondroitin sulphate or chondromucoprotein to 1 ml. of plasma, containing 2 mg. of cholesterol, precipitates 9.8 and 7.7 mg. of complexes A and B respectively, which contain 52 and 50% respectively of the total plasma cholesterol.

Maximum formation of insoluble complexes at pH 5.0–5.6 may have resulted from ionic combination between cationic and anionic groups on the plasma proteins and chondromucoprotein respectively, negligible formation of complexes at pH 3 resulting from suppression of the ionization of the chondromucoprotein carboxyl groups. Sulphate groups appear to be inactive in the formation of complexes since at pH 3 they are still ionized. No insoluble complexes formed at pH 7, ionic combination being unlikely since plasma proteins and chondromucoprotein function as anions. However, simple salt formation cannot be the only mechanism, since at pH 5.0–5.6 other cationic plasma proteins did not form insoluble complexes. Since complexes A and B had almost identical properties the protein moiety of chondromucoprotein was probably not involved in the formation of complexes, small variations in composition of the complexes being due to differences in the chondroitin sulphate and chondromucoprotein moieties rather than to the combination of these mucopolysaccharides with different plasma components. Sialic acid residues would appear to be inactive since neither orosomucoid (12% of sialic acid) nor a urine glycoprotein fraction (4.4% of sialic acid; Klenk, Faillard & Lempfrid, 1955) formed insoluble complexes with chondromucoprotein. The formation of complexes therefore appears to be restricted to bond formations involving carboxylic acid groups on the mucopolysaccharide moiety of chondromucoprotein.

Although the groups on the plasma proteins involved in the formation of complexes are unknown, a consideration of certain unique properties of fibrinogen and β-lipoproteins may provide alternative mechanisms. The fibrinogen molecule, pictured as a linear array (length 475 Å) or 'triad' of three nodules (diameter 50–70 Å) held together by a thin thread (Hall & Slayter, 1959), is probably less stable than other plasma proteins. Similarly, β-lipoproteins, which have high particle weights,

Table 4. *Effect of the injection of papain on the formation of complexes between rabbit plasma proteins and rabbit chondromucoprotein*

Citrate-treated plasma (1 ml.), obtained from a rabbit (900 g.) 18 hr. after the injection of papain (20 mg. in 2 ml. of 0.9% NaCl), was diluted with water (9 ml.) and adjusted to pH 4.6 with 0.1 N-HCl. The euglobulin precipitate (PE) was centrifuged down and washed with 0.1 M-acetate buffer, pH 4.6. Rabbit chondromucoprotein (5 mg.), dissolved in 0.9% NaCl (0.5 ml.), was added to the supernatant fluid. The precipitated complex (PC) was washed with acetate buffer and retained. An identical experiment was performed with normal rabbit plasma, yielding a euglobulin precipitate (NE) and a chondromucoprotein-plasma colloid complex (NC). Protein, cholesterol and sialic acid were determined in the four precipitates.

Plasma	Plasma chondroitin sulphate* (mg./100 ml.)	Precipitate	Amount of constituent in the precipitates		
			Protein (mg.)	Sialic acid (μg.)	Cholesterol (mg.)
Normal	0.01	NE	0.16	13.7	0.10
		NC	3.20	46.0	0.41
18 hr. after injection of papain	2.70	PE	3.38	157.0	0.15
		PC	0.07	7.3	0.04

* Determined by a method involving mucopolysaccharide precipitation by cetyltrimethylammonium bromide (S. Y. Ali, personal communication).

Table 5. *Formation of complexes of rabbit plasma proteins with the chondromucopeptides released from rabbit-ear-cartilage chondromucoprotein after incubation in vitro with rabbit plasmin*

After the additions listed below to each flask, 0.9% NaCl (4 ml.) was added and the volumes were made up to 50 ml. with 0.2 M-phosphate buffer, pH 6.8. The cartilage was then added and the flasks were incubated in a rotator at 37° for 24 hr.

Flask no.	Additions	Cartilage added (g.)
1	None	0.75
2	Rabbit plasminogen (5.71 ml.; 1000 units)	0.74
3	Rabbit plasminogen (5.71 ml.; 1000 units) activated with human urokinase (1.0 ml.; 1000 units) at 37° for 10 min.	0.75
4	Human urokinase (1.0 ml.; 100 units)	0.73
5	ε-Aminocaproic acid (10 ml.; 2M)	0.74
6	ε-Aminocaproic acid (10 ml.; 2M) followed by rabbit plasminogen (5.71 ml.; 1000 units) and human urokinase (1.0 ml.; 100 units); this mixture was incubated at 37° for 10 min.	0.75

probably exist as relatively unstable micellar emulsions in which a protein film surrounds a lipid sphere (Cook & Martin, 1962), combination of the protein film with chondromucoprotein conceivably resulting in a less hydrophilic structure.

