

## The Sedimentation Constant, Diffusion Constant and Molecular Weight of Lactoglobulin

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In a previous paper (Cecil & Ogston, 1948) we have described experiments directed to improving and testing the accuracy of the ultracentrifuge, using lactoglobulin as a test substance. We satisfied ourselves that we could measure sedimentation constants correct to 0.5%. Lactoglobulin was used as a working substance in those experiments; we describe here some further experiments on its sedimentation and diffusion.

### EXPERIMENTAL

**Materials.** Three preparations of lactoglobulin were made from different samples of milk. Preparations 1 and 2 were made by the method of Palmer (1934). Preparation 3, made by a combination of the methods of Palmer and of Sørensen (1939), was found to be as homogeneous as preparations 1 and 2, but was obtained in greater yield and with less labour. The method was as follows:

Fresh milk, warmed to 30°, was cleared of cream by means of a de Laval centrifuge, using the 38 mm. gravity ring; 39.2 g. of solid  $(\text{NH}_4)_2\text{SO}_4/100$  ml. of milk were added. The precipitate, containing the casein and some whey globulins, was granular and filtered easily. A further 11.6 g.  $(\text{NH}_4)_2\text{SO}_4$  were added per 100 ml. of filtrate, and the precipitate, after leaving to settle overnight, was then easily separated by filtration. It was dissolved in the minimal volume of water. Dialysis at pH 5.8 was found to give no precipitate; the pH was, therefore, adjusted directly to 5.2 by dialysis against acetate buffer. Dialysis against glass-distilled water then led to crystallization.

Recrystallization was in all cases performed by dissolving the crystals in dilute NaCl solution and dialyzing against glass-distilled water. Insoluble material, when present, was removed by centrifuging and any adjustment of pH required was obtained by dialysis against buffer. The preparation of solutions and determination of protein concentration have been described in the previous paper (Cecil & Ogston, 1948).

**Sedimentation measurements.** These were made over a range of concentrations using preparation 1, in 0.1 M-NaCl, 0.1 M-Na acetate and 0.04 M-acetic acid, and measurements were made also in buffers containing 0.1 M-NaCl, 0.1 M-Na acetate and 0.01 M- and 0.2 M-acetic acid respectively. Assuming a pK of 4.74 for acetic acid, the pH values of these buffers were 5.14, 5.74 and 4.44. All measurements were made by the new standard procedure, and the results (Table 1) have been corrected for the error of the rotor thermocouple. Fig. 1 shows a plot of sedimentation constant against protein concentration, and includes values obtained by Johnston & Ogston (1946) on a different preparation of lactoglobulin.

**Diffusion constant.** Diffusion runs were made in the pH 5.14 buffer with samples of three different preparations of lactoglobulin by the method of Coulson, Cox, Ogston & Philpot (1948). None of these proved to be quite homogeneous, by the criteria described by Ogston (1949) which were based on the relative positions of the interference bands arising from the diffusion boundary, in spite of exhaustive recrystallization and dialysis against buffer. However, Ogston (1949) has shown that it is possible to correct for the effects of heterogeneity on the apparent diffusion constant. Table 2 gives the apparent and corrected diffusion constants obtained in buffer at 20°, and the values of the diffusion constant corrected for the effect of buffer salts ( $D_{20}$  corr.).

### RESULTS AND DISCUSSION

**The sedimentation constant.** The mean value of  $S_{20}$  (corr.) (Cecil & Ogston, 1948) for a protein concentration of 1 g./100 ml. was  $2.81 \times 10^{-13}$ ; extrapolation to zero concentration gives a value of  $2.83 \times 10^{-13}$  (Fig. 1). These values agree with previous measurements on another preparation of lactoglobulin made in this laboratory, but they disagree seriously with the results of Pedersen (1936).

Pedersen obtained mean values of  $3.12 \times 10^{-13}$  in the range 'pH 5.2-7' and  $2.95 \times 10^{-13}$  in the range 'pH 1-5' at concentrations of 1 g./100 ml. and less. He does not give the exact compositions of his buffers, but in describing diffusion measurements, he quotes 5.0 as the pH of a buffer containing 0.2 M-sodium chloride, 0.038 M-sodium acetate and 0.012 M-acetic acid. On this scale, our 'pH 5.14' buffer would have a pH of 4.9. This difference stresses the inconvenience of quoting pH values without giving the exact compositions of the solutions used (see Ogston, 1947). However, since we have found a fall of sedimentation constant on lowering the pH and no increase on raising it, it appears that our 'pH 5.14' falls within the range of conditions 'pH 5.2-7' of Pedersen, and that our value of the sedimentation constant is, therefore, to be compared with his value of  $3.12 \times 10^{-13}$ .

