Molecular Size, Shape and Aggregation of Soluble Feather Keratin

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The keratins are epidermal proteins which contain a high percentage of cystine and, in the solid state, can form macroscopic structures in which there is a high degree of order in the arrangement of the polypeptide chains. Because of the last characteristic they have been an invaluable material to study the structure of proteins by the diffraction, or scatter, of X-rays. It was considered that measurements of the size and shape of keratin in solution would be of value in the interpretation of the X-ray diagrams, and the present work was undertaken to provide such data for feather keratin. To some extent the interaction between keratin particles in solution has also been considered since feather keratin is a suitable material for forming regenerated fibres (Lundgren, 1949), and a knowledge of the way keratin molecules can polymerize will be of value in devising ways of forming such fibres.

It has been known from the work of those interested in the mechanical properties of keratin fibres that the stability of the protein in the solid state is due to cross-linking by the cystine residues, to hydrogen bonds and to salt linkages (Lundgren, 1949). The addition of reagents which break these bonds decreases the strength of the fibres, and it is possible to dissolve keratin by mixtures of substances which break the —S—S— bonds and the hydrogen bonds, as was first shown by Goddard & Michaelis (1934), and later by Jones & Mechem (1943). The latter work is of particular value since solution was achieved under conditions that do not cause irreversible changes to the cystine residues.

In this study of the physical properties of feather-keratin solutions, the measurements of particle size and shape have been correlated with the cystine and cysteine content of the protein, the working hypothesis being that the keratin monomer would be a particle in which there is no cross-linking by —S—S— bonds.

Mercer & Oloffson (1951) and Ward (1952) observed the behaviour in the centrifugal field of wool keratin dissolved in solutions of urea and bisulphite but did not record the extent to which cross-linking by —S—S— bonds was present.

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Ward, High & Lundgren (1946) dissolved feather keratin in solutions of bisulphite and detergent and then removed the bisulphite by dialysis. The physical properties of the resultant solution were examined, but the cystine content of the dissolved protein was not recorded. It is known from the work of Middlebrook & Phillips (1942) that the reaction of wool keratin with bisulphite is partly reversible, and it has been found in the course of the present investigation that the removal of bisulphite from solutions of feather keratin in urea and bisulphite also results in loss of free —SH groups. It can therefore be presumed that the material studied by Ward *et al.* (1946) contained —S—S— bonds and was polymerized to an unknown extent.

MATERIALS

Starting material

White body feathers were obtained from Leghorn hens from the Hollywood Poultry Co. They were extracted with benzene at room temp. till the extract no longer gave a residue on distillation, and then dried in air and extracted again with distilled water (10×2 l./200 g. feathers), dried in air again, and kept *in vacuo* over P_2O_5 at room temp.

Extraction of soluble keratin

1 g. of feathers was suspended in 20 ml. of solvent and extracted at 40° for 20–24 hr., during which period any necessary adjustments to the pH of the extract were made. The suspension was filtered and the filtrate kept. The residue was washed with fresh solvent and then with distilled water till the washings were free from urea. The residue was then dried at 105° and weighed. Most extractions were made in 10M urea, 0.1M-NaHSO₃, 0.05M sodium phosphate buffer at various pH's. Some extractions were made in 10M urea, 0.5M sodium thioglycollate, 0.05M sodium phosphate buffer (pH 6).

When a solution in 5M urea, 0.1M bisulphite, 0.05M phosphate (pH 6–8.5) or in 5M urea, 0.5M thioglycollate, 0.05M phosphate (pH 6) was required, the extract made in 10M urea was diluted with an equal volume of 0.1M bisulphite, 0.05M phosphate buffer of the appropriate pH, or 0.5M thioglycollate, 0.05M phosphate buffer (pH 6), respectively. The dilution was made through a fine-bored pipette immersed in the solution, which was stirred during the dilution.

The protein in these solvents will be referred to as 'SH-keratin'.

Treatment with performic acid (cf. Sanger, 1949)

Solutions of SH-keratin in 10M urea, 0.1M bisulphite, 0.05M phosphate buffer (pH 6-8.5) were dialysed against running tap water and then distilled water. An opalescent solution, or, when the protein concentration was above about 3%, a clear gel was produced, frozen, and dried by sublimation. 1 g. of this solid was suspended, and allowed to swell, in 30 ml. formic acid at room temp. After 30 min. 7 ml. H_2O_2 (30%, w/v) were added and the suspension was stirred. After about 15 min. a yellow solution was obtained and left at room temp. for a further 15 min. and then filtered through a sintered-glass filter. The filtrate was cooled to 5°, 5 vol. of acetone were added, and the resulting precipitate, which flocculated in about 30 min., was collected by centrifugation. The solid was washed with acetone, suspended in distilled water, frozen, and dried by sublimation. Yield, 95%.

This material will be known as 'cysteic acid-keratin'.

If the solution in the performic acid was diluted with water, incomplete precipitation occurred. Addition of acetone to the supernatant produced an opalescent solution from which the addition of NaCl (final concn. about 1M) produced complete precipitation.