The complexes did not contain plasmin or plasminogen activators. Although labile plasminogen activators in blood (Fearnley, Balmforth & Fearnley, 1957) are present in the precipitates formed by the rapid addition of heparin to fresh cold plasma (Green, 1962), their presence in the complexes described in the present paper would not

Fig. 9. Relationship between breakdown of rabbit-cartilage chondromucoprotein *in vitro* by plasmin and the ability of the resulting chondromucopeptides to form complexes with rabbit plasma proteins. Rabbit-ear cartilage was incubated, as described in Table 4, with: Δ , rabbit plasminogen; $\square$, rabbit plasmin (formed by urokinase activation of plasminogen); $\bullet$, urokinase; $\blacktriangle$, ε-aminocaproic acid (final concn. 0.4M); $\blacksquare$, ε-aminocaproic acid (final concn. 0.4M), followed by urokinase and rabbit plasminogen. No additions were made to the flask designated by $\circ$. The amounts of chondromucoprotein liberated, expressed as hexuronide, were determined in centrifuged samples (0.2 ml.) of each digest. The ability of each centrifuged sample to form complexes with rabbit plasma proteins was investigated as follows. Rabbit plasma (20 ml.) was diluted with water (180 ml.), the pH adjusted to 5.6 with N-HCl and the euglobulin precipitate centrifuged down. To portions of the supernatant fluid (10 ml.) was added a centrifuged sample (0.2 ml.) of each digest. After incubation at 18° for 30 min., the insoluble complexes were centrifuged down, washed with 0.2 M-acetate buffer, pH 5.6, dissolved in 0.1 N-NaOH (5 ml.) and their protein contents determined.

be expected since no precautions were taken to stabilize them. Plasminogen, however, was detected, the plasmin formed by activation by streptokinase being fibrinolytic and caseinolytic. Since activation of human plasminogen by streptokinase first involves interaction of streptokinase with a human serum component (termed proactivator) to form an activator (Troll & Sherry, 1955), it is concluded that complex B contains the proactivator. The presence of plasminogen in complex B was not unexpected since the method of preparation resembled those used for plasminogen. These methods probably rely on the precipitation of complexes containing plasminogen by dilution and acidification of plasma (Blix, 1961), serum (Milstone, 1941) or Cohn's plasma fraction III (Kline, 1953). The solubility of complex B in ϵ -aminocaproic acid is also shared by plasminogen preparations (Alkjaersig *et al.* 1958, 1959). Further, the high affinity of plasminogen for fibrinogen (Blombäck & Blombäck, 1956) indicates that complexes containing fibrinogen would also contain plasminogen. In conformity with the findings that complex B contained fibrinogen, plasminogen and streptokinase proactivator, incubation with plasminogen activators (streptokinase and urokinase) resulted in fibrinogenolysis owing to the formation of plasmin.

Chondromucopeptides released *in vitro* from cartilage by plasmin (Lack & Rogers, 1958) formed complexes with plasma proteins. Inhibition of the activation of plasminogen by ϵ -aminocaproic acid resulted in negligible release of chondromucopeptide and no formation of complexes, whereas plasmin formed by cartilage activator or urokinase liberated complex-forming chondromucopeptides. In the absence of plasmin the unexpected release of complex-forming chondromucopeptides may have resulted from the action of a cartilage enzyme, inhibited by ϵ -aminocaproic acid, which breaks down chondromucoprotein at pH 6.8. This enzyme, with optimum activity at pH 5.0 (S. Y. Ali, personal communication), may be related to the protease liberated under hypo-osmotic conditions from embryonic chick cartilage (Lucy, Dingle & Fell, 1961).

Complexes containing plasma fibrinogen (with closely associated plasminogen and perhaps plasminogen activator), β -lipoproteins, chondromucoprotein and chondromucopeptides may normally circulate in a soluble form, acidification *in vitro* causing insolubility. Soluble complexes containing β -lipoproteins and fibrinogen have, in fact, been observed electrophoretically after the addition of heparin to plasma at physiological pH (Bernfeld *et al.* 1957), acidification to pH 6.8 causing precipitation (Green, 1962). A raised concentration of one or more plasma components, followed by passage of complexes through permeable blood vessel walls

