The Properties of a Complex of the Mucopolysaccharide and Proteins of the Cornea

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It has been established that the acid mucopolysaccharide of the cornea is always extracted in combination with protein (Woodin, 1952). Several complexes are produced but one of them, the phenol-insoluble mucoid, is particularly stable, and the properties of this substance have been investigated further to determine if the protein component is a collagen derivative and in an attempt to identify the linkage between the protein and the mucopolysaccharide. In approaching the latter problem it was decided to use methods which might reveal electrostatic linkage before looking for covalent bonds.

Although there is little information on the mucoids formed in connective tissues from mucopolysaccharides which contain ester sulphur, two classes can be distinguished (Kellgren, 1952; Meyer, 1953). The mucoid from cartilage, which was described by Partridge (1948), contains a collagen derivative which is separated at pH 11 or on electrophoresis, whilst those present in tendon, heart valves and aorta do not contain collagen and are stable under these conditions (Meyer, 1953). It will appear from the results to be described here that the corneal mucoid is of the latter class.

The mucins formed, in acid solution, from chondroitin sulphate and proteins were shown by Meyer, Palmer & Smyth (1937) to be salts formed from the acid groups of the mucopolysaccharide and the basic groups of the protein, but the interaction in the mucoids which are stable at pH values where both components would have a net negative charge has not been considered before. Interaction under physiological conditions will be of this sort and so is fundamental to the understanding of the structure of the ground substance of connective tissues.

A preliminary account of this work has appeared (Woodin, 1953).

METHODS

Preparation of material

The phenol-insoluble mucoid. This was prepared from the water-insoluble sections of ox corneas by the methods described previously (Woodin, 1952). This mucoid represents 2.2% of the dry wt. of the cornea and contains 32% of the ester sulphate. Some physical measurements were made on samples of the phenol-insoluble mucoid after pre-

cipitation from solution in formamide. The phenol-insoluble mucoid was suspended in formamide; all but about 10% dissolved to give a clear solution. Ethanol (2 vol.) was added and the precipitate collected by centrifuging, suspended in water, dialysed in cellophan against water and the solution freeze-dried. This material represented 1.8% of the dry wt. of the cornea. A small variable amount of the phenol-insoluble mucoid was insoluble after freeze-drying and some experiments were made on a sample which had not been dried but was dialysed against the solvents used in the physical measurements. The properties of these solutions did not differ from those of solutions of dried mucoid.

The mucopolysaccharide. This was prepared from the phenol-insoluble mucoid by digestion with trypsin in the manner already described (Woodin, 1952), except that after the digestion the solution was dialysed against NaCl ($I=0.18$), sodium phosphate buffer ($I=0.02$, pH 7), and the fast component separated on electrophoresis. There were two components, of which the slower moved but little from the initial position of the boundary. The yield of the mucopolysaccharide depends on the length of the electrophoretic separation, but as only one component has an appreciable mobility it is considered that this component is representative of all the material which contains ester sulphur in the mucoid.

Gelatin. Gelatin from the cornea was prepared from the sections which had been extracted with water and 10% (w/v) aqueous CaCl_2 . The residues were washed free from CaCl_2 , suspended in water in a sealed tube and heated at 110° for 18 hr. The insoluble material was centrifuged off and the solution kept frozen. This substance contained 16% N; its homogeneity was not investigated. It represented 60% of the dry wt. of the cornea.

The yields of these materials are based on the dry wts. obtained by heating at 105° . The corneal sections were weighed before and after extraction and samples taken for the determination of dry weight and inorganic material. The volumes of the solutions of the mucoids and gelatin, obtained from known weights of the corneal sections, were noted and samples dialysed against 0.001N-HCl and then distilled water, the volumes noted again, and samples dried at 105° . It was assumed that the inorganic material was removed by the dialysis.

Analytical methods

Absorption spectra. These were measured in a Unicam spectrophotometer in the range $\lambda=230-350\text{ m}\mu$. A line was constructed through the points in the range $350-320\text{ m}\mu$ and extrapolated to shorter wavelengths to give a base line from which the absorption curve corrected for scatter could be calculated (Beaven & Holiday, 1952). The corrected

curve was used to obtain the tyrosine and tryptophan contents using the equations of Goodwin & Morton (1946).

Hydroxyproline was estimated by the method of Neuman & Logan (1950).