METHODS

Nitrogen was determined by micro-Kjeldahl, using selenium catalyst and digesting for 8 hr.

Cystine and cysteine were determined by the methods of Shinohara (1935). The apparent cystine content of feather was the same when it was hydrolysed in 5N-HCl for 4, 6, 8, or 12 hr., and as a routine method the solid keratin was hydrolysed in 5N-HCl for 6 hr. under reflux. When the cysteine and cystine content of keratin in urea/bisulphite solution were required, the solution was pipetted into an equal volume of 10N-HCl which was vigorously stirred. The resultant solution was boiled under reflux for 6 hr. There was 95-102% recovery of cystine added to these solutions or to the feathers.

It is shown below that the relative apparent amount of cysteine and cystine in the soluble keratin dissolved in urea/bisulphite is dependent on the pH of the solution, the ratio of cystine to cysteine increasing as the pH is changed from 6 to 9. There is doubt whether the ratios found for these amino acids combined in keratin, dissolved in urea/bisulphite, at a certain pH, are indeed the true ones since by this analytical method there will be a large pH change on addition of the keratin solution to 10N-HCl. Cystine can be recovered quantitatively if it is added to 5N-HCl containing bisulphite but there may be a conversion of combined cystine into combined cysteine at the moment at which the keratin solution is added to the acid. However, this possibility will not affect the validity of the conclusions to be drawn from the cysteine/cystine ratios since these conclusions are based on the presence of cystine and the action of the acid would be to reduce that cystine.

Moisture contents were determined by drying to constant weight at 105°.

Ash was determined gravimetrically as the sulphate.

Determination of protein concentration. The concentration of keratin in solution can be determined from the refractive increment, the cystine content, or from the non-diffusible N content. The specific refractive increment of cysteic acid-

keratin, dissolved in and dialysed against 0.05M- Na_2CO_3 , 0.2M-NaCl was found to be 0.00183, the concentration being determined from the N content, which was taken as 16.5% for dry solid keratin. The value of the specific refractive increment of cysteic acid-keratin in 5M urea, 0.1M bisulphite, 0.05M phosphate was 0.00208, concentration being determined from the non-diffusible N content. It is presumed that this value applies to SH-keratin dissolved in this solvent.

In the determination of the non-diffusible N content, 1 ml. samples of the keratin solution were pipetted into cellophan sacs and dialysed against running tap water for 3 days. The keratin molecules are small enough to pass through the pores of the sac, and it was necessary to apply a correction for this loss. To that end a solution of cysteic acid-keratin was made in 0.05M- Na_2CO_3 and its N content determined. The solution was diluted with an equal volume of 10M-urea and samples of this placed in sacs and the non-diffusible N content determined. 10-12% of the keratin was lost after dialysis in this way for 3 days. The daily loss of keratin was constant, and it was therefore ascribed to the small size of the keratin molecule and not to a diffusible fraction.

Osmotic pressure measurements were made in toluene osmometers at 30°. Visking cellophan was used as the membrane (Visking Corp. Chicago, U.S.A.). About 5 ml. of protein solution and 125 ml. of solvent were used. The loss of cystine-containing material from the inside of the sac was 1%/day and as the corresponding decrease in the height of the column of toluene was small compared with the total height, this loss was neglected. The osmometers came to a steady state after 4 days. The ratio P/C was plotted against C and the values at zero concentration used to calculate the number-average molecular weight. (P =pressure in mm. Hg; C =concentration of keratin in g./100 ml. solution.)

Refractive indices were measured on a differential refractometer (Brice & Halwer, 1951), which was sensitive to a refractive index change of 0.000005. The temperature was 25° and the wavelength 546 m μ .

Viscosity measurements were made in Ostwald viscometers at 30°. The flow time for 4 ml. of distilled water was 277 sec. at 20°. Kinetic energy corrections were not made. The keratin solutions were filtered through ultrafine sintered-glass filters and then dialysed against the solvent. Before being placed in the viscometer they were centrifuged. Flow times were reproducible to 0.5 sec.

Ultracentrifuging was done in a Spinco Model E analytical centrifuge. The synthetic-boundary cell described by Pickels, Harrington & Schachman (1952) was used. The relative positions of the boundary, meniscus, and indices were measured on the photographic reproduction of the schlieren diagram by a travelling microscope, and measurements were reproducible to 0.005 cm. Sedimentation constants were obtained from the slope of the plot of $\Delta \log x$ against Δt , where x is the distance from the boundary to the centre of rotation and Δt the time between the exposures.

The density of 5M urea, 0.1M bisulphite, 0.05M phosphate (pH 8.3) relative to water was 1.09 that of 5M urea, 0.1M-NaCl, 0.05M phosphate (pH 6), 1.079, and that of 10M urea, 0.1M bisulphite, 0.05M phosphate (pH 9), 1.18. The viscosities of the solvents were measured at different temperatures relative to water at 20° and used together with the density measurements to obtain the sedimentation constants

corrected to the value they would have if the solvent were water at 20°. The partial specific volume was taken as 0.727 cm.³/g., this being the value found by Ward (1952) for wool keratin.

