OSMOTIC PRESSURE AND DIFFUSION CONSTANT OF PROTEIN EXTRACTED FROM WOOL WITH UREA-BISULPHITE SOLUTIONS

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Summary

Wool treated with 8M urea, 0.4M $NaHSO_3$ at 60°C forms a polydisperse soluble protein of number-average molecular weight 12,000-16,000 as deduced from osmotic pressure measurements. The values lie within this range whether 20 or 70 per cent. of the wool is dissolved. The pH of measurement or of extraction has no effect on the molecular weight of the solute over the range 5.6-8.0. The diffusion constant, $D_{20,w}$, of the soluble protein is 4.5×10^{-7} cm^2sec^{-1}.

I. INTRODUCTION

Solutions containing sodium bisulphite and high concentrations of urea have been widely used to render keratin partly soluble in an aqueous medium (Jones and Mecham 1943). The bisulphite causes fission of the disulphide bond of cystine with the formation of sulphydryl and S-cysteine sulphonate groups (Middlebrook and Phillips 1942) and the urea breaks internal hydrogen bonds in the protein.

Several workers (Mercer and Olofsson 1951; Olofsson 1951; Woods 1952; Ward 1952) have published determinations of molecular constants of wool dispersed in this solution but the results do not agree. By sedimentation and diffusion measurements Mercer and Olofsson found molecular weights for the wool molecule of 42,000 and 84,000 at pH 7 and 8 respectively, and axial ratios of 65:1 and 90:1, whereas Ward found, again from sedimentation and diffusion measurements, a molecular weight of 12,000-17,000 and an axial ratio of 45:1. From viscosity measurements Woods found that the molecule had an axial ratio of 10-16:1 and the molecular weight determined osmometrically was 30,000.

The present paper describes a study of the osmotic pressures and diffusion constants of wool extracts in urea-bisulphite solutions over the concentration range 0.5-3.5 per cent. of protein.

II. EXPERIMENTAL

(*a*) *Preparation of Wool Extracts*

The wool extracts were prepared by the following procedures:

(i) Solvent-scoured 64's Merino wool (50 g) was immersed at 60°C for 48 hr in 8M urea (300 ml) containing 0.4M $NaHSO_3$ (Merck Reagent Grade).

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The protein solution was then separated by centrifugation, the residue was extracted with a further 200 ml of urea-bisulphite solution, and the extracts were combined. During extraction the pH of the solution rose from the initial value of 5.6 to 7.8. About 15-20 per cent. of the wool was dissolved under these conditions. The extract was dialysed for 2 days against 2 l. of urea-bisulphite solution (8M urea, 0.4M $NaHSO_3$, 0.1N CH_3COONa) and then centrifuged at 25,000*g* for 1 hr to remove a small amount of aggregated material. By this means an optically clear solution was obtained. The pH of this solution was maintained above 5, as otherwise the solution became turbid, indicating gross aggregation of the protein.

(ii) Merino wool (64's, 6 g) was immersed for 8 hr at 60°C in 100 ml of 8M urea, 0.4M bisulphite solution. The mixture was homogenized in a Waring Blendor for 20 min and the extraction continued at 60°C for a further 16-24 hr. With this method more than 60 per cent. of the wool dissolved; with repeated extraction and homogenization at least 85 per cent. could be dissolved, as determined by estimation of the protein in solution. The increase in solubility was almost certainly due solely to the mechanical disintegration of the swollen fibre whereby the solubilizing agents could penetrate the cortex much more readily. It is interesting in this connection that wool ground in a Wiley mill prior to extraction was not significantly more soluble in the reagent than the intact fibre.

For diffusion measurements 20 ml of the stock solution of protein, prepared by the first of the above methods, was dialysed against 2 l. of urea-bisulphite solution containing 0.1M CH_3COONa, 0.1M CH_3COOH, and 0.05M NaCl. More dilute solutions were obtained by dilution of the stock. Osmotic pressure measurements were carried out using similar solutions or solutions prepared by the second method. pH's were all measured using a glass electrode assembly and refer to values obtained in 8M urea.

(*b*) *Estimation of Protein Concentration*

The concentration of protein was determined by dialysing 5-ml samples against running water and weighing the precipitated protein after drying at 105°C for 18 hr. Attempts were made to use the modified Folin-Wu colorimetric method (Lowry *et al.* 1951) since a similar method was found satisfactory by Burk and Greenberg (1930) for various proteins dissolved in urea solutions, but the presence of sodium bisulphite caused irregular colour formation. Destruction of the bisulphite by boiling with acid, by oxidation with bromine water followed by boiling, or by oxidation with hydrogen peroxide followed by destruction of excess peroxide with a minute amount of catalase did not satisfactorily overcome this difficulty.

(*c*) *Measurement of Viscosity*

Viscosities were measured in Ostwald viscometers (British Standard Nos. 0 and 1) and densities in a Sprengel pyknometer.