Hydrolysis by collagenase. The *Clostridium welchii* collagenase was a gift from Dr C. L. Oakley. It had been prepared by the method of Bidwell & van Heyningen (1948) and was the final product of that fractionation, dried by sublimation after dialysis against borate buffer. It was dissolved in Palitzsch borate buffer pH 7.1 (Clark, 1928) to give a solution containing 15 Q units/ml. (Bidwell & van Heyningen, 1948). The activity of the corneal mucoid and gelatin as substrates for the enzyme was assessed from the amount of non-diffusible N remaining after incubation. Solutions of the mucoid (0.3%) and of gelatin (1.0%) were made in Palitzsch borate buffer, pH 7.1, and 3 ml. samples put in tubes with 1 ml. of the enzyme. The N contents of the mixed solutions and of the enzyme solution were measured and then the mixtures incubated at 37° for 7 hr., 1 ml. withdrawn, dialysed in cellophane against running tap water and the non-diffusible N content measured.

Electrophoresis. This was done in the Tiselius apparatus. The conditions have been described (Woodin, 1952).

Ultracentrifuging. A model E Spinco analytical centrifuge was used. In some cases the balance cell in the rotor was replaced by a 'wedge' cell so that two measurements could be made simultaneously. All measurements were made at 22–25° and at a speed of 59780 rev./min. (250 000 g).

Osmotic pressures. These were measured in toluene osmometers. Membranes were made from nitrocellulose; a solvent containing 50 ml. ethanol, 50 ml. ether and 7 ml. ethylene glycol gave membranes of suitable permeability. After the osmometers had reached equilibrium at 3–4° the concentration of the mucoid was measured from the N content. When urea was present it was first removed by dialysis.

Other analytical methods were those described by Woodin (1952).

Fractionation of the phenol-insoluble mucoid

Extraction with organic solvents. The dry-matter content of a solution of the phenol-insoluble mucoid was determined and appropriate volumes were dried by sublimation in tubes. Samples were then extracted with formamide, pyridine or ethylene glycol. The solutions were removed and the dry wt. and N content determined after dialysis. The insoluble fraction was dissolved in water and the dry weight and N content were determined after dialysis.

Treatment with trichloroacetic acid and phenol (Aminoff, Morgan & Watkins, 1950). A 0.5% solution of the phenol-insoluble mucoid in water was treated with trichloroacetic acid (final concentration 5%, w/v), left at room temp. for 24 hr., dialysed and freeze-dried. The solid was then extracted with aqueous phenol (92%, w/v). The solution was dialysed and the dry wt. and N content determined. The material remaining insoluble after the phenol treatment was dissolved in water and the dry wt. and N content were determined after dialysis.

Partition between organic solvents and aqueous solution. A solution of the phenol-insoluble mucoid, 0.5% in 0.2M-NaCl (pH 7) was shaken with *n*-butanol and the interfacial precipitate collected. The aqueous phase was shaken again with *n*-butanol and after six extractions removed, dialysed, and the dry wt. and N content were determined. The com-

bined interfacial precipitate was collected, suspended in water, dialysed and the dry wt. and N content were determined. The same procedure was used with the phenol-insoluble mucoid in saturated aqueous $(\text{NH}_4)_2\text{SO}_4$ with pyridine as the organic solvent and with the phenol-insoluble mucoid in 5M urea, 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 7) with CHCl_3 -amyl alcohol (4:1, v/v) as the organic solvent.

Treatment with Zeo-Karb 215. A column was set up with 10 ml. wet volume Zeo-Karb 215 (Permutit Co. Ltd., London) in the acid form, and 4 ml. of a solution in water of the phenol-insoluble mucoid were pressed in and washed through with water. The eluate was collected and the hexosamine/N ratio measured.

Fractionation from formamide solution. A solution of the phenol-insoluble mucoid was made in formamide and the non-diffusible N content determined. The solution was treated with ethanol and the precipitates were collected after adding 1.25 and 2.0 vol. ethanol. The precipitates were dissolved in water and the dry wt. and N contents determined after dialysis.

In all cases the dialysis before analysis was against 0.001N-HCl and then distilled water. The volume after dialysis was noted and measured portions were dried in weighed ampoules at 105°. The solid and the ampoule were then incinerated in a Kjeldahl flask and the N content was determined.

