The Effect of Chondromucoprotein on the Solubility and Peroxidatic Properties of Haemoglobin and Methaemalbumin in Aqueous Solution and Blood

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1. Insoluble complexes, formed by electrostatic interaction between chondromucoprotein and chromoproteins (haemoglobin, methaemalbumin), were studied by measurement of precipitated pigment and by decrease in peroxidatic activity, maximum formation from aqueous solution occurring at pH 4.0–4.6. 2. Chondromucoprotein did not form complexes with plasma haptoglobins and haptohaemoglobins under these conditions, and high concentrations had no significant effect on colorimetric estimates of serum haptoglobin, although the peroxidatic activity of haemoglobinaemic serum was depressed owing to formation of chondromucoprotein–methaemalbumin complex. 3. The complexes formed by interaction between chondromucoprotein and plasma proteins contain two protein-bound biologically active components (plasminogen, haematin), as a result of co-precipitation after interaction between their carriers and chondromucoprotein. The possible presence of other biologically active trace components is discussed. 4. The results are related to complex-formation between other plasma proteins and chondromucoprotein, and possible implications arising from the complex-forming properties of tissue and urine chondromucoprotein are referred to. It is concluded that the inability of chondromucoprotein to form complexes with normal urine proteins is due to a deficiency of fibrinogen, β -lipoproteins and chromoproteins, which, in plasma, form a large proportion of the proteins involved in complex-formation.

The addition to plasma of a solution of human-cartilage chondromucoprotein (Anderson, 1961, 1962) precipitates material consisting mainly of mucopolysaccharide, fibrinogen, lipoprotein, γ -globulins and certain components of the fibrinolytic system (plasminogen, streptokinase proactivator). The precipitates are termed 'chondromucoprotein–plasma-protein complexes', since a major factor in their formation appears to be an electrostatic interaction between negatively charged groups on the chondromucoprotein molecule and proteins that acquire net positive charges when the plasma pH is below their isoelectric points (Anderson, 1963).

It has been proposed that the ill-defined euglobulin fraction, which contains similar complexes and is precipitated spontaneously from acidified plasma, results from a similar interaction, the small amount of chondromucoprotein normally present *in vivo* acting as the precipitating anion. Euglobulins that are precipitated from plasmas with high chondromucoprotein concentrations, such as are produced after papain injection into rabbits, are

therefore correspondingly bulkier. The composition and amount of the euglobulins are therefore predictable and depend on the same conditions of pH and chondromucoprotein concentration that decide the formation of the chondromucoprotein–plasma-protein complexes (Anderson & Lack, 1964).

During this work it was observed that chondromucoprotein added to an extensively haemolysed plasma precipitates complexes containing red pigment with peroxidatic activity, no such precipitation occurring from normal plasma. The presence of chromoproteins with peroxidatic properties, which include haemoproteins (haemoglobin) and haematin-containing proteins (methaemalbumin), in plasma euglobulin fractions and in chondromucoprotein–plasma-protein complexes does not appear to have been established previously, and the present paper describes the effect of chondromucoprotein on these pigments, both in aqueous solution and blood.

The results are related to the formation of other chondromucoprotein–plasma-protein complexes re-

ported elsewhere, and to the significance of tissue and urine chondromucoprotein in relation to complex-formation *in vivo*. Interactions between chondromucoprotein, albumin and haematin, the last-named being a major catabolic product of extracellular plasma haemoglobin with peroxidatic activity, were also investigated and the results are discussed.

