undergo this reaction in the absence of the other protein. This association is of a rapidly reëquilibrated type, and the polymeric species is greater than a dimer. The exact degree of association, however, cannot be deduced from the ultracentrifugal and electrophoretic data alone. Light scattering measurements aimed at that information will be described in the next paper of this series.

**Acknowledgments.**—The authors would like to thank Dr. W. G. Gordon for the samples of pooled milk β-lactoglobulin preparations, Dr. T. L. McMeekin for a sample of the "$\beta_2$-enriched" Polis fraction, Dr. R. Aschaffenburg for samples of β-lactoglobulins A and B and Dr. R. F. Peterson for giving us β-lactoglobulin fractions prepared by the paper curtain electrophoresis technique. We would also like to thank Dr. Aschaffenburg and Dr. G. A. Gilbert for most stimulating discussions of this work. We would like to thank Mrs. P. DeLucia for performing ultracentrifugal measurements.

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[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY,[1] PHILADELPHIA 18, PENNA.]

# Molecular Interactions in β-Lactoglobulin. III. Light Scattering Investigation of the Stoichiometry of the Association between pH 3.7 and 5.2[2]

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A light scattering investigation of the association of β-lactoglobulin between *p*H 3.7 and 5.2 has been carried out. The data can be best described in terms of a monomer–tetramer equilibrium. At the *p*H of maximal association $\Delta F^0 = -14.4 \pm 0.4$ kcal./mole, $\Delta H^0 = -53 \pm 1$ kcal./mole, $\Delta S^0 = -138 \pm 6$ e.u. From the thermodynamic parameters obtained in light scattering as a function of *p*H, area distributions of ultracentrifugal patterns have been calculated using the Gilbert theory. These are in quantitative agreement with the experimental sedimentation patterns. Comparison of samples of different compositions show that 90% of β-A and 30% of β-B can enter into this reaction.

## Introduction

In the previous paper,[3] ultracentrifugal and electrophoretic studies have been described, showing that β-lactoglobulin undergoes a reversible aggregation at cold temperatures between *p*H 3.7 and 5.2, maximal between *p*H 4.40 and 4.65. This association yields species greater than a dimer, but the exact degree of aggregation could not be deduced, as trimer, tetramer and pentamer formation were equally compatible with the sedimentation data.

In order to establish the stoichiometry of this reaction, a light scattering investigation was carried out between *p*H 3.7 and 5.1 in the temperature interval of 4.5 to 30°. It is the purpose of this paper to present the results of this study.

## Experimental

**Materials.**—The proteins used were the samples of pooled β-lactoglobulin (Prep II) and "Polis-$B_2$ enriched" protein described in the previous two papers.[3,4]

**Light Scattering.**—The light scattering measurements were carried out at 436 mμ in the Brice–Speiser photometer,[5] using 2 mm. slit optics. Stock concentrated solutions (*ca.* 100 g./l.) of β-lactoglobulin were made up in an acetate buffer of proper *p*H (r/2 = 0.1), dialyzed overnight against a large excess of the same buffer and cleared for light scattering by centrifuging in a Spinco Model L[6] centrifuge at 40,000 r.p.m. for 30 min., with filtration through an ultrafine sintered glass filter of special design[7,8] The working solutions were then made up by dilution into Dintzis-type cells,[9] using an ultra-micro burette. A blank light scattering measurement was first carried out in each cell on the diluting buffer used in it. Mixing was accomplished by gentle inversion and rocking of the teflon-stoppered cell. The temperature of the solutions was controlled by keeping the cells in a constant temperature bath adjusted to the desired temperature. The room was kept at a temperature close to the working temperature to minimize temperature changes during the actual measurements. Water from the constant temperature bath was circulated through the cell holder table and through a coil in the cell compartment of the photometer.

The solutions were prepared at the highest temperature used in a particular experiment, and then the temperature was decreased stepwise for each set of measurements. In this way, leakage out of the cell due to air expansion was avoided. Concentrations were measured on the stock solution and the cell contents were weighed at the beginning and end of a series of measurements to check for evaporation or leakage.

Concentrations were measured by ultraviolet absorption at 278 mμ, using a value of 0.96 l./cm. g. for the absorptivity.[3] The value for $dn/dc$ used was 0.1890.[10] All *p*H's were measured on a Beckman Model G[6] *p*H meter at 25°.

