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JOHNSTON–OGSTON EFFECT IN THE ULTRACENTRIFUGATION OF SOLUTIONS OF DENATURED COLLAGEN

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SUMMARY

Denatured collagen, made by raising the temperature of acid-soluble collagen dissolved in sodium chloride–acetic acid to 40°, consists of two main protein species, α and β, and a small quantity of a third species, γ-collagen. It is shown that the ratio of the amounts of the α- and β-proteins may be calculated from the ratio of the areas of the schlieren α- and β-peaks and the sedimentation coefficients of the peaks. The method is tested by comparing the $\alpha/\beta/\gamma$ ratios of two denatured collagen fractions, quantitatively separated by salt gradient elution from a carboxymethylcellulose column, with the ratio in the collagen solution which was the starting material on the column.

INTRODUCTION

In the ultracentrifuge the rate of sedimentation of a protein in solution decreases as the protein concentration increases. SCHACHMAN[1] points out that, if the solution contains a neutral salt to minimize effects due to migration of molecules with a net charge, then there remain at least three contributing causes of the concentration dependence of sedimentation rate: the influence of protein concentration on the viscosity of the medium, on the solution density, and on the counterflow of displaced liquid as the protein moves forward. Because of the complex causes of the concentration dependence, any relationship between sedimentation coefficient and concentration must be empirical.

When two proteins, α and β, with different sedimentation coefficients are sedimenting together in the ultracentrifuge cell part of the slower sedimenting protein, α, is sedimenting in the presence of the faster sedimenting protein, β, and part is sedimenting in the absence of the faster component. JOHNSTON AND OGSTON[2] showed that this would cause the concentration of the α-protein to be greater behind the β-protein boundary than in front of it, if the rate of sedimentation were related to the total protein concentration, irrespective of species. The areas under the schlieren peaks then cease to represent the concentrations of the α- and β-proteins. The apparent and actual states of affairs are shown in Fig. 1.

When acid-soluble collagen, dissolved in 0.05% acetic acid – 0.1 M NaCl, is

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denatured by raising the temperature to 40°, the tropocollagen molecules dissociate to give two main protein species, α- and β-collagen, with a small amount of a third species, γ-collagen. The three species have molecular weights[3] in the ratio 1:2:3, and the ultracentrifuge will clearly reveal three peaks corresponding to the three species (Fig. 2). This paper will show how the true α/β ratio can be calculated from the apparent ratio, the ratio of the areas of the schlieren α- and β-peaks, using an empirical relation between the sedimentation coefficient of the α-species and the total protein concentration.

The sedimentation coefficients of the α-, β- and γ-species were measured using an acid-soluble tropocollagen at different concentrations in acetic acid–NaCl solution.

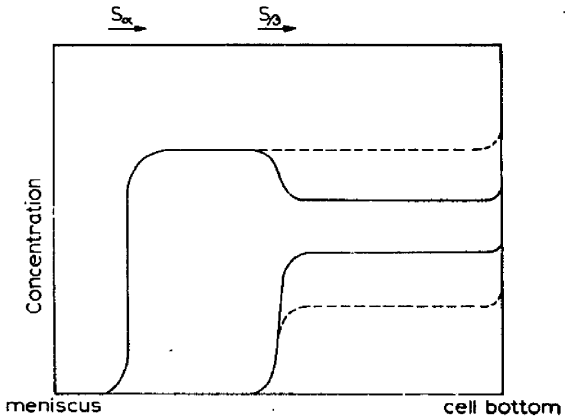

Fig. 1. Full lines, the actual state of affairs in the ultracentrifuge cell, after the separation of two proteins with different sedimentation constants; broken lines, the apparent state of affairs.