to tissues, may result in precipitation *in vivo* in regions of acid pH. Alternatively, free β -lipoproteins and fibrinogen may be transported to mucopolysaccharide-containing tissues, the formation of complexes occurring *in situ*. The insoluble deposits in connective tissue (fibrinoid, sclerotic, hyaline and amyloid material), sometimes seen in certain diseases and which are qualitatively similar to the complexes described in the present paper, probably originate from precipitation of plasma fibrinogen or β -lipoprotein by connective-tissue mucopolysaccharides (Altshuler & Angevine, 1951; Brunson, Davis & Thomas, 1955; Schalloek & Schmidt-Matthiesen, 1956; Spector, 1960; Kennedy, 1962). A similar mechanism may explain the fibrinoid deposits sometimes observed in kidney glomeruli and blood vessels that follow the raised concentration of plasma chondromucoprotein in rabbits injected with papain or with plasmin (Spicer & Bryant, 1958; Lack, 1961). Breakdown of intercellular material may also be involved in the pathogenesis of atherosclerosis, since lipid deposits contain several plasma proteins, including β -lipoproteins (Faber, 1949; Gerö *et al.* 1961; Newman & Zilversmit, 1962), mucopolysaccharides (Taylor, 1953) and substances resembling fibrin (O'Neal, Curran & Thomas, 1959; Duguid, 1959).

SUMMARY

1. Chondroitin sulphate and chondromucoprotein, isolated from cartilage, formed insoluble complexes with blood proteins, mainly fibrinogen and β -lipoproteins, their composition depending on whether plasma or serum was used, the pH and the mucopolysaccharide concentration.

2. Complexes isolated from human plasma contained α -, β - and γ -globulins, β -lipoproteins, cholesterol, mucopolysaccharides, sialoproteins and components of the fibrinolytic system (fibrinogen, plasminogen and streptokinase proactivator). Incubation of the complexes with plasminogen activators (streptokinase and urokinase) formed caseinolytic, fibrinolytic and fibrinogenolytic plasmin.

3. Partially degraded chondromucoprotein (for which the name 'chondromucopeptide' is proposed), released *in vitro* and *in vivo* from rabbit cartilage by plasmin and papain respectively, formed insoluble complexes with rabbit plasma proteins.

4. The complexes were qualitatively similar to certain pathological tissue deposits (fibrinoid, sclerotic, hyaline and amyloid material), and possible mechanisms for deposition are discussed.

The author is grateful to Dr R. J. Winzler, Dr W. H. Boyce and Dr A. Courts for gifts of *N*-acetylneuraminic acid and orosomucoid, urine colloid fractions and collagen

respectively. Thanks are due to Dr C. H. Lack and Dr H. J. Rogers for helpful discussions. This research was partly aided by grants for equipment from the Dowager Countess Eleanor Peel Medical Research Trust and from the University of London.