The very large difference between these results—nearly 10%—requires explanation. It might possibly arise from differences in the samples of lactoglobulin used by us and by Pedersen. Thus Bull (1946a) has found erroneous values for the surface pressure of lactoglobulin which was recrystallized after dissolving the crystals in dilute

Table 1. Variation of  $S_{20}$  with protein concentration and pH

Run	Conc. of lactoglobulin (g./100 ml.)	pH	Cell (mm.)	$S_{20}$ (corr.) $\times 10^{13}$	Standard deviation of $S_{20}$ (corr.) $\times 10^{13}$
584	0.28	5.14	12	2.836	0.009
585	0.28	5.14	12	2.821	0.009
583	0.53	5.14	12	2.823	0.006
*	1.0	5.14	12	2.809	0.014
592	1.52	5.14	3	2.762	0.014
591	2.11	5.14	3	2.759	0.013
590	2.65	5.14	3	2.700	0.004
586	c. 1.1	5.74	12	2.776	0.011
594	1.0	4.44	12	2.723	0.009

\* From Cecil & Ogston (1948).

sodium hydroxide; the history, in this respect, of Pedersen's sample is not quoted; our samples were never exposed to a pH higher than that of native fresh milk.

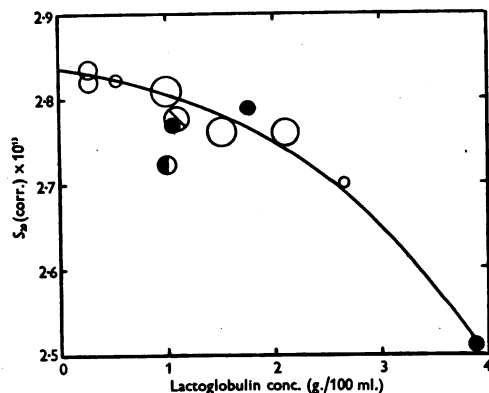


Fig. 1. Fully corrected values of  $S_{20}$  (corr.) against protein concentration. Filled circles: data of Johnston & Ogston (1946), corrected for effects of hydrogen pressure and thermocouple error. Open circles: data at pH 5.14; barred circle: value at pH 5.74; half-filled circle: value at pH 4.44. The radii of these circles are the estimated standard deviations.

On the other hand, the difference might arise from errors of one or both measurements. This explanation is supported by the result of a measurement kindly

made for us on one of our solutions (1 g./100 ml. in the pH 5.14 buffer) by Mr C. J. Bradish, using the Svedberg ultracentrifuge at the Lister Institute, from which he obtained a value of  $3.05 \pm$  (s.d.)  $0.04 \times 10^{-13}$  for  $S_{20}$  (corr.), in agreement with Pedersen's values. The discrepancy between this value and ours obtained on the same solution cannot be accounted for by errors in the measurement of speed. It therefore seems likely that the thermocouple readings in the Lister Institute ultracentrifuge are  $2.5^\circ$  lower than ours and, in our view,  $3.5^\circ$  below the actual cell temperature, and that a similar error has affected the Uppsala measurements. We have given reasons for believing (Cecil & Ogston, 1948) that we know the error of our rotor thermocouple ( $-1.0^\circ$ ) to within  $0.1^\circ$  and that no other systematic error affects our results.