METHODS AND MATERIALS

Chromoproteins were determined by the alkaline-haematin method (Clegg & King, 1942), with as standard either human haemoglobin (L. Light and Co. Ltd., Colnbrook, Bucks.) or haematin hydrochloride (containing 8.86% of Fe) (British Drug Houses Ltd., Poole, Dorset). Protein and hexuronic acid were determined as described by Anderson (1962). Peroxidatic activity was estimated by the method of Lupovitch & Zak (1964), as described in Fig. 5, with *o*-dianisidine (L. Light and Co. Ltd.) as the hydrogen donor. Activity was expressed as extinction values of the transient green oxidation product measured at 690 m μ in a Unicam SP.500 spectrophotometer with cells of 1 cm. light-path. Electrophoresis was carried out with 5 cm.-wide strips of Whatman no. 3 paper with barbitone buffer, pH 8.6 and *I* 0.06, or phosphate buffer, pH 7.0 and *I* 0.10. A potential gradient of 6–7 v/cm. was applied for 17 hr. The strips were dried (at 70° for 15 min.) and stained both for protein (with an azocarmine stain) and peroxidatically active components (with an *o*-dianisidine stain). The azocarmine stain was prepared by dissolving 0.75 g. of Azocarmine B in 50 ml. of methanol and 10 ml. of acetic acid, the volume being made up to 100 ml. with water. Staining was carried out for 10 min., and the strips were washed in methanol-acetic acid (9:1, v/v) mixture followed by 10% (v/v) acetic acid. The *o*-dianisidine stain was prepared from the following three solutions: (i) a mixture of 5 ml. of 1N-NaOH, 50 ml. of 0.2 M-sodium acetate and 55 ml. of ethanol; (ii) a solution of *o*-dianisidine (10 mg.) in 2.5 ml. of 5 N-acetic acid; (iii) a solution of 0.5 ml. of 30% (w/v) hydrogen peroxide in 10 ml. of water. Solutions (ii) and (iii) were freshly prepared. Immediately before use the solutions were mixed by adding (ii) to (i), followed by (iii). The paper strips were dipped briefly into this reagent and air-dried. Regions of peroxidatic activity stained orange on a white background.

Human chondromucoprotein. Solutions of chondromucoprotein, isolated from human cartilage (Anderson, 1962), were prepared in 0.9% NaCl as described by Anderson & Lack (1964). Chondromucoprotein concentrations were expressed as μ g. of hexuronic acid/ml.

Human haemoglobin. Twice-crystallized native haemoglobin (L. Light and Co. Ltd.) was used as a solution in 0.9% NaCl, any trace of insoluble material (which did not amount to more than 5%, by wt.) being removed by centrifugation.

Human methaemalbumin. This was prepared from crystallized serum albumin (Armour Pharmaceuticals Ltd., Eastbourne, Sussex) by a modification of the method of Fairley (1941), as used by Allison & ap Rees (1957). An equal volume of haematin hydrochloride solution (0.1% in 0.01N-NaOH) was added to a human albumin solution

(1%, w/v, in 0.2 M-phosphate buffer, pH 7.4). Methaemalbumin remained soluble at pH values above 3.3.

Human plasma and serum. These were unhaemolysed bulk samples from normal individuals. They were stored

Fig. 1. Effect of pH on the interaction of chondromucoprotein with haemoglobin. Into each of six tubes were measured 2.5 ml. of haemoglobin solution (4.25 mg.), 2.5 ml. of 0.2 M-acetate buffer, pH as indicated, and 1 ml. of chondromucoprotein solution (200 μ g.). The complexes were centrifuged down, washed twice with 5 ml. of the appropriate buffer, dissolved in 3 ml. of 0.1N-NaOH and the solutions analysed for chromoprotein.

Fig. 2. Effect of pH on the interaction of chondromucoprotein with methaemalbumin and albumin. In one experiment, each tube received 0.4 ml. of methaemalbumin and 5 ml. of 0.2 M-acetate buffer, pH as indicated, followed by 0.2 ml. of chondromucoprotein solution (96 μ g.). The complexes were centrifuged down, washed twice with 5 ml. of the appropriate buffer and dissolved in 2 ml. of 0.1N-NaOH, and the solutions were estimated for haematin ($\circ$) and total protein (Δ). A second identical experiment was performed except that methaemalbumin was replaced by 0.2 ml. of 0.01N-NaOH and 0.1 ml. of human albumin solution (1.0 mg.). The turbidities formed on adding chondromucoprotein, measured by the extinction at 400 m μ , represent total precipitated protein ($\square$).

Fig. 3. Effect of chondromucoprotein concentration on the interaction of chondromucoprotein with haemoglobin. Into each of four tubes were measured 2.5 ml. of haemoglobin solution (4.25 mg.) followed by increasing amounts of chondromucoprotein (60 to 480 $\mu\text{g.}$) dissolved in 1 ml. of 0.9% NaCl. A fifth tube contained 1 ml. of 0.9% NaCl in place of chondromucoprotein solution. To each mixture was added 3.5 ml. of 0.2 M-acetate buffer, pH 4.6, the precipitated complexes were treated as described in Fig. 1 and the resulting solutions were estimated for chromoprotein.