## Results

Since, for the proper interpretation of the electrophoretic, ultracentrifugal and light scattering data, it is essential to know whether the association is rapidly re-equilibrated, light scattering experiments were carried out to determine this rate. A 92.1 g./l. solution of pooled β-lactoglobulin was prepared in a *p*H 4.65 acetate buffer of 0.1 ionic strength, dialyzed against the same buffer and filtered for light scattering at 4.5°. It was then diluted rapidly with buffer to 10.6 and 3.2 g./l. with no change in temperature and its turbidity was measured as a function of time, starting less than one minute after dilution. It was found that no changes occurred after three minutes, while readings taken at times less than three minutes were not

(1) Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

(2) This work was presented in part at the 131st National Meeting of the American Chemical Society, Miami, April 1957 and at the 132nd Meeting, New York, September 1957.

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(5) B. A. Brice, M. Halwer and R. Speiser, *J. Opt. Soc. Amer.*, **40**, 768 (1950).

(6) Mention of specific firms and products does not imply endorsement by the Department to the possible detriment of others not mentioned.

(7) F. F. Nord, M. Bier and S. N. Timasheff, THIS JOURNAL, **73**, 289 (1951).

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(9) S. N. Timasheff, H. M. Dintzis, J. G. Kirkwood and B. D. Coleman, THIS JOURNAL, **79**, 782 (1957).

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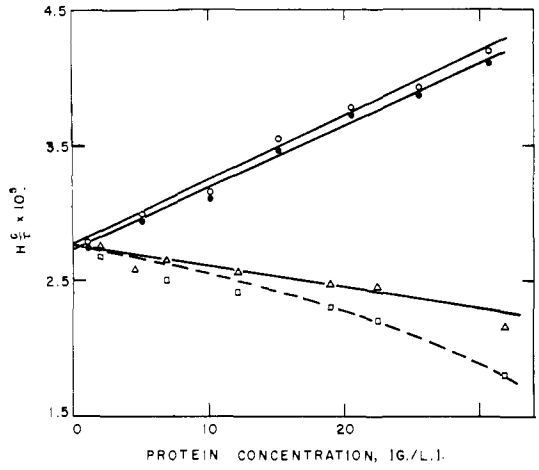


Fig. 1.—Light scattering data on pooled β-lactoglobulin at *p*H 3.7 and 5.1: O, *p*H 3.7, 25°; ●, *p*H 3.7, 8°; △, *p*H 5.1, 30°; □, *p*H 5.1, 4.5°. The dashed line at *p*H 5.1, 4.5° was calculated from the equilibrium constant and the slope at 30°.

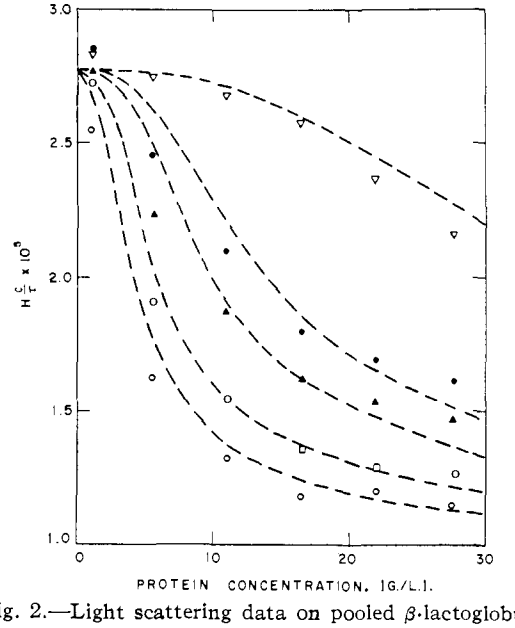


Fig. 2.—Light scattering data on pooled β-lactoglobulin at *p*H 4.4: O, 4.5°; □, 8°; ▲, 12°; ●, 15°; ▽, 25°. The curves have been calculated from the equilibrium constants of Table II, as described in the text.

much different from the final equilibrium values. It was concluded, then, that no significant changes in turbidity occur over the period during which measurements are taken and that equilibrium is established within one or two minutes. Since this is very short compared to the length of ultracentrifugal and electrophoretic runs, the system may be considered as an instantaneously re-equilibrated one for the purpose of data analysis, and the use of the Gilbert theory[11] in the previous paper[3] is fully justified. For light scattering studies, one can assume that the solutions are in a state of equilibrium at the time of measurement, which is of the order of 20–30 minutes after mixing or after reaching the experimental temperature.