RESULTS

Fractionation of the phenol-insoluble mucoid. It was known that the mucopolysaccharide could not be separated from the proteins in the mucoid by shaking with chloroform and amyl alcohol or by extraction with phenol. The chemical fractionations listed in Table 1 also do not dissociate the mucoid. The reagents used would be expected to separate the mucopolysaccharide if its linkage to protein were due solely to hydrogen bonds. Zeo-Karb 215 was used because of the possibility that the interaction was due to the Ca^{2+} used in the extraction of the mucoid. In this case its removal by the resin could enable preferential absorption of the protein on the surface of the resin particles.

The products obtained in these procedures differ in composition by more than the experimental error and this suggests that the mucoid is not a homogeneous substance.

Composition of the mucoid. The mucoid preparations contained 8.3–8.6% N and 20–25% hexosamine. If the non-hexosamine N of the mucoid were protein (16% N), then the mucoid contains 42% protein.

The ultraviolet absorption spectrum of the mucopolysaccharide and of the mucoid are shown in Fig. 1; solutions made in distilled water and in 0.2M-NaCl had identical absorption. The curves for the mucoid at pH 6 and at pH 13, corrected for scatter, show maxima at 275 $m\mu$. and at 290 $m\mu$. respectively, and intersect at 281–282 $m\mu$. Beaven & Holiday (1952) consider an intersection in the

Table 1. *Fractionation of phenol-insoluble mucoid*

The starting material had 8.4–8.6% N and hexosamine/N=2.6.

Treatment (see text)	Result	Composition of products
Extraction with phenol after tri-chloroacetic acid treatment	92% recovered in insoluble phase	Insoluble fraction, 8.5% N
Extraction with pyridine	95% recovered in insoluble phase	Insoluble fraction, 8.5% N
Extraction with ethylene glycol	93% recovered in insoluble phase	Insoluble fraction, 8.5% N
Extraction with formamide	80–90% soluble	Insoluble fraction, 8.5% N; soluble fraction, 8.5% N
Solution in 0.2M-NaCl shaken with <i>n</i> -butanol	After 6 extractions 30% recovered in precipitate, 60% recovered in solution	Soluble fraction, 8.8% N; precipitate, 7.9% N
Solution in sat. (NH ₄) ₂ SO ₄ shaken with pyridine	After 4 extractions 20% recovered in precipitate, 72% recovered in solution	Precipitate 8.7% N; soluble fraction 8.1% N
Solution in 5M urea shaken with chloroform and amyl alcohol	After 6 extractions 34% in precipitate, 54% in solution	Precipitate, 8.9% N; soluble fraction, 8.2% N
Solution in formamide treated with ethanol	75% precipitated by 1.25 vol. ethanol; 15% precipitated on increasing ethanol to 2 vol.	First precipitate 8.8% N, 2nd precipitate, 7.9% N
Solution in distilled water passed down column of Zeo-Karb 215	80% recovered in eluate	Eluate had hexosamine/N=2.8

Fig. 1. Ultraviolet absorption spectra of the mucoid and mucopolysaccharide: x—x, phenol-insoluble mucoid in distilled water; Δ—Δ, phenol-insoluble mucoid in 0.1N-NaOH; O—O, mucopolysaccharide in distilled water.

range 276–281 mμ. to be evidence that only tyrosine and tryptophan are contributing significantly to the absorption in the region. The tyrosine content of the mucoid on this assumption is 2.4–2.5% and the tryptophan content 0.8–0.9%. If the

mucoid contains 40% protein, then that protein contains 6% tyrosine and 2% tryptophan. The absorption curve of the gelatin analysed in the same way gave, tyrosine 0.8%, tryptophan 0.25%. It is not claimed that these figures are those for pure corneal collagen, but it is clear that the mucoid protein contains more tyrosine and tryptophan than does the collagen.

The hydroxyproline content of the mucoid was 0.5%, of the gelatin 10%.

After 7 hr. incubation with collagenase 90% of the corneal gelatin, but less than 15% of the mucoid diffused through the dialysis membrane.

Electrophoresis. If the protein and the mucopolysaccharide are combined by a specific interaction of their charged groups, the anionic groups of the polysaccharide combining with the cationic groups of the protein, then at a pH where the ionization of the latter is suppressed dissociation should occur. The pK for the second stage of the dissociation of sulphuric acid is given by Glasstone (1942) as 1.02. It is to be expected, therefore, that the ionization of the sulphuric ester groups of the mucopolysaccharide will be complete above pH 3. The effect of increasing the pH above this value will be to induce the ionization of the carboxyl groups and then, successively, to suppress the ionization of the imino, α-amino, ε-amino, and guanidino groups. This will have the effect of increasing the overall repulsion between the protein and the polysaccharide and will also tend to abolish any specific interaction between the cationic groups of the protein and the sulphuric ester groups.