Fig. 4. Effect of chondromucoprotein concentration on the interaction of chondromucoprotein with methaemalbumin and albumin. Methaemalbumin, as in Fig. 1, was added to each of five tubes. To each tube was added 5 ml. of 0.2 M-acetate buffer, pH 4.0, followed by increasing amounts of chondromucoprotein (12 to 288 $\mu\text{g.}$) dissolved in 1 ml. of 0.9% NaCl. A sixth tube contained 1 ml. of 0.9% NaCl in place of chondromucoprotein solution. The precipitated complexes were treated as in Fig. 2, and the resulting solutions estimated for haematin ($\circ$). A second identical experiment was performed with 0.1 ml. of 0.01 N-NaOH and 0.1 ml. of albumin solution (1.0 mg.) in place of methaemalbumin. Total precipitated protein was determined (Δ) as in Fig. 2.

frozen, and immediately before use were thawed, incubated at 37° for 30 min. and centrifuged. Samples frozen and thawed more than once were not used.

EXPERIMENTAL AND RESULTS

Interaction in aqueous solution between chondromucoprotein and chromoproteins

Effect of chondromucoprotein on the precipitation of haemoglobin and methaemalbumin. Chondromucoprotein was added to chromoprotein solutions at different pH values and the amount of pigment in the precipitates was estimated. Precipitation of complexes containing haemoglobin was maximum at pH 4.6 (Fig. 1). At concentrations of 0.01M, 0.1M and 1M, ϵ -aminohexanoic acid (ϵ -aminocaproic acid) inhibited the precipitation of chondromucoprotein-haemoglobin complex by 0, 17 and 97% respectively. The precipitation of chondromucoprotein-methaemalbumin complex (Fig. 2) closely paralleled the formation of chondromucoprotein-albumin complex, maximum precipitation of both complexes occurring at pH 4.0. At these

Fig. 5. Effect of incubation time on the peroxidatic activity of haemoglobin in the absence and presence of chondromucoprotein. Into each tube of two series (A and B) were measured 0.05 ml. of haemoglobin solution (12.5 $\mu\text{g.}$). To each tube of series A was added 0.05 ml. of 0.9% NaCl, and to each tube of series B 0.05 ml. of chondromucoprotein solution (2.25 $\mu\text{g.}$). The contents were thoroughly mixed. A control tube contained 0.1 ml. of 0.9% NaCl. The tubes were placed in an ice bath at 3°, and to each 5 ml. of cold *o*-dianisidine reagent was added, followed by 1 ml. of cold 0.4 N-H₂O₂, with thorough mixing after each addition. Periodically the clear mixtures were quickly transferred to a cuvette and extinctions read immediately against the contents of the control tube. The peroxidatic activities (E_{690mμ}) of haemoglobin in the absence ($\circ$) and presence (Δ) of chondromucoprotein were thus measured. The percentage inhibition of peroxidatic activity by chondromucoprotein is also shown ($\square$).

Fig. 6. Effect of chondromucoprotein concentration on the peroxidatic activity of haemoglobin. Into each tube of a series were measured 0.5 ml. of haemoglobin solution (100 μ g.) and 0.5 ml. of chondromucoprotein solution (2 to 60 μ g.). A control tube contained 0.5 ml. of 0.9% NaCl in place of chondromucoprotein solution. Peroxidatic activities were estimated in 0.1 ml. samples. The peroxidatic activity of haemoglobin in the presence of increasing amounts of chondromucoprotein was thus measured ($\circ$), and the calculated percentage decrease in catalytic activity is also shown (Δ). Under these conditions chondromucoprotein inhibited the peroxidatic activity of methaemalbumin also.

optimum pH values increasing amounts of complexes formed as chondromucoprotein was added to the chromoprotein solutions, amounts in excess of certain concentrations resulting in diminished precipitation (Figs. 3 and 4).

Effect of chondromucoprotein on the peroxidatic activity of haemoglobin and methaemalbumin. The effect of incubation time on the peroxidatic activity

Fig. 7. Effect of chondromucoprotein on the peroxidatic activity of increasing concentrations of haemoglobin. Haemoglobin solutions (0.5 ml., containing 20 to 100 μ g.) were measured into each tube of two series (A and B). To each tube of series A was added 0.5 ml. of 0.9% NaCl, and to series B 0.5 ml. of chondromucoprotein solution (20 μ g.). Peroxidatic activities were estimated in 0.1 ml. samples. The peroxidatic activities of haemoglobin in the absence ($\circ$) and presence (Δ) of chondromucoprotein were thus measured, and the calculated percentage decrease in peroxidatic activity is also shown ($\square$).