(11) G. A. Gilbert, *Discussions Faraday Soc.*, **No. 2**, 68 (1955).

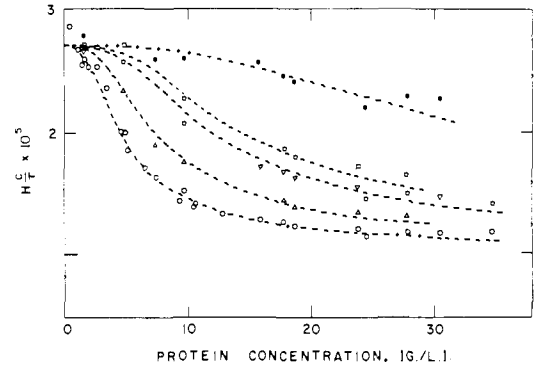


Fig. 3.—Light scattering data on pooled β-lactoglobulin at *p*H 4.65: O, 4.5°; △, 8°; ▽, 12°; □, 15°; ●, 25°. The curves have been calculated from the equilibrium constants of Table II, as described in the text.

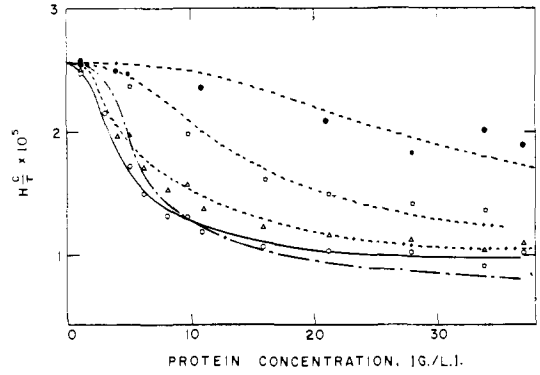


Fig. 4.—Light scattering data on "Polis $\beta_2$-enriched" β-lactoglobulin at *p*H 4.65: O, 4.5°; △, 8°; □, 15°; ●, 25°. ———, tetramer at 4.5°; — · —, pentamer at 4.5°; — —, tetramer at other temperatures, using equilibrium constants of Table I.

Complete light scattering experiments were carried out on the Prep II β-lactoglobulin between the temperatures of 4.5 and 30° at *p*H's 3.7, 3.9, 4.14, 4.4, 4.65, 4.9 and 5.1 in the concentration range of 0.5 to 35 g./l. The results of some of these are presented in Figs. 1–4. It can be seen in Fig. 1 that at *p*H 3.7 no significant change in turbidity occurs with decrease in temperatures, as the points obtained at 25 and 8° almost coincide over the entire concentration range studied. The slope at both temperatures is positive and almost identical. At *p*H 5.1, the function $H\frac{C_2}{\Delta\tau}$ decreases with concentration at all temperatures between 4.5 and 30° and at each concentration the low temperature points fall significantly below the corresponding room temperature ones, indicating the presence of a real effect at *p*H 5.1.

At the *p*H's of 4.4 and 4.65 (Fig. 2 and 3), there is a sharp decrease in the function $H\frac{C_2}{\Delta\tau}$ with increasing concentration at all temperatures. At the lower temperatures, the points first drop strongly and tend asymptotically toward a limit at higher concentrations. Thus, at *p*H 4.65, the values of $H\frac{C_2}{\Delta\tau}$ measured at a protein concentration of 56 g./l. (not

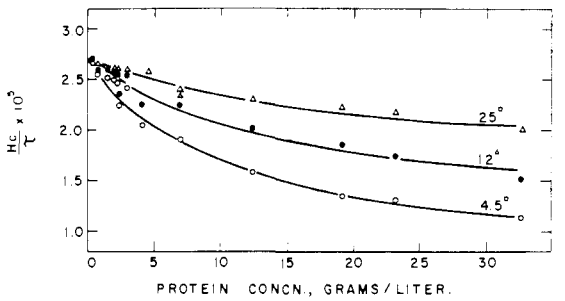


Fig. 5.—Light scattering data on pooled β-lactoglobulin at *p*H 4.9: O, 4.5°; ●, 12°; △, 25°.

shown in the figure) are not much different from those obtained between 20 and 35 g./l. At higher temperatures, the decrease in the values of $H\frac{C_2}{\Delta\tau}$ with increasing concentration becomes pronounced only in the higher concentration region, following an initial very slow decrease. The ultimate value of $H\frac{C_2}{\Delta\tau}$ approached decreases with a decrease in temperature.