Fig. 2 shows the pH/mobility curve for the phenol-insoluble mucoid and Fig. 3 the schlieren diagrams for the phenol-insoluble mucoid migrating under various conditions. Below pH 4 the solutions became increasingly opalescent and for measurements below pH 2 it was necessary to use a mucoid concentration of 0.2 %. Electrophoresis at a pH where ionization of the sulphuric ester groups would be suppressed was considered impracticable because of this and of the high conductivity of the solution.

The phenol-insoluble mucoid migrated with a single boundary in the range pH 2–11.5 in buffers containing 0.18M-NaCl or in 0.5M-NaCl (pH 6.7). In the presence of guanidine hydrochloride (0.5M) at pH 6.6 the peak becomes asymmetric and the mobility is reduced to about half the value it has in 0.5M-NaCl. These two solvents have almost the same conductivity. At pH 12 the peaks in the two sides of the cell are no longer identical, the σ boundary is larger than at lower pH and there is partial resolution into two components. There is also evidence of dissociation at pH 1.4, but because of the low concentration of mucoid and the opalescence of the solutions, this is less evident than at pH 12. Moreover, at pH 1.4 the mucopolysaccharide and protein would probably be oppositely charged and

Fig. 2. pH/mobility curve for the phenol-insoluble mucoid. The mobilities are the means of the values for the boundaries in both sides of the cell. The solvents were:

pH	Composition of solvent			
1.48	0.16M-NaCl	0.04M-HCl	—	
1.60	0.17M-NaCl	0.03M-HCl	—	
1.93	0.186M-NaCl	0.0142M-HCl	—	
2.62	0.18M-NaCl	0.02M-HCl	0.04M glycine	
3.8	0.18M-NaCl	0.02M sodium acetate	0.13M acetic acid	
4.1	0.18M-NaCl	0.02M sodium acetate	0.07M acetic acid	
6.1	0.18M-NaCl	0.0027M-Na ₂ HPO ₄	0.0122M-NaH ₂ PO ₄	
7.1	0.18M-NaCl	0.0057M-Na ₂ HPO ₄	0.0029M-NaH ₂ PO ₄	
8.25	0.18M-NaCl	0.0066M-Na ₂ HPO ₄	0.0006M-NaH ₂ PO ₄	
10.65	0.18M-NaCl	0.007M-Na ₂ CO ₃	—	
11.4	0.18M-NaCl	0.0017M-Na ₂ HPO ₄	0.0026M-Na ₃ PO ₄	

their interaction different from that at a higher pH.

Samples of the residues from the osmotic-pressure measurements made at pH 12 and also in 5M urea (pH 7) were dialysed against NaCl ($I=0.18$), sodium phosphate buffer ($I=0.02$, pH 6). On electrophoresis a single symmetrical boundary was observed.

Osmotic pressure. A straight line is not obtained by plotting P/C (P =osmotic pressure in mm. Hg, C =concn. as %, w/v) against concentration. The plot of $\log_{10} P/C$ against concentration gives a better approximation to a straight line and this method was used to obtain, by extrapolation, the values of P/C at infinite dilution. Because of the heterogeneity of the mucoid these values are useful only as a measure of the number of particles in unit weight of mucoid.

Fig. 4 gives the osmotic-pressure data for a sample of the mucoid precipitated from formamide in salt solution, in alkali and in urea and also for the residues from the urea and alkali returned to the

Fig. 3. Schlieren diagrams for the phenol-insoluble mucoid migrating on electrophoresis. The ascending boundary is on the left-hand side and the arrows indicate the direction of migration which in all cases was towards the anode. The scale line represents 1 cm. in the electrophoresis cell for all diagrams. The light source was a mercury vapour lamp with a filter giving light at 546 m μ . A: mucoid in NaCl (0.5M), NaH₂PO₄ (0.0029M), Na₂HPO₄ (0.0057M), pH 6.75, after 400 min. at 0.8 v/cm. B: mucoid in guanidine hydrochloride (0.5M), NaH₂PO₄ (0.0029M), Na₂HPO₄ (0.0057M), pH 6.6, after 1100 min. at 0.85 v/cm. C: mucoid in NaCl (0.18M), NaOH (0.02M), pH 12.1, after 200 min. at 1.7 v/cm. D: mucoid in NaCl (0.17M), HCl (0.03M), pH 1.58, after 90 min. at 1.17 v/cm. and 180 min. at 1.95 v/cm.

salt solution. Table 2 gives the range of values obtained with different samples of mucoid. The mucoid is dissociated by urea and at pH 12; these effects are partly reversible.