Table 1. *Inhibition of the peroxidatic activity of haemoglobin by various colloid fractions*

To a solution of the colloid (200 μ g. in 0.5 ml. of 0.9% NaCl) was added 0.5 ml. of haemoglobin solution (100 μ g.). A control tube contained 100 μ g. of haemoglobin in 1 ml. of 0.9% NaCl. Peroxidatic activities were estimated in 0.1 ml. samples. Results are expressed as the percentage decrease in the catalytic activity of free haemoglobin in the presence of the colloid. None of the colloids had catalytic activity in the absence of haemoglobin.

Colloid	Reference	Decrease in peroxidatic activity (%)
Normal human urine glycoprotein	Anderson & MacLagan (1955)	0
Bovine fibrinogen	—	0
Human serum orosomucoid	—	0
Ovomucoid	—	1
Crystallized bovine plasma albumin	—	4
Cohn's plasma fraction VI (containing 50% of albumin and 50% of glycoprotein)	—	5
Urine colloid fraction UF-0*	Boyce, King, Little & Artom (1958)	11
Urine colloid fraction RS1†	Boyce <i>et al.</i> (1958)	13
Human cartilage chondromucoprotein	Anderson (1962)	35
Chondroitin sulphate A complex from human aorta	Anderson (1961)	37
Total normal human urine colloids‡	Anderson, Lepper & Winzler (1960)	38
Chondroitin sulphate C	—	49

* Containing 1.3% of hexuronic acid.

† Containing less than 0.5% of hexuronic acid plus some proteins originating from serum.

‡ Containing 4.3% of hexuronic acid plus proteins originating from serum.

of free aqueous haemoglobin is shown in Fig. 5. The inhibition of catalytic activity in the presence of chondromucoprotein is also shown in Fig. 5. At 1M concentration ϵ -aminohexanoic acid abolished this inhibition. The peroxidatic activities of mixtures containing increasing amounts of chondromucoprotein and haemoglobin are shown in Figs. 6 and 7 respectively. Heating a chondromucoprotein solution for 10 min. at 100° did not affect its ability to form a complex with haemoglobin or diminish its peroxidatic activity. Table 1 shows the effect of various protein, glycoprotein and muco-

polysaccharide fractions on the peroxidatic activity of haemoglobin.

Interaction in blood between chondromucoprotein and chromoproteins

Effect of chondromucoprotein on the peroxidatic activity of normal plasma. The conditions described above that led to the precipitation of chondromucoprotein-chromoprotein complexes from aqueous solution were applied to plasma. Increasing amounts of chondromucoprotein were added to

Table 2. *Peroxidatic activity of normal plasma after removal at pH 4.6 of the euglobulin fraction, and of the proteins precipitating with added chondromucoprotein*

Into each of six tubes was measured 1 ml. of centrifuged human plasma. To tube 1 was added 1 ml. of 0.9% NaCl and to tubes 2-5 were added increasing amounts of chondromucoprotein (12-240 μ g.) dissolved in 1 ml. of 0.9% NaCl. To tube 6 was added 3 ml. of 0.9% NaCl. To tubes 1-5 were added 2 ml. of 0.2 M-acetate buffer, pH 4.6, and the insoluble euglobulin fraction (in tube 1) and the insoluble chondromucoprotein-plasma-protein complexes (in tubes 2-5) centrifuged down. The peroxidatic activities of 0.4 ml. samples of the supernatant fluids were determined. The peroxidatic activity of a similar sample from tube 6 was also determined. Both the euglobulin fraction (tube 1) and the chondromucoprotein-plasma-protein complexes (tubes 2-5) gave negative results in the *o*-dianisidine reaction.

Tube no.	Chondromucoprotein added to plasma (μ g.)	Peroxidatic activity of supernatant fluid	
		($E_{690m\mu}$)	(% of that of untreated plasma)
1	0	0.542	97
2	12	0.570	102
3	60	0.602	106
4	120	0.620	109
5	240	0.628	112
6	Untreated plasma	0.560	—

Table 3. *Haemoglobin-binding proteins in chondromucoprotein-plasma-protein complexes formed at different pH values from normal plasma*