Light-scattering measurements were made in a light-scattering photometer (Brice, Halwer & Speiser, 1950) at 546 mμ. The keratin solutions were dialysed against the solvent and then centrifuged at 80 000 *g* for 1 hr. They were then filtered through ultrafine sintered-glass filters (average pore diameter, 1.2 μ.) directly into the cell. Distilled water filtered directly through these filters had a turbidity of 2×10^{-5} cm.⁻¹, 5 M urea, 0.1 M bisulphite, 0.05 M phosphate (pH 8.5) had a turbidity of 9×10^{-5} cm.⁻¹. The concentrations of keratin were obtained after the measurements from the non-diffusible N contents. The values of HC/τ were calculated and plotted against concentration and molecular weights obtained from the value of HC/τ at zero concentration. H is the refraction constant, τ , the turbidity, is the logarithm of the fractional decrease in the transmitted intensity. C is the concentration of keratin in g./ml. (Precise definitions of H and τ are given by Oster (1948).

$$H = \frac{32\pi^3 n_0^2 (\Delta n/c)^2}{3N\lambda^4},$$

where n_0 is the refractive index of the solvent, $\Delta n/c$, the specific refractive increment, λ the wavelength of the incident light and N is Avagadro's number. The turbidity is defined by

$$I = I_0 e^{-\tau r},$$

I_0 is the intensity of the incident light, I the intensity of the transmitted light, and r the path length in the scattering system.)

RESULTS

Solubility and composition of soluble keratin

Fig. 1 illustrates the fractionation procedure.

With 10 M urea, 0.1 M bisulphite, 0.05 M phosphate as the solvent, extraction in the range pH 6–9.2 dissolved 80–85% of the feather. 60% was dissolved when 10 M urea, 0.5 M thioglycollate, 0.05 M phosphate (pH 6) was the solvent.

The extracts were yellow in colour. When the urea concentration at pH 6–8.5 was decreased to 5 M, the solutions developed a bluish opalescence due to the precipitation of a material which could be removed by high-speed centrifuging or passage through an ultrafine filter, and which represented a negligible fraction of the total weight of the keratin.

Decreasing the urea concentration in the extract made at pH 9 to 5 M, caused precipitation of part of the keratin as a fibrous clot. Rapid precipitation could be induced by overlaying the extract with an equal volume of 0.1 M bisulphite, 0.05 M phosphate

Fig. 1. Diagram of fractionation of feather keratin.

(pH 9), when an interfacial precipitate was produced, and then gently mixing the two layers. If, however, the solution of bisulphite and phosphate was added slowly and with stirring, a clear solution was obtained which developed an opalescence and then a precipitate during several hours. Precipitation in this way was always observed when the extracts were diluted immediately after separation from the insoluble residue. After standing at room temperature for several days, these extracts did not give a precipitate.

The extracts made in 10M urea, 0.1M bisulphite, 0.05M phosphate, (pH 6-8.5), when dialysed against water, gave a clear gel if the protein concentration was above about 3%, but an opalescent solution at lower protein concentration. The extract at pH 9 when dialysed in this way gave an opaque clot. The dialysed solutions and gels were frozen and dried by sublimation. The solid obtained from the gels, produced on dialysis of the extracts at pH 6-8.5, was soluble in 5M urea, 0.1M bisulphite, 0.05M phosphate (pH 6), but it was necessary to use 10M urea, 0.1M bisulphite, 0.05M phosphate, to induce solution at pH 8.5.

The cystine content of the solid recovered from the dialysed extracts was determined and the results are given in Table 1. There was loss of some of the cystine in the feather, but the loss was not de-

pendent on the pH of the extraction. It is therefore unlikely that the precipitation observed on dilution of the extracts made at pH 9 is due to formation of thio-ether or other stable cross-links from the cystine residues. Middlebrook & Phillips (1942) found a small loss of combined cystine on treatment of wool with bisulphite at temperatures above 40°.

Table 2 lists the ratio of combined cysteine to combined cystine in soluble keratin under various conditions. The reaction with bisulphite is largely reversible; thus removal of the bisulphite from solutions of SH-keratin in 5M urea, 0.1M bisulphite,

Table 1. *Cystine contents of feather keratin and its derivatives*

In all cases the cysteine content was less than 0.3%. The percentages are calculated on the basis of the dry weight determined by heating at 105° but are not corrected for inorganic material remaining after the dialysis. The cystine content of the original feather is corrected for the moisture content and for the inorganic material present.