Similar light scattering measurements were carried out on the "Polis $\beta_2$-enriched" protein at *p*H 4.65. The results obtained, shown in Fig. 4, are similar to those of the Prep II protein (Fig. 3). At each concentration, however, the values of $H\frac{C_2}{\Delta\tau}$ are lower than the corresponding value for the Prep II β-lactoglobulin. Therefore, at every temperature, the ultimate value of $H\frac{C_2}{\Delta\tau}$ attained by the "$\beta_2$-enriched" protein is lower than that in the case of the pooled milk preparation.

The results at *p*H 4.9 (Fig. 5) for the Prep II protein exhibit the same effects. At this *p*H, the data at 30° (not in figure) show an increase in turbidity with increase in concentration. The difference between the points at 30° and those at lower temperatures, however, demonstrates that at *p*H 4.9 also there is a strong temperature dependence of light scattering.

## Discussion

A detailed analysis of the light scattering data was undertaken in the following manner. In a three component system,[12] the light scattering equation[9,13-15] can be written in the form (component 0 = water, component 1 = diffusible solute, component 2 = protein)

$$H\frac{C_2}{\Delta\tau} = \frac{1}{1+D}\left\{\frac{1}{M_m} + C_2\left[\frac{1}{RT}\frac{\partial\mu_2^{(e)}}{\partial C_2} - F\left(\frac{\partial\mu_2}{\partial C_1}, \frac{\partial\mu_1}{\partial C_1}\right)\right]\right\} \quad (1)$$

$$D = \phi\left(\frac{\partial\mu_2}{\partial C_1}, \frac{\partial\mu_1}{\partial C_1}\right)$$

$$H = \frac{32\pi n^2(\partial n/\partial C_2)^2}{3\lambda^4 N}$$

$$u_i = RT\log C_i + \mu_i^{(e)} + \mu_i^0(T,P)$$

(12) In the present case, the system is really a four component one. However, the buffer (sodium acetate and acetic acid) will be considered for simplicity as a single component.

(13) J. G. Kirkwood and R. J. Goldberg, *J. Chem. Phys.*, **18**, 54 (1950).

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(15) H. C. Brinkman and J. J. Hermans, *ibid.*, **17**, 574 (1949).

where $C_2$ is the protein concentration in g./ml., $\Delta\tau$ is the excess turbidity of the solution over the solvent, $M_m$ is the molecular weight of the protein monomer, $n$ the refractive index of the solvent, $\lambda$ the wave length of the light, and $\mu_2^{(e)}$ is the excess chemical potential of the protein. $R$, $T$ and $N$ have their usual significance. The terms $F$ and $\phi$ are functions of the protein-diffusible component interaction and that of diffusible component with itself. $\phi$ also depends on the ratio of the refractive increments of the protein and the diffusible component.

Under the conditions of the present experiments, the contributions of $F$ and $\phi$ can be expected to be small. The term $D$ can be set equal to zero by analogy with serum albumin,[16,17] since β-lactoglobulin binds small ions[18,19] to a lesser extent than BSA.[20] The term $F$ should not be affected, as a first approximation, by aggregation. Thus, the light scattering equation for an associating system can be written in the form

$$H\frac{C_2}{\Delta\tau} = \frac{1}{M_m}\left[1 + C_2\left(\frac{\partial\log\gamma_m}{\partial C_2} + \frac{\partial\log f_m}{\partial C_2} + F\right)\right] \quad (2)$$

where $\gamma_m$ is the activity coefficient of the non-aggregated protein and $f_m$ is the fraction unaggregated.