Into each of three tubes were measured 1 ml. of 0.9% NaCl, 0.5 ml. of normal plasma and 1 ml. of chondromucoprotein solution (200 μ g.). To one of these tubes was added 2 ml. of 0.9% NaCl and to each of the other two tubes was added 2 ml. of 0.2 M-acetate buffer, pH 5.3 or 4.6. The complexes were centrifuged down and peroxidatic activities in 0.15 ml. samples of each supernatant fluid were estimated (column 2). Untreated plasma (16.6 μ l.) gave an extinction value of 0.140 in the *o*-dianisidine reaction. The complexes were washed twice with 5 ml. of 0.9% NaCl or the appropriate buffer, dissociated into free chondromucoprotein and plasma proteins by dissolving them in 1.5 ml. of 0.2 M-phosphate buffer, pH 8.0, and peroxidatic activities in 0.15 ml. samples were estimated (column 3). The final incubation mixtures, although slightly turbid, had no visible colour. The presence of haemoglobin-binding proteins in the complexes was detected as follows. To 0.6 ml. of each of the above solutions of complex in phosphate buffer, pH 8.0, was added 0.2 ml. of haemoglobin solution (40 μ g.), and peroxidatic activities in 0.2 ml. samples were determined (column 4). Free haemoglobin (10 μ g.) gave an extinction value of 0.643 in the *o*-dianisidine reaction.

pH at which complexes were precipitated	Peroxidatic activity ($E_{690m\mu}$)			Decrease in peroxidatic activity of free haemoglobin (%)
	Supernatant fluid	Complexes in absence of haemoglobin	Complexes in presence of haemoglobin	
4.6	0.150	0.090	0.325	49
5.3	0.150	0.035	0.470	27
7.4	0.140	0.025	0.540	16

normal plasma at pH 4.6, the precipitated light-yellow chondromucoprotein-plasma-protein complexes centrifuged down and the supernatant fluids estimated for peroxidatic activity (Table 2).

When the chondromucoprotein-plasma-protein complexes, precipitated from normal plasma at pH 4.6, 5.3 and 7.4, were incubated with haemoglobin, peroxidatic activity was decreased, indicating the presence of haemoglobin-binding proteins, those formed at pH 4.6 being the most active (Table 3).

In a separate experiment the complexes were precipitated from 3 ml. of plasma as described in Table 3 and dissolved in 5 ml. of barbiturate buffer, pH 8.6. Mixtures containing 0.3 ml. of each solution of complex and 0.3 ml. of a haemoglobin solution (1.5 mg.) were incubated at 37° for 2 hr., and samples (10–50 μ l.) were subjected to electrophoresis on paper. After staining for protein and peroxidatic activity, free haemoglobin (migrating as $\gamma\beta_2$ -globulin) was detected in all the complexes. Haptohaemoglobins (migrating as β_1 -globulins) were absent. Methaemalbumin (migrating as albumin) was only present in complexes formed at pH 4.6.

Effect of chondromucoprotein on the peroxidatic activity of haemoglobinaemic plasma. The haemoglobin-binding capacity of the haptoglobins was exceeded by adding haemoglobin to normal plasma to a concentration of 200 mg./100 ml. Electrophoresis of this mixture at pH 8.6 showed the absence of free haemoglobin and the presence of haptohaemoglobins and methaemalbumin. Complexes were then precipitated by adding chondromucoprotein at pH 4.6 and the peroxidatic activity was estimated (Table 4).

The peroxidatic activity and haemoglobin-binding capacity of normal serum in the presence of increasing amounts of haemoglobin are shown in Fig. 8. The effect of adding chondromucoprotein to

Fig. 8. Effect of chondromucoprotein on peroxidatic activity of serum. Samples (0.2 ml.) of a mixture containing 0.5 ml. of human serum, 1.5 ml. of chondromucoprotein solution (720 μ g.) and 8 ml. of 0.9% NaCl were measured into a series (A) of nine tubes. To each of eight of these tubes was added 0.5 ml. of a haemoglobin solution (containing 1 to 8 mg. of haemoglobin/100 ml. of 0.9% NaCl), and to the ninth tube was added 0.5 ml. of 0.9% NaCl. Samples (0.2 ml.) from another mixture, containing 0.5 ml. of serum and 9.5 ml. of 0.9% NaCl, were measured into a series (B) of nine tubes, and the same additions made as to series A. The peroxidatic activities of samples from each tube were then determined: Δ , series A; $\square$, series B. The significance of points A–E is given in the text.

Table 4. *Presence of chromoproteins in complexes formed by adding chondromucoprotein to haemoglobinaemic plasma*

Normal human plasma (0.5 ml.) was measured into each of two tubes. To one was added 1 ml. of a haemoglobin solution (1.0 mg.), and to the other, which served as a control, was added 1 ml. of 0.9% NaCl. After 0.5 ml. of a chondromucoprotein solution (200 μ g.) had been added to each tube, the chondromucoprotein-plasma-protein complexes were precipitated by the addition of 2 ml. of 0.2 M-acetate buffer, pH 4.6. The complexes were centrifuged down, and 0.1 ml. samples of each supernatant fluid were estimated for peroxidatic activity (column 3). The complexes were washed with buffer, dissolved in 2 ml. of 0.2 M-phosphate buffer, pH 8.0, and 0.05 ml. samples of each solution were estimated for peroxidatic activity (column 4).