REFERENCES

- Alkjaersig, N., Fletcher, A. P. & Sherry, S. (1958). *J. biol. Chem.* **233**, 86.
- Alkjaersig, N., Fletcher, A. P. & Sherry, S. (1959). *J. biol. Chem.* **234**, 832.
- Altshuler, C. H. & Angevine, D. M. (1951). *Amer. J. Path.* **27**, 141.
- Amenta, J. S. & Waters, L. L. (1960). *Yale J. Biol. Med.* **33**, 122.
- Anderson, A. J. (1961). *Biochem. J.* **78**, 399.
- Anderson, A. J. (1962a). *Biochem. J.* **82**, 372.
- Anderson, A. J. (1962b). *Biochem. J.* **83**, 7p.
- Anderson, A. J. (1962c). *Biochem. J.* **84**, 48p.
- Anderson, A. J. (1962d). *Biochim. biophys. Acta*, **57**, 154.
- Anderson, A. J., Lepper, M. H. & Winzler, R. J. (1960). *Biochem. J.* **77**, 581.
- Anderson, A. J. & MacLagan, N. F. (1955). *Biochem. J.* **59**, 638.
- Badin, J. & Schubert, M. (1955). *J. clin. Invest.* **34**, 1312.
- Bernfeld, P., Donahue, V. M. & Berkowitz, M. E. (1957). *J. biol. Chem.* **226**, 51.
- Bernfeld, P. & Nisselbaum, J. S. (1956). *Fed. Proc.* **15**, 220.
- Bernheim, F., Bernheim, M. L. C. & Wilbur, K. M. (1948). *J. biol. Chem.* **174**, 257.
- Bitter, T. & Ewins, R. (1961). *Biochem. J.* **81**, 43p.
- Blix, S. (1961). *Scand. J. clin. Lab. Invest.* **13** (Suppl. 58), 3.
- Blombäck, B. & Blombäck, M. (1956). *Ark. Kemi*, **10**, 415.
- Boyce, W. H., King, J. S., Little, J. M. & Artom, C. (1958). *J. clin. Invest.* **37**, 1658.
- Brunson, J. G., Davis, R. L. & Thomas, L. (1955). *Amer. J. Path.* **31**, 669.
- Bryant, J. H., Leder, I. G. & Stetten, D. (1958). *Arch. Biochem. Biophys.* **76**, 122.
- Burstein, M. (1956). *C.R. Acad. Sci., Paris*, **243**, 527.
- Cook, W. H. & Martin, W. G. (1962). *Canad. J. Biochem. Physiol.* **40**, 1273.
- Dische, Z. (1955). *Meth. biochem. Anal.* **2**, 313.
- Duguid, J. B. (1959). In *Connective Tissue, Thrombosis and Atherosclerosis*, p. 13. Ed. by Page, I. New York: Academic Press Inc.
- Durum, E. L. (1950). *J. Amer. Chem. Soc.* **72**, 2943.
- Faber, M. (1949). *Arch. Path.* **48**, 342.
- Fearnley, G. R., Balmforth, G. & Fearnley, E. (1957). *Clin. Sci.* **16**, 645.
- Flynn, F. V. & De Mayo, P. (1951). *Lancet*, ii, 235.
- Gerö, S., Gergely, J., Jakab, L., Székely, J. & Virag, S. (1961). *J. Atheroscler. Res.* **1**, 88.
- Godal, H. C. (1961). *Scand. J. clin. Lab. Invest.* **13**, 550.
- Green, J. (1962). *Biochem. J.* **84**, 74p.
- Hall, C. E. & Slayter, H. S. (1959). *J. biophys. biochem. Cytol.* **5**, 11.
- Kennedy, J. S. (1962). *J. Path. Bact.* **83**, 165.
- Kerby, G. P., Taylor, S. M. & Langley, N. M. (1961). *J. clin. Invest.* **40**, 1900.
- Klenk, E., Faillard, H. & Lempfrid, H. (1955). *Hoppe-Seyl. Z.* **301**, 235.
- Kline, D. L. (1953). *J. biol. Chem.* **204**, 949.
- Lack, C. H. (1961). *Ann. phys. Med.* **6**, 93.
- Lack, C. H. (1962). *Proc. roy. Soc. Med.* **55**, 113.
- Lack, C. H., Anderson, A. J. & Ali, S. Y. (1961). *Nature, Lond.*, **191**, 1402.
- Lack, C. H. & Rogers, H. J. (1958). *Nature, Lond.*, **182**, 948.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Lucy, J. A., Dingle, J. T. & Fell, H. B. (1961). *Biochem. J.* **79**, 500.
- Milstone, H. (1941). *J. Immunol.* **42**, 109.
- Morrison, P. R. (1947). *J. Amer. chem. Soc.* **69**, 2723.
- Muir, H. (1958). *Biochem. J.* **69**, 195.
- Newman, H. A. I. & Zilversmit, D. B. (1962). *J. biol. Chem.* **237**, 2078.
- Norman, P. S. (1957). *J. exp. Med.* **106**, 423.
- Oncley, J. L., Walton, K. W. & Cornwell, D. G. (1957). *J. Amer. chem. Soc.* **79**, 4666.
- O'Neal, R. M., Curran, J. & Thomas, W. A. (1959). *Fed. Proc.* **18**, 497.
- Ouchterlony, O. (1953). *Proc. 6th int. Congr. Microbiology, Rome*, p. 546.
- Partridge, S. M., Davis, H. F. & Adair, G. S. (1961). *Biochem. J.* **79**, 15.
- Schallock, G. & Schmidt-Matthiesen, H. (1956). In *Contemporary Rheumatology*, p. 121. Ed. by Goslings, J. & Van Swaay, H. Amsterdam: Elsevier Publishing Co.
- Serafini-Cessi, F. (1959). *Sperimentale*, **109**, 535.
- Shatton, J. & Schubert, M. (1954). *J. biol. Chem.* **211**, 565.
- Smith, R. T. & Korff, R. W. von (1957). *J. clin. Invest.* **36**, 596.
- Spector, W. G. (1960). In *Progress in the Biological Sciences in Relation to Dermatology*, p. 295. Ed. by Rook, A. Cambridge University Press.
- Spicer, S. S. & Bryant, J. H. (1958). *Amer. J. Path.* **34**, 61.
- Taylor, H. E. (1953). *Amer. J. Path.* **29**, 871.
- Thomas, L. (1956). *J. exp. Med.* **104**, 245.
- Troll, W. & Sherry, S. (1955). *J. biol. Chem.* **213**, 881.
- Walton, K. W. (1954). *Brit. J. Pharmacol.* **9**, 1.
- Warren, L. (1959). *J. biol. Chem.* **234**, 1971.
- Winzler, R. J. (1960). In *The Plasma Proteins*, vol. 1, p. 309. Ed. by Putman, F. W. London: Academic Press Inc. Ltd.
- Zlatkiss, A., Zak, B. & Boyle, A. J. (1953). *J. Lab. clin. Med.* **41**, 486.