(*d*) *Measurement of Osmotic Pressure*

The osmotic pressure was measured in the osmometers of Adair (1925) and Bull (1941) and in a modified Hepp osmometer designed by Scatchard *et al.* (unpublished data). All measurements were made in a constant-temperature bath at 27.2°C. "Cellophane" tubing (Visking 18/32 in.) identical with that used for dialysis of the protein solution was used in the Adair and Bull osmometers, and a period of 1 wk was usually required for equilibrium to be established. In the Hepp osmometer "Cellophane 300 PT" was found more satisfactory than other membranes such as "Cellophane 600 PT" and collodion. Measurements by both dynamic and static methods were completed within 3-4 hr.

(*e*) *Measurement of Diffusion Constant*

Diffusion constants were measured in a standard LKB 3021 Tiselius apparatus equipped with a diagonal-slit Schlieren system. The 10-cm intermediate cell sections were used. The boundaries were moved mechanically to the centre of the cell and then sharpened according to the technique of Kahn and Polson (1947). Because of the high viscosity of the solutions, the sharpening was not as successful as it might have been, but the "zero time" of the experiments was reduced considerably from that found without sharpening the boundaries. Diffusion was allowed to proceed for *c.* 7 days at 7.5°C. At the conclusion of each experiment tracings of enlarged images of the boundaries were made on squared paper for measurement.

III. Results

(*a*) *Osmotic Pressure Measurements*

The molecular weights were calculated from the extrapolated value of P/C (P = osmotic pressure, C = concentration of protein) using the formula

$$M = RT/(P/C)_0,$$

where M is molecular weight, R the gas constant, and T the absolute temperature. If P is in cm H_2O and C in g/l the expression becomes, at 27.2°C,

$$M = 25{,}475/(P/C)_0.$$

The range of protein concentration covered was 4-20 g/l, the lower limit being set by the sensitivity of the analytical method used. The values of P/C obtained with the Adair and Bull type osmometers showed a considerable spread (Table 1) and led to a mean P/C of 2.06 ± 0.22 (S.D.) with no certain concentration dependence. The corresponding molecular weight is 12,400. On the other hand the values obtained with the Hepp osmometer showed very little spread, no concentration dependence, and a $(P/C)_0$ value of 1.57 ± 0.04 (S.D.), which gives a molecular weight of 16,200. It was noticed with the Adair and Bull type osmometers that after some days, when apparent equilibrium had been reached, there was often a slight fall in pressure. With the Hepp osmometer there was a slow fall of osmotic pressure with time but this did not affect the results as measurements were usually completed in 3-4 hr. Scatchard (1952)

reported similar effects using a Hepp osmometer with isinglass and stated that extrapolation to zero time gave accurate values for molecular weights. A similar procedure was found to be valid in the present instance. The slight decrease in osmotic pressure is attributed to a slow diffusion of protein through the membrane. Some of the molecules of such polydisperse systems certainly lie in the critical range for diffusion through the "Cellophane."

TABLE 1

OSMOTIC PRESSURE MEASUREMENTS

In experiments 1-4 about 20 per cent. of the wool was dissolved but in experiments 5 and 6 the amount was 70 per cent.

	Experiment No.	Concentration (g/l)	pH	*P/C*
Adair and Bull osmometers	1	11·4	5·4	2·22
		11·5		2·17
		8·0		2·43
		7·9		2·38
		4·2		1·89
	2	14·66	5·6	1·91
		14·56		1·92 Mean 2·06±0·22*
		7·90		1·83 M = 12,400
		7·78		1·82
		4·74		1·84
	3	7·80	5·7	2·00
		3·90		2·32
		3·90		2·08
Hepp osmometer	4	8·86	5·6	1·57
		8·86		1·60
		8·86		1·58
	5	18·1	5·6	1·55
		9·05		1·49 Mean 1·57±0·04*
		4·53		1·56 M = 16,200
	6†	18·8	5·8	1·57
		17·5	7·9	1·61

*Standard deviations.

† The initial pH of the urea-bisulphite was 5·8 but during extraction of the wool the pH rose to 7·8. The two measurements were carried out on samples dialysed against buffers of pH 5·8 and 7·9 respectively.

(*b*) *Diffusion Measurements*

The shortcomings of the Tiselius apparatus for measuring diffusion constants are well known. They arise chiefly from the facts that the cylindrical lens is uncorrected and that the boundary has to be moved after its formation. In

the present work the use of a boundary-sharpening device partly compensated for the second difficulty. However, the maxima of the diffusion curves were not particularly well defined, especially near the beginning of an experiment.

Diffusion constants were usually calculated from both limbs of the apparatus by both the height-area (D_h) and second moment (D_m) methods. In Table 2 the two values from each experiment have been averaged. All values were corrected to diffusion in water at 20°C. For calculating D_h a mean area was used. It will be seen that there is no significant concentration dependence in the values of D_h. The values for both D_m and D_h were derived from the gradient of the root mean square line through about six points over the interval 0-10,000 min. The points for D_h fitted these closely but there was a tendency for D_m to diminish somewhat in the later stages of the diffusion process ($t > 5000$ min). This effect has been observed previously (Neurath 1942); in the present investigation at least it may be due to slow aggregation of the protein. However, all points were given equal weight in the calculation of D_m, which no doubt contributed largely to the wide variation encountered in the results.