*The diffusion constant.* The values which we have obtained (Table 2) are not quite as consistent as might be desired, although some of the variation is due to variation of the degree of homogeneity in different samples, for which correction has been made; this correction in no case amounts to more than 2% and is believed to be a reliable one. The mean value of  $D_{20}$  (corr.) at 1 g./100 ml. is  $7.70 \times 10^{-7}$  and at infinite dilution  $7.82 \times 10^{-7}$ . These values differ considerably from those of Polson (1939) who obtained values of  $D_{20}$  (corr.) between 7.10 and  $7.27 \times 10^{-7}$  at a concentration of 1 g./100 ml. Our method (Coulson *et al.* 1948) has given values in good

Table 2. Variation of  $D_{20}$  with concentration of lactoglobulin

Preparation	Conc. of lactoglobulin (g./100 ml.)	Apparent $D_{20} \times 10^7$	Corrected $D_{20} \times 10^7$	$D_{20}$ (corr.) $\times 10^7$
1	0.96	7.15	7.22	7.57
2	1.16	7.29	7.10	7.45
2	1.01	7.42	7.35	7.71
2	0.50	7.40	7.32	7.68
2	0.25	7.55	7.47	7.84
2	0.18	7.48	7.41	7.77
3	1.0	7.57	7.41	7.77
3	1.0	7.46	7.38	7.74

Mean value at 1 g./100 ml.

$7.70 \pm 0.09$

Value extrapolated to zero concentration

7.82

agreement with those obtained by the method of Lamm (used by Polson), not only for glycine but for material of much lower diffusion constant. It seems likely, therefore, that the difference may depend on the sample of lactoglobulin and on the method of its preparation.

*The molecular weight.* Estimates of the molecular weight of lactoglobulin by various methods range from 33,000, by X-ray diffraction on wet crystals of the orthorhombic form (McMeekin & Warner, 1942), to about 42,000, by sedimentation velocity and diffusion (Pedersen, 1936). The more reliable determinations by X-ray diffraction on dry crystals gave values about 36,000 (McMeekin & Warner, 1942). Pedersen's (1936) measurements on sedimentation equilibrium gave a mean value about 39,000; the variation of the value obtained with the method of computation suggests that the material used by him (and by Polson, 1939) was not homogeneous. Measurements of osmotic pressure have given values of 37,800 (Gutfreund, 1945),  $37,300 \pm$  (s.d.) 300 (Johnston & Ogston, 1946) and  $35,020 \pm$  (s.d.) 140 (Bull, 1946*a*). Bull (1946*b*) obtained a value of  $2 \times 17,100$  from measurements of surface pressure.

The present values of the sedimentation and diffusion constants, using Pedersen's (1936) value of 0.751 for the partial specific volume, give values

of 35,600 at 1 g./100 ml. and 35,400 at infinite dilution.

This wide variation of values is unsatisfactory. Errors may in some cases (as that of the sedimentation constant) have arisen from technical factors in the measurements; in others, variation may have been due to the treatment of the material during preparation, as is suggested by the data of Pedersen (1936) and the findings of Bull (1946*a*). It is difficult to decide which is the true value for the molecular weight; the most reliable determinations by X-ray diffraction favour a value near to 36,000, and our measurements on material which was very nearly homogeneous support this. The possibility exists, however, that lactoglobulin is not a material whose physical constants have unique values.

### SUMMARY

1. Measurements of the sedimentation and diffusion constants of lactoglobulin are described. The values, extrapolated to infinite dilution, are  $S_{20}(\text{corr.}) = 2.83 \times 10^{-13}$  and  $D_{20}(\text{corr.}) = 7.82 \times 10^{-7}$ ; these differ considerably from other data but combine to give a molecular weight of 35,400. Other reliable values of the molecular weight agree with this.

2. The causes of differences between measured values of the constants of lactoglobulin are discussed.

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## The Estimation of Peroxidase Activity

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The standard method for the estimation of the catalytic activity of peroxidase is that initially published by Willstätter & Stoll (1918). They allowed a measured quantity of the enzyme preparation dissolved in 2 l. distilled water to react with fixed amounts of hydrogen peroxide and pyrogallol for exactly 5 min. The reaction was then stopped by addition of sulphuric acid and the yellow purpurogallin transferred to ether in a separating funnel. The concentration of the ethereal solution was esti-

mated by comparison with standard purpurogallin solutions. The activity of the enzyme, Purpurogallin number (*P.N.*) or *Purpurogallinzahl* (*P.Z.*) of Willstätter, was defined as the weight of purpurogallin in mg. formed by 1 mg. of the enzyme preparation. Willstätter & Weber (1926) showed that the *P.Z.* values obtained varied over a wide range with changes in the quantities of the ingredients and in the volume of solution used for the test. A similar method for estimation of *P.N.* was