Tube no.	Haemoglobin added (mg./100 ml. of plasma)	Peroxidatic activity ($E_{690m\mu}$)	
		Supernatant fluid	Complexes
1	200	1.054	0.411
2	0	0.094	0

this serum, to a concentration of 144 mg./100 ml., is also shown in Fig. 8.

DISCUSSION

Other than its role as a structural cartilage component and its possible role in calcification (Bywaters & Glynn, 1964), the functions of chondromucoprotein are largely unknown. In plasma, however, it is unique in being negatively charged over a wide pH range, owing to the presence of dissociated carboxyl and sulphate groups on the mucopolysaccharide moiety. The simultaneous presence of positively charged proteins leads to precipitation of chondromucoprotein-plasma-protein complexes (Anderson, 1963). At pH 6.5 only plasma γ -globulins (isoelectric point near pH 7.2) are available for interaction, albumin interacting at pH values below its isoelectric point of pH 4.8. The complexes dissociate at pH values above those at which they are precipitated, the freed proteins suffering no irreversible change in solubility or in mobility, as judged by electrophoresis in starch gel at pH 8.6 (Anderson & Lack, 1964). The significance of these potentially insoluble blood components has been discussed by Anderson (1963) and Anderson & Lack (1964). Although other negatively charged macromolecules behave similarly (Nitschmann, Rickli & Kistler, 1958), chondromucoprotein is of special importance since it is the naturally occurring form of chondroitin sulphate, the predominant mammalian mucopolysaccharide in blood and tissues.

Chondromucoprotein-haemoglobin complex is precipitated to a maximum extent from aqueous solution between pH 3.7 and 4.7 (Figs. 1 and 3). This wide pH range is probably due to the presence of different haemoglobin types (Huisman, 1963) with slightly varying charges or to dissociation of haemoglobin, known to occur below pH 5.0, into differently charged α_2 - and β_2 -sub-units (Huehns, Shooter & Beaven, 1964). The inhibition of the peroxidatic activity of haemoglobin by chondromucoprotein (Figs. 5-7) is related to complex-formation, since the estimation, in common with other colorimetric methods (Crosby & Furth, 1956; Connell & Smithies, 1959; Smith & Owen, 1961), was performed under acid conditions. No turbidities were encountered when normal plasma and serum were assayed, in conformity with Lupovitch & Zak (1964), even though pH conditions were conducive to euglobulin precipitation. The small amounts of chondromucoprotein-protein complexes that formed were probably stabilized in colloidal suspension by protective colloids. The property of ϵ -aminohexanoic acid to prevent the formation of chondromucoprotein-chromoprotein complexes has

been observed in the formation of other complexes (Anderson, 1963).

Chondromucoprotein does not affect the absorption spectrum (Liang, 1957) or the electrophoretic mobility (Nyman, 1959) of haemoglobin at pH 7.0-8.6, and cannot therefore be considered to be a haptoglobin (Polonovski & Jayle, 1940; Smithies, 1955), since, by definition, the latter form soluble complexes, the haptohaemoglobins, with haemoglobin at neutral pH (Laurell & Grönvall, 1962). However, when plasma fractions are examined for haptoglobins by colorimetric measurement of haemoglobin-binding capacity, the presence of chondromucoprotein may give false positive results owing to the formation of chondromucoprotein-chromoprotein complexes. In such cases electrophoresis should be carried out under neutral conditions, which, although less precise, detect alterations in haemoglobin mobility due to specific interaction with haptoglobins.

Only mucopolysaccharides suppress the peroxidatic properties of haemoglobin (Table 1), whereas haptoglobins contribute to the inhibitory effect of the total urine colloids (Berggård, 1961). Most glycoproteins were inactive; this agrees with the view that only those present in the plasma α_2 -globulin fraction, the haptoglobins, combine with haemoglobin. Complex-formation by chondromucoprotein was unaffected by heat treatment, the high carbohydrate content protecting it from denaturation.