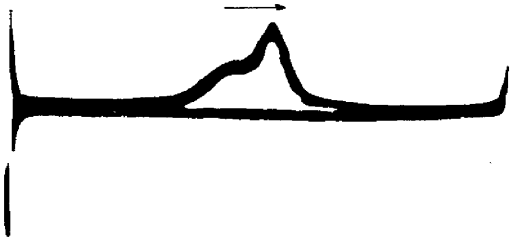

Fig. 2. Schlieren diagram from a denatured 0.35% acid-soluble collagen solution in 0.1 M NaCl–0.05% acetic acid; 56100 rev./min, 40°, 90 min after reaching maximum speed, sedimentation from left to right, bar angle 60°.

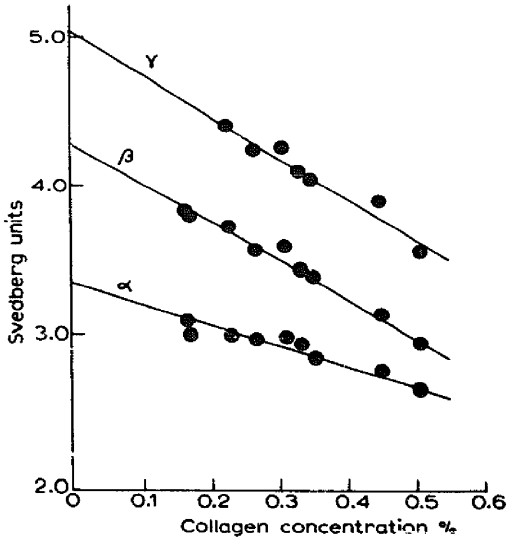

Fig. 3. Variation of the α-, β- and γ-sedimentation coefficients with total collagen concentration. Experimental conditions as in Fig. 2.

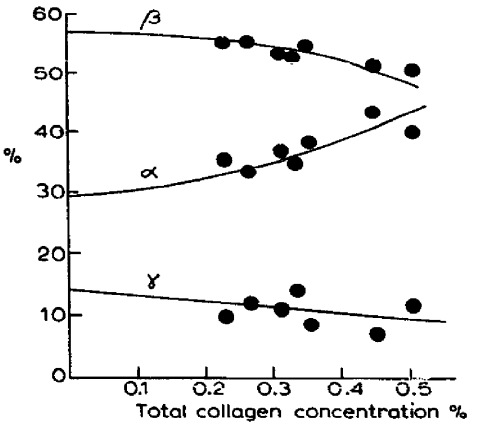

Fig. 4. Variation in the apparent ratios by weight of the α-, β- and γ-protein with total collagen concentration. Experimental conditions as in Figs. 2 and 3.

Fig. 3 shows that the fit of the points to a straight line is satisfactory if the sedimentation coefficients are plotted directly against total collagen concentration. At the same time, by constructing the α-, β- and γ-curves under the schlieren peaks, photographed when they have become clearly defined in the run, and correcting for radial dilution during the run, the apparent $\alpha/\beta/\gamma$ ratio can be measured. Fig. 4 shows the results plotted against total collagen concentration.

In JOHNSTON AND OGSTON's treatment the assumption is made that the sedimentation coefficient, S, of a protein at any point in the ultracentrifuge cell is linearly related to the total concentration of all species of protein at that point, C.

$$S = S_{\infty} + kC$$

where S_{∞} is the sedimentation coefficient at infinite protein dilution. Then it can be shown[2] that the real concentration of α-material, C_{α}, is related to the apparent concentration, C_{α}', by the equation,

$$\frac{C_{\alpha}'}{C_{\alpha}} = \frac{S_{\beta} - S_{\alpha,0}}{S_{\beta} - S_{\alpha}} \quad (1)$$

where S_{α} and S_{β} are the sedimentation coefficients of the α- and β-peaks, and $S_{\alpha,0}$ is the sedimentation coefficient of the α-protein in the presence of the β-protein.

$$S_{\alpha,0} = S_{\alpha,\infty} + k_{\alpha}C \quad (2)$$

where $S_{\alpha,\infty}$ is the sedimentation coefficient of the α-protein at infinite protein dilution, and k_{α} is the proportionality constant relating the sedimentation coefficient of the α-protein to the total protein concentration, C. In applying this theory to collagen solutions the JOHNSTON–OGSTON effect between β- and γ-molecules is taken to be negligible because of the small concentration of γ-protein, and C is the total collagen concentration, including γ-protein.