TABLE 2

DIFFUSION CONSTANTS OF WOOL PROTEIN

Experiment No.	Concentration (g/l)	$D_m \times 10^7$	$D_h \times 10^7$
7	17	4·39	4·38
7*a*	35	4·98	4·65
7*b*	9	4·09	4·50
7*c*	9	—	4·52
		Mean 4·49	Mean 4·51

IV. DISCUSSION

The molecular weights 12,000 and 16,000 derived from osmotic pressure measurements appear to agree with the value of 14,000 reported by Ward (1952). However, Ward employed values of sedimentation (S) and diffusion constants (D) determined at 3 per cent. concentration in his calculations. The validity of this procedure depends upon S and D having similar concentration dependence. In the present investigation it was shown that D_h varied little with concentration whereas Ward found a strong concentration dependence of S upon C. When this is taken into account it appears that Ward's molecular weights should be approximately doubled. This would be much more reasonable, since if Ward's weight-average value and our number-average value were approximately the same the protein solution would be fairly homogeneous in molecular size, which is most unlikely.

The values reported in the present paper are also lower than the approximate value of 30,000 reported by Woods (1952), but this would be accounted for by his use of a more porous membrane, since he found that during the

attainment of equilibrium some 30 per cent. of the protein passed through the collodion. Ward found that 10 per cent. of his wool extract was dialysable through "Cellophane." In the present study the amount of protein passing through "Cellophane" was too small to be detected with protein precipitants.

The value 12,000-16,000 is much lower than Mercer and Olofsson's value of 84,000 obtained at pH 8 and Olofsson's value of 42,000 at pH 7. Olofsson (1951) stated that this high-molecular-weight fraction was limited to 10 per cent. or less of the total weight of wool. If this were so, it would not appreciably affect the number-average molecular weight. Mercer and Olofsson carried out their sedimentation and diffusion measurements in 25 per cent. urea (approximately 4M), although they had used 8M urea for the extraction. When the urea concentration of our extracts was reduced below *c.* 6M a considerable increase in the turbidity of the solution invariably occurred, suggesting that the protein had aggregated. This conclusion is supported by the low value reported by Mercer and Olofsson for the diffusion constant ($D_h = 1.61 \times 10^{-7}$) compared with the present value of 4.5×10^{-7}.

It is further shown that there was no difference in molecular weight of the protein extracted at pH 5.6 and at pH 8.0, which is contrary to Olofsson's results.

Osmotic pressure measurements gave the same molecular weight whether 20 or 70 per cent. of the wool protein dissolved and thus revealed no obvious fractionation of the protein in the extraction process. The mean diffusion constant, 4.5×10^{-7} cm^2 sec^{-1}, is only slightly lower than Ward's value of 4.9×10^{-7}. Because of the high degree of polydispersity of the wool protein solution, axial ratios calculated from molecular weights and diffusion constants using Perrin's equation are of doubtful significance. Various authors have applied such treatments and obtained results indicating extremely asymmetric molecules. Recent theoretical developments (Fessler and Ogston 1951; Scheraga and Mandelkern 1953)* make it probable that many axial ratios calculated in the literature are too large, and more exact data on actual hydration of well-defined proteins or protein fractions are necessary before the question of axial ratios of protein molecules in solution can be finally settled. Applying the theory of Scheraga and Mandelkern to these urea-bisulphite extracts of wool, and using the viscosity results of Woods together with the diffusion constant and corrected sedimentation molecular weight of Ward (approx. 28,000), it was found that values of the parameter β are obtained which correspond to molecules of low asymmetry.

Reduction to normal coordinates of several diffusion curves from experiment 7 showed very little deviation from a standard Gaussian curve. Experiment 7*a*, however, showed rather more. No definite conclusions can therefore be reached concerning the homogeneity of the protein.

The small difference in average D_m and D_h values in Table 2 does not indicate that the extracted wool protein is monodisperse. Gralén (1941, 1947) has shown that a solute must be grossly polydisperse to make D_m and D_h differ by more than 10 per cent., and the individual values of D_m in Table 2 vary so much that no great reliance can be placed upon the average.

* We are indebted to Mr. E. F. Woods for drawing our attention to these papers.

Experiments by several workers (Olofsson and Gralén 1947; Mercer and Olofsson 1951; Ward 1952) as well as some unpublished experiments from this laboratory showed that treatment of wool at high pH (> 12) led to the extraction of fragments having a mean molecular weight similar to that reported in the present paper.* Preliminary results showed that the diffusion constant was somewhat higher ($D_h = 5.7 \times 10^{-7}$; $D_m = 5.3 \times 10^{-7}$). The diffusion curves when reduced to normal coordinates deviated considerably from Gaussian curves, indicating a high degree of polydispersity. It is inherently unlikely, of course, that urea-bisulphite and strong alkali would have the same chemical effect on the wool fibre. Useful comparisons between different chemical treatments of wool cannot be made until chemical analyses of the different soluble products have been made.

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