The peroxidatic properties of normal plasma are unaffected by chondromucoprotein at pH 4.6 (Table 2), the precipitated light-yellow complexes lacking catalytic activity. Extracellular haemoglobin in normal plasma is bound to haptoglobins and circulates entirely as haptohaemoglobulins. It is thus evident that the latter, in contrast with free haemoglobin, cannot form complexes with chondromucoprotein, perhaps because amino groups on the haemoglobin, being combined with carboxyl groups on the haptoglobins (Nyman, 1959), are unavailable for interaction.

When the binding capacity of haptoglobins was exceeded by adding an excess of haemoglobin to plasma, the precipitates formed at pH 4.6 were red and had peroxidatic activity (Table 4). Although haptoglobins stabilize haemoglobin in normal plasma, free plasma haemoglobin rapidly loses haem groups, which are oxidized to haematin and bind with albumin to form methaemalbumin (Fairley, 1938; Allison & ap Rees, 1957; Allison, 1958; Shinowara & Walters, 1963). This was confirmed by electrophoresis of haemoglobinaemic plasma, in which free haemoglobin (normally migrating with $\gamma\beta_2$ -globulins) is absent, whereas methaemalbumin (migrating with albumin; Fišer-Herman & Davorin, 1953) and haptohaemoglobins

(migrating with β_1 -globulins; Jayle, Boussier & Badin, 1952) are present.

The component with peroxidatic properties in complexes precipitated from haemoglobinaemic plasma is therefore methaemalbumin, and it was established that chondromucoprotein can, in fact, interact with this haematin-containing protein as well as with free albumin (Fig. 2). The decreased precipitation above an optimum chondromucoprotein concentration (Fig. 4) has been observed in the formation of other complexes (Anderson, 1963; Anderson & Lack, 1964). In common with haemoglobin, the peroxidatic activity of methaemalbumin is decreased by chondromucoprotein, owing to complex-formation (Fig. 7).

Haematin, a major catabolic product of extracellular haemoglobin with peroxidatic activity in haemoglobinaemic plasma, thus assumes not only the electrophoretic behaviour of albumin but also its solubility characteristics in the presence of chondromucoprotein, and will appear in euglobulin fractions and chondromucoprotein-plasma-protein complexes only under conditions of pH and chondromucoprotein concentration that precipitate the chondromucoprotein-albumin complex.

Maximum amounts of chondromucoprotein-plasma-protein complexes are precipitated at pH 4.4-4.7, whereas those formed at higher pH values lack albumin (Anderson & Lack, 1964). Although these complexes lacked haptoglobins (Table 2), haemoglobin-binding components were present since the peroxidatic activity of added haemoglobin was suppressed (Table 3). Complexes formed at pH 4.6 were the most active since they contained larger amounts of chondromucoprotein and methaemalbumin, the latter being absent from those formed at higher pH values. The inability of chondromucoprotein to form complexes with haptoglobins was expected since, with isoelectric points below pH 4.3, their negative charge precludes electrostatic interaction.

The peroxidatic properties of serum therefore depend on complex interactions involving chondromucoprotein, haemoglobin, haptoglobins and catabolic products of haemoglobin. Small amounts of haemoglobin added to serum bind specifically to haptoglobins, the rise in peroxidatic activity (Fig. 8, *A* to *B*) being unaffected by chondromucoprotein, since the latter cannot form complexes with, or dissociate, the haptoglobins. At *B* (Fig. 8), the conventional index of haptoglobin concentration, haptoglobins are saturated and free haemoglobin forms methaemalbumin with higher peroxidatic activity than haptoglobins. Thus serum peroxidatic activity rises abruptly (Fig. 8, *B* to *C*). In the presence of added chondromucoprotein, the chondromucoprotein-methaemalbumin complex that forms had less catalytic activity than free

methaemalbumin, and the peroxidatic activity of serum rises less abruptly (Fig. 8, *D* to *E*).

High chondromucoprotein concentrations therefore do not significantly affect serum haptoglobin estimations by colorimetric methods. This is in agreement with others (Robert, Mombelloni & Crosti, 1961; Robert & Crosti, 1964), who have concluded that raised haptoglobin concentrations in rabbit sera after papain injection are not due to simultaneously raised chondromucoprotein concentrations, since injection of other enzymes (elastase and hyaluronidase) elevates only the concentration of haptoglobins.

Although urine also contains mucopolysaccharides, and in addition has an acid pH conducive to complex-formation *in vivo*, it is particularly deficient in those plasma proteins, namely fibrinogen, lipoproteins and chromoproteins (Berggård, 1961), that form a large part of chondromucoprotein-plasma-protein complexes. For this reason normal urine is deficient in euglobulins (Varley, 1963), and only traces of chondromucoprotein-urine-protein complexes are precipitated when chondromucoprotein is added to normal urine colloid solutions above pH 4.4 (A. J. Anderson, unpublished work).