Material	pH of extraction	Cystine (%)
Feathers	—	7.6
Cystic acid-keratin	—	Less than 0.2
SH-keratin from urea/	6	6.9
bisulphite extraction after	8.5	7.1
dialysis against water	9.2	6.9

Table 2. *Cysteine/cystine ratios of soluble keratin under various conditions*

Solutions in which the urea concentration was 5M were made from the extracts by dilution with 0.1M bisulphite, 0.05M phosphate, or with 0.5M thioglycollate, 0.05M phosphate. In all cases the solutions were dialysed against the solvent before the hydrolysis. The solid precipitated on dilution of the extract made in 10M urea, 0.1M bisulphite, 0.05M phosphate (pH 9) was washed in the solvent and suspended in that solvent before hydrolysis. The average number of —SH and —S—S— groups is calculated on the basis that each monomer has mol.wt. 10000 and potentially six —SH groups.

Material	Solvent	pH	Moles cysteine Moles cystine	Average no. —SH groups in monomer	Average no. —S—S— groups in monomer
SH-keratin from urea/bisulphite extraction pH 6-8.5	5M urea	6	10-14	5.2	0.4
	0.1M bisulphite	8.5	1.0-1.4	2.2	1.9
	0.05M phosphate				
	5M urea				
	0.1M bisulphite	8.5	1.4	2.4	1.8
	0.05M phosphate				
	0.2M ethanol				
	10M urea	6	> 25	6	0
	0.1M bisulphite	8.5	4	4	1
	0.05M phosphate	9.2	3.4	3.8	1.1
	5M urea				
	0.1M-NaCl	6	0.08	0.2	2.9
	0.05M phosphate				
SH-keratin remaining in solution after dilution of the extract in 10M urea (pH 9)	5M urea				
	0.1M bisulphite	9.2	2.0	3	1.5
	0.05M phosphate				
Solid precipitated on dilution of the extract in 10M urea (pH 9)	5M urea				
	0.1M bisulphite	9.2	0.23	0.6	2.7
	0.05M phosphate				
SH-keratin from urea/thio- glycollate extraction (pH 6)	5M urea				
	0.1M-NaCl	5.8	0.13	0.4	2.8
	0.05M phosphate				

0.05M phosphate (pH 6) by dialysis against 5M urea, 0.1M-NaCl, 0.05M phosphate (pH 6) resulted in loss of many of the free —SH groups. In an attempt to prepare a solution of SH-keratin with a large number of free —SH groups in the absence of reducing agent, a solution of SH-keratin was prepared in 5M urea, 0.5M thioglycollate, 0.05M phosphate (pH 5.5) and dialysed against 5M urea, 0.1M-NaCl, 0.05M phosphate, 0.1% (pH 5.5), but this too resulted in a product with many —S—S— bonds.

The cysteine/cystine ratio of SH-keratin in urea and bisulphite solution is dependent on the pH and the urea concentration. While the effect of both could be to change the reactivity of the —S—S— groups, it is probable that the effect of the pH, in part at least, is to change the concentration of the HSO_3^- ion which, according to Lugg (1932), reacts with disulphides by the reaction

Solutions of bisulphite titrate in the range pH 4–9. The reaction with alkali is

and there is very little HSO_3^- at pH 9. Elsworth & Phillips (1932) found that the number of free —SH groups in wool, suspended in buffered solutions of bisulphite, was similarly dependent on the pH and concluded that it was due to a change in the concentration of the HSO_3^- ion.

Cysteic acid-keratin was soluble in 5M urea, 0.1M-NaCl, 0.05M phosphate (pH 6–8.5) and in 0.05M- Na_2CO_3 or in 0.05M- Na_2CO_3 , 0.2M-NaCl. A 2% (w/v) solution of cysteic acid-keratin in 0.05M- Na_2CO_3 migrated with a single boundary on electrophoresis. This is consistent with there being an equal distribution of cysteic acid side chains, and hence the cystine, among the keratin monomers.

Osmotic pressure

Table 3 gives the values of P/C at zero concentration, the number-average molecular weight, and also the ratio of P/C at zero and 1% concentrations, for soluble keratin under various conditions. Figs. 2–4 record the experimental data for some of these conditions.

Table 3. *Results of osmotic pressure measurements*

When the urea concentration was 5M, the extract was diluted with 0.1M bisulphite, 0.05M phosphate of the appropriate pH and this solution was then dialysed against the solvent used in the osmometer. P = pressure in mm. Hg; C = concentration of keratin in g./100 ml. solution.