Ultracentrifuge data. Fig. 5 shows the schlieren diagrams of the mucoid precipitated from formamide and the mucopolysaccharide prepared from this material, sedimenting in various solvents. Base lines were constructed to these diagrams and the peaks reduced to this as origin. The constructed curves are shown in Fig. 6. Two samples of the mucoid precipitated from formamide and one sample of the phenol-insoluble mucoid have given essentially the same results.

In 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 7), and in 2.2M-NaCl, 0.05M sodium phosphate buffer (pH 7), the mucoid has three components but

Fig. 4. Osmotic pressure of the phenol-insoluble mucoid. P = Pressure in mm. Hg; C = concentration as g. mucoid/100 ml. solution. (a) ○—○, mucoid in 0.5M-NaCl, 0.05M sodium phosphate buffer (pH 7); ×—×, mucoid in 0.5M-KCl, 0.02M-NaOH (pH 12.1); △—△, mucoid, after exposure to pH 12.1, in 0.5M-NaCl, 0.05M sodium phosphate buffer (pH 7). (b) ○—○, mucoid in 5M urea, 0.5M-NaCl, 0.05M sodium phosphate buffer (pH 7); △—△, mucoid, after exposure to 5M urea, in 0.5M-NaCl, 0.05M sodium phosphate buffer (pH 7).

in 5M urea, 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 7), the mucoid sediments with a single asymmetric boundary. After standing in 5M urea solution for several days followed by dialysis against 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 7), the mucoid shows three components but the contribution of the fastest to the total refraction is smaller than in the mucoid before treatment with urea.

Fig. 5. Schlieren diagrams of the mucoid precipitated from formamide and mucopolysaccharide sedimenting on ultracentrifuging. A and B upper diagram: mucoid in 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 8). Lower diagram: mucoid in 5M urea, 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 8). A, 70 min. after reaching full speed. C, upper diagram: mixture of mucopolysaccharide and mucoid in 5M urea, 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 8). Lower diagram: mucopolysaccharide in 5M urea, 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 8), 163 min. after reaching full speed. D, upper diagram: mucopolysaccharide in 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 8). Lower diagram: mucoid in 2.2M-NaCl, 0.05M sodium phosphate buffer (pH 8), 161 min. after reaching full speed. E, residues from urea treatment: 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 7), 170 min. after reaching full speed.

Table 2. *Osmotic-pressure data on mucoid preparations*

'Phosphate' signifies sodium phosphate buffer. P = pressure in mm. Hg; C = concn. as % w/v.

Solute	Solvent	pH	P/C ($C \rightarrow 0$)
Mucoid precipitated from formamide	NaCl 0.5M } Phosphate 0.05M }	7	0.9-1.05
Mucoid precipitated from formamide	NaCl 0.5M } Na_2CO_3 0.05M }	10.5	0.9
Mucoid precipitated from formamide	Urea 5M } NaCl 0.5M } Phosphate 0.05M }	7	2.5-2.8
Mucoid precipitated from formamide	KCl 0.5M } NaOH 0.02M }	12.1	2.0-2.15
Mucoid precipitated from formamide (after NaOH treatment)	NaCl 0.5M } Phosphate 0.05M }	7	1.2
Mucoid precipitated from formamide (after urea treatment)	NaCl 0.5M } Phosphate 0.05M }	7	1.2
Phenol-insoluble mucoid	NaCl 0.5M } Phosphate 0.05M }	7	0.95
Phenol-insoluble mucoid	Urea 5M } NaCl 0.5M } Phosphate 0.05M }	7	2.95
Phenol-insoluble mucoid (after urea treatment)	NaCl 0.5M } Phosphate 0.05M }	7	1.15

Fig. 6. Schlieren diagrams of the mucoid and mucopolysaccharide sedimenting in the ultracentrifuge. The curves are corrected for the variation in the refractive index of the solvent in the direction of sedimentation. A, mucopolysaccharide in 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 8); B, mucopolysaccharide in 5M urea, 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 8); C, mixture of mucoid and mucopolysaccharide in 5M urea, 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 8); D, mucoid in 5M urea, 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 8). The broken lines are drawn through the mid-points of the bases of the diagrams and their departure from the vertical is a measure of the asymmetry.