This and previous studies (Anderson, 1963; Anderson & Lack, 1964) show that chondromucoprotein-plasma-protein complexes contain at least two protein-bound biologically active trace components (plasminogen and haematin) due to coprecipitation after the formation of complexes of their carriers with chondromucoprotein, even though they may lack, when free, the charge necessary for electrostatic interaction. Since they have the potential to affect the chemical and physical integrity of the complexes, they assume significance if present in complexes deposited under abnormal conditions in body fluids and tissues. Other trace substances may also be present, notably keto steroids and enzymes associated with plasma lipoproteins (Oertel & Kaiser, 1962; Lawrence & Melnick, 1961), protein-bound metals (Laurell, 1960) and γ -globulin antibodies.

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REFERENCES

- Allison, A. C. (1958). *Proc. R. Soc. Med.* **51**, 641.
- Allison, A. C. & ap Rees, W. (1957). *Brit. med. J.* **2**, 1137.
- Anderson, A. J. (1961). *Biochem. J.* **78**, 399.
- Anderson, A. J. (1962). *Biochem. J.* **82**, 372.
- Anderson, A. J. (1963). *Biochem. J.* **88**, 460.
- Anderson, A. J. & Lack, C. H. (1964). *Clin. Sci.* **26**, 97.
- 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.
- Berggård, I. (1961). *Clin. chim. Acta*, **6**, 413.
- Boyce, W. H., King, J. S., Little, J. M. & Artom, C. (1958). *J. clin. Invest.* **37**, 1658.
- Bywaters, E. G. L. & Glynn, L. E. (1964). In *Biochemical Disorders in Human Disease*, p. 809. Ed. by Thompson, R. H. S. & King, E. J. London: J. and A. Churchill Ltd.
- Clegg, J. W. & King, E. J. (1942). *Brit. med. J.* ii, 329.
- Connell, G. E. & Smithies, O. (1959). *Biochem. J.* **72**, 115.
- Crosby, W. H. & Furth, F. W. (1956). *Blood*, **11**, 380.
- Fairley, N. H. (1938). *Nature, Lond.*, **142**, 1156.
- Fairley, N. H. (1941). *Quart. J. Med.* **10**, 115.
- Fišer-Herman, M. & Davorin, P. (1953). *Biochem. Z.* **324**, 96.
- Huehns, E. R., Shooter, E. M. & Beaven, G. H. (1964). *Biochem. J.* **91**, 331.
- Huisman, T. H. J. (1963). *Advanc. clin. Chem.* **6**, 231.
- Jayle, M. F., Boussier, G. & Badin, J. (1952). *Bull. Soc. Chim. biol., Paris*, **34**, 1063.
- Laurell, C. B. (1960). In *The Plasma Proteins*, vol. 1, p. 349. Ed. by Putnam, F. W. New York: Academic Press Inc.
- Laurell, C. B. & Grönvall, C. (1962). *Advanc. clin. Chem.* **5**, 135.
- Lawrence, S. H. & Melnick, P. J. (1961). *Proc. Soc. exp. Biol., N.Y.*, **107**, 998.
- Liang, C. C. (1957). *Biochem. J.* **66**, 552.
- Lupovitch, A. & Zak, B. (1964). *Clin. chim. Acta*, **9**, 49.
- Nitschmann, H., Rickli, E. & Kistler, P. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, section 16, p. 194.
- Nyman, M. (1959). *Scand. J. clin. Lab. Invest.* **11** (suppl.), 39.
- Oertel, G. W. & Kaiser, E. (1962). *Clin. chim. Acta*, **7**, 463.
- Polonovski, M. & Jayle, M. F. (1940). *C.R. Acad. Sci., Paris*, **211**, 517.
- Robert, L. & Crosti, P. (1964). *Clin. chim. Acta*, **9**, 56.
- Robert, L., Mombelloni, P. & Crosti, P. (1961). *Proc. Soc. exp. Biol., N.Y.*, **107**, 499.
- Shinowara, G. Y. & Walters, M. J. (1963). *Amer. J. clin. Path.* **40**, 113.
- Smith, H. & Owen, J. A. (1961). *Biochem. J.* **78**, 723.
- Smithies, O. (1955). *Nature, Lond.*, **175**, 307.
- Varley, H. (1963). In *Practical Clinical Biochemistry*, p. 94. London: William Heinemann Ltd.