Solute	Solvent	pH	P/C ($C=0$)	Mol.wt.	$\frac{P/C (C=0)}{P/C (C=1)}$
Cysteic acid-keratin	5M urea	6	18.2	10 200	1*
	0.1M-NaCl				
	0.05M phosphate				
	5M urea	8.5	18.2	10 200	1
	0.1M bisulphite				
	0.05M phosphate				
SH-keratin from extraction in urea/bisulphite	5M urea	6	18.2	10 200	1.08
	0.1M bisulphite				
	0.05M phosphate				
	5M urea	8.5	19.7	9 500	1.45
	0.1M bisulphite				
	0.115M phosphate				
	5M urea	8.5	19.8	9 500	1.54
	0.1M bisulphite				
	0.05M phosphate				
	5M urea	8.5	19.8	9 500	1.58
	0.1M bisulphite				
	0.05M phosphate				
	0.2M ethanol	8.5	19.2	9 700	1.09
	5M urea				
	0.1M bisulphite				
	0.05M phosphate	8.5	18.5	10 100	1.08
	0.2M mercaptoethanol				
	0.1M bisulphite				
	0.05M phosphate	9.2	18.5	10 100	1.25
	0.2M thioglycollate				
	0.1M bisulphite				
SH-keratin from urea/thioglycollate extraction	10M urea	5.8	19.0	9 800	1.4
	0.1M-NaCl				
	0.05M phosphate

Fig. 2. Osmotic pressure of cysteic acid-keratin. $\circ$, 5M urea, 0.1M-NaCl, 0.05M phosphate (pH 6); $\times$, 5M urea, 0.1M bisulphite, 0.05M phosphate (pH 8.5). P =pressure in mm. Hg; C =concn. of keratin in g./100 ml. solution.

Fig. 3. Osmotic pressure of SH-keratin in 5M urea, 0.1M bisulphite, 0.05M phosphate (pH 6). P =pressure in mm. Hg; C =concn. of keratin in g./100 ml. solution.

Fig. 4. Osmotic pressure of SH-keratin in 5M urea, 0.1M bisulphite, 0.05M phosphate (pH 8.5). P =pressure in mm. Hg; C =concn. of keratin in g./100 ml. solution.

Fig. 5. Osmotic pressure of SH-keratin. $\circ$ — $\circ$, 5M urea, 0.1M bisulphite, 0.05M phosphate, 0.2M ethanol (pH 8.5). $\times$ — $\times$, 5M urea, 0.1M bisulphite, 0.05M phosphate, 0.2M β -mercaptoethanol (pH 8.5). P =pressure in mm. Hg; C =concn. of keratin in g./100 ml. solution.

The graphs representing the dependence of P/C on concentration all extrapolate, with different slopes, to the same value of $P/C = 19 \pm 1$ at zero concentration. Thus the number-average molecular weight of soluble keratin is constant in the urea concentration range 5–10M, in the range of ionic strength 0.17–0.64 and for preparations which have 0–2 moles of cystine/10000 g.

Cysteic acid-keratin gives values of P/C which are independent of concentration in the range studied. SH-keratin deviates from ideality, indicating that association occurs with increasing concentration. The extent of the association is represented by the ratio of P/C at zero to that at 1% concentration and comparison with the cystine content of the material suggests that the association only occurs when —S—S— bonds are present. However, cysteic acid-keratin, SH-keratin at pH 6, and SH-keratin at pH 8.5, are not strictly comparable, as they will have different net charges, and the ionic strength of the media in which the measurements were made was not constant. It was possible that the association was due to a charge effect. However, the addition of β -mercaptoethanol decreases the association of SH-keratin at pH 8.5, while the addition of ethanol does not (Fig. 5), and so the association probably requires the presence of —S—S— bonds.

SH-keratin associates with increasing concentration in 5M urea, 0.1M bisulphite, 0.115M phosphate (pH 8.5), but in this solution, which has the highest ionic strength which has been used, precipitation of part of the keratin occurred when the protein concentration was above 2%.

Viscosity

Fig. 6 shows the relation between the specific viscosity, $(\eta_{rel} - 1)/C$, and concentration for cysteic acid-keratin in 5M urea, 0.1M-NaCl, 0.05M phosphate (pH 6) and for SH-keratin in 5M urea, 0.1M bisulphite, 0.05M phosphate (pH 8.5). Extrapolation

Fig. 6. Viscosity results. $\circ$ — $\circ$, SH-keratin in 5M urea, 0.1M bisulphite, 0.05M phosphate (pH 8.5). $\times$ — $\times$, Cysteic acid-keratin in 5M urea, 0.1M-NaCl, 0.05M phosphate (pH 6).

of these graphs to zero concentration gives the same value for the intrinsic viscosity (specific viscosity at zero concentration) for both preparations. Taking the partial specific volume as 0.727, the viscosity increment is 20.6. The viscosity increment is $(\eta_{rel} - 1)/\phi$; $\phi = 0$, where ϕ is the volume fraction of the keratin. If the keratin particle is a prolate ellipsoid and unsolvated, then the corresponding value for the axial ratio is 13.2 (Simha, 1940). If the shape is spherical, the viscosity increment should be 2.5 (Einstein, 1906, 1911) and it would be necessary to assume that the keratin is solvated to a volume 8 times that of the partial specific volume. Pasynskii & Chernyak (1950) have measured the absorption of water and urea by wool keratin, using the technique of equilibrium dialysis, and give the values 0.15 and 0.08 g./g. keratin, respectively. It is therefore probable that the viscosity of keratin is determined mainly by an asymmetric shape.