The mucopolysaccharide sediments with a single boundary in both 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 7), and in 5M urea, 0.2M-NaCl, 0.05M sodium phosphate buffer (pH 7). A mixture of the mucopolysaccharide and the mucoid in urea solution sediments with a single boundary.

DISCUSSION

During sedimentation in the centrifugal field the mucoid in salt solution separates into three components but the mucopolysaccharide under the same conditions moves with a single boundary. The heterogeneity of the mucoid could be due to combination of the mucopolysaccharide with proteins of different weight or shape or to polymerization of different proportions of mucopolysaccharide and protein particles. The fact that the dissociation of the fastest-sedimenting component by urea is not completely reversible suggests that it is such a polymer.

The tyrosine, tryptophan and hydroxyproline contents, and the reaction with collagenase, indicate that the mucoid contains proteins other than collagen derivatives. The heterogeneity of the mucoid makes impossible the rigorous exclusion of the presence of collagen, but the suggestion made previously, that the mucoid protein is derived from corneal collagen can no longer be accepted (Woodin, 1952).

The mucoid migrates in the electrical field with a single boundary in the range pH 2-11.5. At a higher pH there is partial resolution into two components but the sharp appearance of the ascending boundary and the incomplete resolution of the descending boundary suggest rather, that the mucoid is migrating as an equilibrium mixture with its dissociation products. If the values of the dissociation constants for the α -amino, carboxyl, imino, and lysine ϵ -amino groups, given by Cohn & Edsall (1943), are those which apply to these groups combined in the mucoid, then their participation in

a specific electrostatic linkage can be excluded on account of the single boundary observed on electrophoresis. However, in absence of knowledge of the effect of the negative charge of the mucopolysaccharide on these dissociation constants, it is possible that the ϵ -amino group of lysine still has a positive charge at pH 11.5 and the dissociation observed at pH 12 could be due to suppression of that charge. It is clear, from the relative contribution of the two components at pH 12 to the total refraction, that the slower cannot account for all the protein combined in the mucoid. That protein still combined above pH 12, if it is combined electrostatically, must be so by virtue of the charge on the amidine group of the arginine and a more probable explanation of the dissociation at pH 12 is that it is due to partial suppression of the ionization of these groups.

In guanidine hydrochloride solutions the mucoid migrates with a markedly asymmetric boundary. This condition could arise if the mucoid were migrating as an equilibrium mixture with its dissociation products. There is also a reduction in the mobility of the mucoid in this solvent, a condition which is taken as evidence for a strong interaction of the guanidine and the sulphuric ester groups.

Implicit in this analysis of the electrophoretic behaviour is the assumption that the protein and mucopolysaccharide will differ sufficiently in their net charge to permit separation where dissociation occurs. This is likely, as the mucoid has a normal distribution of amino acids (Woodin, 1952) and the protein will have normal amphoteric properties.

The osmotic-pressure measurements provide confirmation of the reversible dissociation of the mucoid by alkali or the amidinium ion. In the presence of urea or at pH 12, but not at pH 10.5, there is an increase in (number of particles/unit weight of mucoid) over the value observed at pH 7 in salt solutions. If the action of urea and alkali is, as is suggested here, to set up an equilibrium between the mucoid and its products of dissociation, then further dissociation should be favoured by dilution and that would reveal itself by values of P/C which increase with dilution. The deviation of the mucoid under all conditions from ideal behaviour is so great, that it cannot be said if this occurs, but the fact that $\log_{10} P/C$ is a linear function of concentration may be of significance.

The single boundary observed when the mucoid sediments in urea solution could arise if the products of dissociation have identical sedimentation rates or if there were set up an equilibrium of the mucoid and its dissociation products. The boundary is asymmetric but there is no tendency to separation into separate peaks, suggesting a continuous distribution of sedimentation rates. This could arise if there

were, in urea solution, an equilibrium of the type:

combination of A with B , C , or D being an equally probable event. The single boundary observed in the mixture of the mucopolysaccharide and mucoid in urea could then arise if the added mucopolysaccharide participated in that equilibrium.