Thus, it was necessary to fit a line, using Eqns. 1 and 2, to the experimental points in the curve of apparent concentration of α-protein against total collagen concentration (Fig. 4). There are two unknowns, C_{α} and k_{α}, and these were calculated by choosing two total collagen concentrations, 0.3 % and 0.5 %, where the experimental points defined the values of C_{α}' quite clearly, and solving two simultaneous equations for k_{α} (= — 1.66) and C_{α}, the real concentration of α-protein. Then the line relating C_{α}' to total collagen can be constructed, a straight line can be drawn through the C_{γ}' points, the apparent concentrations of γ-protein, and the C_{β}' curve can be constructed by difference.

With Eqns. 1 and 2, the value of k_{α} determined above, and $S_{\alpha,\infty}$ from Fig. 3, the α/β ratio in any collagen solution can be determined from the apparent ratio and the values of S_{α} and S_{β} for the solution. The assumptions involved are (a) that no large error is introduced by treating the small amount of γ-material in the manner described (b) that the sedimentation coefficient of the α-protein is linearly related to the total protein concentration, irrespective (c) of whether this is α-protein or a mixture of α and β.

EXPERIMENTAL

Acid-soluble collagen was prepared at 5° by the extraction of fresh calf skin by 0.15 M citrate buffer (pH 3.1–3.7) after the skin had been exhaustively extracted at the same

temperature by 10 % NaCl solution. The collagen solutions were rigorously purified by filtration, low speed centrifugation, and precipitation by dialysis against 1 % NaCl. Sedimentation rates were measured in 0.1 M NaCl–0.05 % acetic acid at 40° in a Spinco Model E ultracentrifuge at 56100 rev./min. The schlieren areas were calibrated by a synthetic-boundary run with a sucrose solution; dn/dc collagen[5] = 0.186, dn/dc sucrose[6] = 0.143. The collagen solutions were denatured for 1 h at 40° before filling the ultracentrifuge cell. The base line in the ultracentrifuge runs was provided by a second normal cell containing the same amount of NaCl–acetic acid solution against which the collagen solution had been dialysed. The concentrations of the collagen solutions were measured in a Zeiss interferometer calibrated with a standard sucrose solution using the same values for dn/dc.

Test of the computation

PIEZ *et al.*[6] fractionated denatured collagen on a carboxymethylcellulose column with a sodium acetate buffer (pH 4.8) falling linearly in ionic strength during the elution. Monitoring the effluent, four peaks could be distinguished; two pairs of peaks were clearly separated.

The ultracentrifuge showed that the combined material responsible for the first two peaks (Fraction I) to be eluted from the column was enriched in α-collagen compared with the original collagen solution, and the material from the second pair of peaks (Fraction II) to leave the column was rich in β-collagen. (I am most grateful to Dr. J. H. HIGHBERGER of the United Shoe Machinery Company, Beverly, Mass. for the gift of samples of these fractions.) The recovery of material from the column was complete. The ratio of the amount of protein under the first pair of peaks, I, to that under the second pair, II, was 1–1.9. The compositions of the original collagen solution, and of Fractions I and II, were calculated by correcting the ratios of the schlieren areas for the JOHNSTON–OGSTON effect in the way described. Table I shows that the agreement between the composition of the original collagen solution and that calculated from the relative proportions of I and II is better than 5 %, except in the case of the γ-protein, where the error is less than 10 %.

TABLE I

	α-Component (%)	β-Component (%)	γ-Component (%)
Original acid-soluble collagen-schlieren areas corrected for JOHNSTON–OGSTON effect	29	57	14
Fraction I (see text) corrected schlieren areas	56	44	0
Fraction II corrected schlieren areas	21	69	10
Original acid-soluble collagen calculated from the composition of Fraction I and Fraction II	33	61	6

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