At concentrations below 1% the specific viscosity of cysteic acid-keratin is independent of concentration but the specific viscosity of SH-keratin at pH 8.5 increases with concentration. It is unlikely that the hydration would vary with protein concentration and so the increase in viscosity is taken as evidence for formation of increasingly asymmetric molecules by association.

A further feature of interest in the dependence of the specific viscosity on concentration is that the curve for SH-keratin at pH 8.5 does not flatten at concentrations above 1%, at which level P/C becomes independent of concentration.

Sedimentation

Fig. 7 shows the schlieren diagram for SH-keratin at pH 8.3 in 5M urea, 0.1M bisulphite, 0.05M phosphate, sedimenting in the centrifugal field (250 000 g). Fig. 8 gives the diagram for

Fig. 7. Schlieren diagrams of SH-keratin sedimenting in the centrifugal field. The solvent was 5M urea, 0.1M bisulphite, 0.05M phosphate (pH 8.3), and the keratin concentration 0.8% (w/v). A, 20 min. after forming the boundary. B, 131 min. after forming the boundary. (Sedimentation from right to left.)

cysteic acid-keratin in 5M urea, 0.1M-NaCl, 0.05M phosphate (pH 6). That for SH-keratin in 10M urea, 0.1M bisulphite, 0.05M phosphate (pH 9) was indistinguishable from these.

Cysteic acid-keratin and SH-keratin sediment with a single symmetrical boundary. The slow rate of sedimentation does not permit this to be taken as evidence for a single sedimenting species, but it is evidence that there is not present in solution a mixture of monomer with stable polymers, unless the polymers have a shape which compensates for their increased weight and so have a sedimentation rate identical with the monomer. Alternatively, since it is known from the osmotic pressure measurements that SH-keratin is associated under these conditions, the appearance of the sedimentation boundary could arise if the protein were sedimenting as an equilibrium mixture of monomer and polymer in which the rate of association and disassociation were fast compared with the rate of sedimentation.

Fig. 9 gives the relation between the sedimentation constant and concentration for cysteic acid-keratin, and Table 4 gives the values of the sedimentation constant at different concentrations of SH-keratin in 5M urea (pH 8.3) and for SH-keratin

Fig. 8. Schlieren diagrams of cysteic acid-keratin sedimenting in the centrifugal field. The solvent was 5M urea, 0.1M-NaCl, 0.05M phosphate (pH 6), and the concentration of the keratin 1.8% (w/v). A, 30 min. after forming the boundary; B, 176 min. after forming the boundary. (Sedimentation from right to left.)

Fig. 9. Sedimentation constants of cysteic acid-keratin in 5M urea, 0.1M-NaCl, 0.05M phosphate (pH 6).

in 10M urea (pH 9). Within the limits of the experimental error, the variation of sedimentation constant with concentration is the same for all three preparations.

The identity of the sedimentation constants of cysteic acid-keratin and SH-keratin at pH 8.3, considered together with the association that the osmotic pressure measurements reveal in solutions of the latter, requires that the association should occur with production of increasingly asymmetric particles. The viscosity measurements give qualitative support for this explanation.

From the sedimentation constant and the frictional ratio the molecular weight can be calculated by the equation given by Svedberg & Pedersen (1940). The frictional ratio of keratin, obtained from the axial ratio by Perrin's equation (Perrin, 1936) is 1.7 and the corresponding value of the molecular weight of cysteic acid-keratin is 10 100.

If, however, the ratio of the molecular weights of SH-keratin and cysteic acid-keratin, at 1% concentration, is calculated from the appropriate frictional ratios the value of 1.15 is obtained. The ratio of the number-average molecular weights at this concentration is 1.5 and this discrepancy cannot be due to experimental error alone.

Light-scattering

Fig. 10 gives the relationship between HC/τ and concentration of SH-keratin in 5M urea, 0.1M bisulphite, 0.05M phosphate at pH 6 and

Fig. 10. Light scattering by SH-keratin. $\times \times \times$, In 5M urea, 0.1M bisulphite, 0.05M phosphate (pH 6). $\bigcirc - \bigcirc$, In 5M urea, 0.1M bisulphite, 0.05M phosphate (pH 8.5).

Table 4. Sedimentation constants for SH-keratin

Solute	Solvent	pH	Concn. (% w/v)	$S_{20,w}$
SH-keratin extracted at pH 8.3	5M urea	8.3	1.5	0.97
	0.1M bisulphite		1.25	0.97
	0.05M phosphate		0.7	1.00
			0.3	0.93
SH-keratin extracted at pH 9	10M urea	9.1	3.0	0.63
	0.1M bisulphite		1.2	0.94
	0.05M phosphate		0.67	0.93

pH 8.5. The weight-average molecular weight is $11\,000 \pm 1000$. This is sufficiently close to the number-average molecular weight (10 000) to warrant the conclusion that there is not a large distribution of particle weights. Deviations of the molecular weight obtained in this way from the true value could occur due to preferential absorption of urea or water (Ewart, Roe, Debye & McCartney, 1946, Doty & Edsall, 1951), but as Pasynskii & Chernyak (1950) found little absorption of either on wool keratin, they probably do not occur with feather keratin.