It is possible that a further analysis of the action of urea and guanidine will permit identification of the linkage between the mucopolysaccharide and protein in the mucoid. The electrophoretic behaviour indicates that an electrostatic linkage, if present, is most probably between the sulphuric ester groups and the arginine side chains. If there were present in the mucoid such a sulphuric ester-arginine linkage, then urea and guanidine might induce dissociation by competing with the arginine for the sulphuric ester groups. That the guanidinium cation could have such an action can be readily understood (Walker, 1949). Harris & Robson (1948) have suggested that the dipolar form of urea should be regarded as a strongly basic substituted amidine, but evidence for the interaction of urea and resonating anions of the sulphuric ester type does not appear to have been sought. An action of urea and guanidine analogous to that suggested here was originally put forward by Tsao, Bailey & Adair (1951) to account for the depolymerization of tropomyosin.

That urea and guanidine do have this action on the corneal mucoid must remain a hypothesis till it is possible to establish that the products of dissociation are indeed the mucopolysaccharide and the protein. The only evidence in favour of this at present is that the mixture of the mucopolysaccharide and dissociated mucoid sediments as a single component.

SUMMARY

1. The tyrosine, tryptophan and hydroxyproline contents and the reaction with collagenase indicate that corneal mucoid contains proteins which are not collagen derivatives.

2. The mucoid migrates with a single boundary on electrophoresis in the range pH 2–11.5. At higher pH, or in presence of guanidine, the peak is asymmetric and the mucoid behaves as an equilibrium mixture with its dissociation products.

3. Osmotic pressure measurements reveal that the mucoid is dissociated by urea or at pH 12. These effects are partly reversible.

4. The derived mucopolysaccharide sediments with a single boundary in the ultracentrifuge. The mucoid has three components in salt solutions, but in urea solution it moves with a single boundary. A mixture of the mucoid and mucopolysaccharide in urea solution sediments with a single boundary.

5. The relevance of these observations to the problem of the protein-mucopolysaccharide linkage is discussed and the hypothesis put forward that it is due to ion-pair formation between sulphuric ester groups and the amidine groups of arginine residues.

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The Isolation of *allo*Hydroxy-L-Proline from Sandal (*Santalum album* L.)

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During the course of investigations on the nitrogen metabolism of plants by the radial ('circular') paper-chromatographic technique, the presence of considerable quantities of free proline in leaves of several plant species and hydroxyproline in the leaves of sandal was observed (Giri, Gopalkrishnan, Radhakrishnan & Vaidyanathan, 1952). Examination of paper chromatograms of an ethanolic extract of the leaves of *Santalum album* showed that the band corresponding to hydroxyproline was quite prominent and an attempt was therefore made to isolate the substance in a pure state. In the present paper an account is given of its isolation and identification as *allo*hydroxy-L-proline.

The procedure of Town (1928) was not found suitable in this case. However, a partial purification was initially effected by absorption over a column of Zeo-Karb H.I., whereby some neutral constituents and a few of the amino acids were removed. By chromatography of the eluates on cellulose powder and copper carbonate-alumina columns, the purification of the substance was sufficient to induce crystallization. Failure to isolate the amino acid by Town's procedure is believed to be due to the presence of an accompanying substance which yields a picrate (m.p. 335°) but is otherwise as yet unidentified.

The purified amino acid gave correct analytical values and melted at 248° (decomp.). The melting point as well as the optical rotation are in good agreement with values previously reported for *allo*hydroxy-L-proline. A mixed melting point with an authentic sample was unchanged. Further, both the samples were indistinguishable in their behaviour on paper chromatograms and also crystallized in the same characteristic broom-like manner on a microscope slide. Additional evidence as to their identity was provided by X-ray powder photographs.

EXPERIMENTAL

Identification of free amino acids in leaves of Santalum album

Extraction. Fresh leaves were dried in an oven at 50° and powdered (200 mesh/in.). A sample (0.2 g.) of the powder was extracted for 1 hr. in the cold with 75% (v/v) aqueous ethanol (2 ml.). After centrifuging, the supernatant was shaken with CHCl₃ (3 vol.). The aqueous layer containing all the amino acids was separated by centrifuging and examined by paper chromatography. Clear separation of the bands could be achieved with 10–20 μl. of the aqueous extract.

Paper chromatography. Whatman no. 1 filter-paper disks (25 cm. diam.) were used and the chromatograms developed