The variation of the turbidity with concentration of SH-keratin at pH 8.5 is taken as confirmation of the association detected by the osmotic pressure measurements.

DISCUSSION

As it is known that keratin in the solid state is aggregated by —S—S— bonds, hydrogen bonds and salt linkages, it is necessary, in order to identify the keratin monomer, to specify the extent to which these forces are present between the keratin particles in the solutions in which the measurements of molecular size were made. It is probable that solutions of cysteic acid-keratin and infinitely dilute solutions of SH-keratin in 5M urea, 0.1M bisulphite, 0.05M phosphate (pH 6–8.5), contain no intermolecular bonding of this sort, for cysteic acid-keratin which has no —S—S— bonds and SH-keratin which does have them have the same value for the number-average molecular weight, and increase of the urea concentration from 5M (pH 8.5), to 10M (pH 9) or variation of the ionic strength from 0.17 (pH 6) to 0.64 (pH 8.5) does not change that value.

With regard to the effect of the ionic strength, in solutions with a pH different from the isoelectric point there will be a repulsive force between the keratin particles and the increase of ionic strength would reduce that force. Alternatively, if there were a considerable distance between electrically positive and negative centres, attraction between the oppositely charged parts of adjacent molecules would be possible and the effect of high ionic strength would be to decrease the interaction. The constancy of the molecular weight under the conditions listed in Table 3 is taken as evidence for the absence of aggregation due to these effects.

It must therefore be concluded that the —S—S— bonds which are present in SH-keratin are, when the solutions are very dilute, intramolecular. The association which occurs with increasing concentration can be due to formation of intermolecular —S—S— bonds at the expense of intramolecular bonds through the intermediary action of the SH

groups, of which only a catalytic amount would be necessary.

Dissociation would be favoured by the mass-action effect of dilution. Association may be favoured by the increased opportunity which polymer formation affords for hydrogen bonding between the polypeptide chains. Indeed, it is probable that some secondary bonding is a necessary condition for the association to occur since it produces molecules of increased asymmetry and there will be a decrease in the entropy of the system. This explanation of the association is analogous to that put forward by Huggins, Tapley & Jensen (1951), to account for the formation of gels by concentrated solutions of serum albumin in urea and thioglycollate.

We have little information on the homogeneity of the polypeptides in soluble feather keratin except that the ultracentrifuging and a single electrophoresis experiment are consistent with there being a narrow distribution of particle weights and charge. However, it has been shown by end-group analysis that wool keratin contains several polypeptides of similar weights (Middlebrook, 1951), and there is no evidence to show that this is not also true of feather keratin. The precipitation of some SH-keratin at high ionic strength or on diminution of the urea concentration at pH 9, cannot be taken as evidence for chemical heterogeneity, as in the former case the precipitation is dependent on the protein concentration and in the latter on the age of the solutions.

The solubility of SH-keratin may be determined by the configuration which the polypeptide chains assume (cf. Blackburn, 1951). Pauling & Corey (1953) have described possible structures for keratins, and the different degrees of hydrogen bonding in these may determine their relative stability in the solid state. Alexander & Earland (1950) found a correlation between the solubility of a product obtained by oxidation of wool and its ability to give an α or β X-ray diagram.

The graph relating the specific viscosity to concentration of SH-keratin at pH 8.5 does not flatten at concentrations above 1% in the way found with the P/C against C relationship. Cysteic acid-keratin is ideal over this concentration range, but this need not be true of the polymers formed by association of SH-keratin. Indeed, because of their increased asymmetry, they would be expected to deviate from ideal behaviour at lower concentrations than the monomer, and the increasing slope of the specific viscosity/concentration relationship indicates that this is so.

For this reason it is not possible from the osmotic pressure measurements to set an upper limit to the association. Molecular weights can only be obtained from osmotic pressure measurements at finite concentrations when the solutions are ideal, that is the entropy of mixing is given by $\Delta S = -R \ln N$, where N is the mole fraction of the solvent. For asymmetric molecules this relationship does not hold and above a certain concentration P/C becomes dependent on concentration (Flory, 1945; Huggins, 1943). For feather keratin, deviations from ideality from this cause would operate in the opposite way from deviations due to the association.

Similar considerations apply to the variation of the turbidity with concentration. The turbidity of a solution of finite concentration is given by

$$\frac{HC}{\tau} = \frac{1}{M} + \frac{2BC}{RT},$$

where H , C and τ have the definition already given, M is the molecular weight, R the gas constant, T the absolute temperature, and B is a constant depending on the shape of the solute molecules, their interaction with the solvent, and with each other. At finite concentrations of asymmetric molecules, B operates to produce an increase of HC/τ with concentration; this is in the opposite sense to the effect of the association.

However, although the extent to which association proceeds cannot be specified from the experiments described here, the formation of gels on dialysis of solutions of SH-keratin indicates that association can proceed beyond the dimer level. If hydrogen bonding with urea and electrostatic repulsion between the keratin monomers tend to decrease the extent of association, then a lower urea concentration and a higher ionic strength may permit the formation of larger, but soluble, polymers than those detected in the solutions used in this work. Physical measurements on such solutions might give additional support to the hypothesis put forward here, that solutions of keratin with both —SH and —S—S— groups contain an equilibrium mixture of monomer and polymer in which the polymerization has produced increasingly asymmetric particles. With such solutions it might be possible to obtain better agreement between the calculated and measured sedimentation constants of keratin at finite concentrations.

SUMMARY

1. The preparation of solutions of feather keratin by dispersion in urea and bisulphite and by oxidation with performic acid is described.

2. The cysteine/cystine ratio of keratin dissolved in solutions of urea and bisulphite depends on the urea concentration and on the pH.

3. The physical properties of keratin in solution have been measured and correlated with the cystine and cysteine contents.

4. Measurements of osmotic pressure, turbidity, sedimentation rate and viscosity indicate that in infinitely dilute solution keratin has a molecular weight of 10000 and an axial ratio of 13.

5. Preparations which contain both free —SH groups and —S—S— bonds associate with increasing concentration. It is concluded that the association involves the formation of intermolecular —S—S— bonds at the expense of intramolecular bonds.

6. Association produces a particle which is more asymmetric than the monomer.

7. Keratin sediments with a single boundary in the ultracentrifuge.

8. The variation of the viscosity, osmotic pressure, sedimentation rate and turbidity with concentration of dissolved keratin is discussed.

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The Effect of Anionic Detergents and some Related Compounds on Enzymes

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Synthetic detergents of all types are now widely used domestically, and in many branches of medicine and industry. The biological effects of these detergents are only partially understood, and it was considered that a systematic examination of the effect of some of these detergents on enzymes would be of value. Most commercial detergents are complex mixtures and often of secret formula, but some classifications have been presented in the literature (e.g. Van Antwerpen, 1943). The present work deals only with those detergents usually referred to as the 'anionic detergents'.

In earlier investigations from this laboratory it was found that the trypanocidal drug Suramin has a strong inhibitory action on a few enzymes at pH 7.5 and a complete or almost complete inhibiting effect on many enzymes on the acid side of their isoelectric points (Wills & Wormall, 1950*a*, *b*; Wills, 1952*a*). As an extension of this work, a study has now been made of the effect of various detergents on enzymes, since preliminary experiments indicated that some detergents have an effect on enzymes somewhat similar to that of Suramin. In the investigations described here a few experiments have been made with commercial detergents, but in most cases sodium dodecyl sulphate (SDS), which is obtainable in a fairly pure state, has been used as a typical anionic detergent.

Several studies have been made of the combination of SDS with proteins (Putnam & Neurath, 1944), but few observations have been made on the effect of SDS or related compounds on enzymes. Marron & Moreland (1939) reported that surface-active substances (including a detergent) had little effect on several enzymes tested. SDS has been observed to precipitate crystalline pepsin at acid pH (Putnam & Neurath, 1944) and inactivate it at pH 4.0 (Cooper

& Bull, 1944) but only slight combination of this enzyme and detergent occurs at pH 6.5 (Lundgren, 1945). More recently, Rabinovitz & Boyer (1950) have shown that SDS inhibits the succinic oxidase system.

In the present work the effect of SDS on enzymes has been compared with the effect of several aromatic sulphonic acids and with that of some commercial detergents which are sulphonated compounds, e.g. Teepol. A preliminary report on some of these investigations has been presented (Wills, 1952*b*, 1953).

EXPERIMENTAL

Enzyme preparations and activity measurements

The methods of preparation and estimation of activity for most of the enzymes used have been previously described (Wills & Wormall, 1950*b*). Other sources and methods are described below. Where SDS or another substance was being tested for inhibitory power, a suitable concentration of this test substance was added to the enzyme mixture and the amount of water reduced accordingly.

Lipase. This was used as an aqueous extract of hog pancreas powder. This powder was prepared by extracting a pancreas with acetone and ether, and drying and pulverizing the solid in a mechanical mortar. A 5 g. sample of the powder was extracted with 50 ml. water in a mechanical shaker, and the supernatant centrifuged.

D-Amino acid oxidase. An aqueous extract of dried acetone-powder of pig kidney prepared as described by Krebs (1935) was used.

Lipase estimations. These were made by three methods.

(i) *Continuous-titration method.* Water (3.8 ml.), triacetin (0.2 ml.) and bromothymol blue (4 drops of a 0.04% solution) were mixed in a boiling tube at 18° and the pH was adjusted to 6.8 with 0.01 N-NaOH. Enzyme solution (1 ml.) was added and the pH kept at 6.8 by addition of 0.01 N-NaOH. Titration readings were taken at intervals of 1 or 2 min. for about 10 min.