For the reaction $nP \rightleftharpoons P_n$, the association equilibrium constant, $K_a$, is given by

$$K_a = \frac{(1-f_m)M_m^{n-1}}{nf_m^n C_2^{n-1}} \qquad f_m = 1 - \frac{\overline{M_w} - M_m}{(n-1)\overline{M_w}} \quad (3)$$

Then

$$\frac{\partial\log f_m}{\partial C_2} = \frac{K_a n(n-1)f_m^n C_2^{n-2}}{M_m^{n-1} + K_a n^2 f_m^n C_2^{n-1}} = \frac{\overline{M_w} - M_m}{\overline{M_w}\,C_2}$$

$$H\frac{C_2}{\Delta\tau} = \frac{1}{M_m}\left[1 + \left(\frac{\partial\log\gamma_m}{\partial C_2} + F\right)C_2\right] + \frac{1 - \overline{M_w}/M_m}{\overline{M_w}} = \frac{1}{\overline{M_w}} + \frac{2B_0C_2}{M_m} \quad (4)$$

Where $\overline{M_w}$ is the weight average molecular weight of the system at any given protein concentration and $(2B_0/M_m)$ is the second virial coefficient of the non-aggregated protein. It has been shown that for several systems $B_0$ does not change with aggregation.[16,21,22]

Since the maximum of aggregation of β-lactoglobulin occurs between *p*H 4.40 and 4.65,[3,23] this region was selected for the detailed analysis of the stoichiometry of this process. At *p*H 4.65, the β-lactoglobulin monomer carries a net average charge of +7[16,24] due to proton binding and it can be shown that at 0.1 ionic strength the light scattering second virial coefficient should be very small. The principal contributions to $2B_0/M_m$ should be

(16) S. N. Timasheff and M. J. Kronman, *Arch. Biochem. Biophys.*, **83**, 60 (1959).

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(19) C. W. Carr, *Arch. Biochem. Biophys.*, **46**, 417 (1953).

(20) G. Scatchard, J. S. Coleman and A. H. Shen, THIS JOURNAL, **79**, 12 (1957).

(21) H. Edelhoch, E. Katchalski, R. H. Maybury, W. L. Hughes, Jr., and J. T. Edsall, *ibid.*, **75**, 5058 (1953).

(22) C. M. Kay and J. T. Edsall, *Arch. Biochem. Biophys.*, **65**, 354 (1956).

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(24) R. K. Cannan, A. H. Palmer and A. C. Kibrick, *J. Biol. Chem.* **142**, 803 (1942).

a positive one from charge–charge repulsion and negative ones from the attractive force due to charge fluctuations[9,20,25-28] and from the ion-binding term $F$.[9,20] The contribution[29] to $2B_0/M_m$ of the repulsive force was estimated by a method[30] similar to that of Stigter and Hill[31] to be *ca.* $2.5 \times 10^{-7}$ l./g. The value of the fluctuating charge due to proton fluctuations, $<\Delta Z_2^2>^{1/2}_{Av.}$, of β-lactoglobulin was calculated according to the method of Kirkwood and Shumaker[25,32] to be 3.5, giving a contribution to $2B_0/M_m$ of *ca.* $-0.8 \times 10^{-7}$ l./g. The small negative contribution due to fluctuation of other bound ions cannot be calculated in the absence of detailed binding data.

The term $F$ was estimated[20] from data on potassium binding[16] to be *ca.* $-0.5 \times 10^{-7}$ l./g., assuming that sodium and potassium binding is not drastically different. Thus, $2B_0/M_m$ is the sum of small positive and negative quantities of similar magnitude and is itself small. The variation of $2B_0/M_m$ with temperature should not be significant if the principal contributions are electrostatic in nature.

Data analysis at *p*H 4.65 in 0.1 ionic strength acetate buffer is greatly facilitated by taking $2B_0/M_m$ to be zero, an assumption which the above arguments indicate as reasonable. The function $HC_2/\Delta\tau$ can be considered then to be equal to the reciprocal of the weight average molecular weight at any given concentration, and the equilibrium constant can be calculated directly from the data, using equation 3. Such an assumption would result in an error of not more than 5% at a protein concentration of 20 g./l.

Values of the molecular weights obtained at the higher protein concentrations at *p*H 4.65 rule out completely the previously proposed concept of a dimer.[23,33,34] This is in full agreement with the Gilbert[11] analysis[3] of the ultracentrifugal data. Thus, at 4.5° and a protein concentration of 30 g./l., the weight average molecular weight is 87,000 in the case of the Prep II protein and 103,000 in the "Polis $\beta_2$-enriched" material. The monomer molecular weight obtained in these light scattering studies is *ca.* 37,000.[35]

If it is considered, as shown in the previous paper,[3] that the protein consists of two fractions, one which can aggregate ($\beta_{Ag}$) and the other one which cannot ($\beta_{un}$), then, for the association

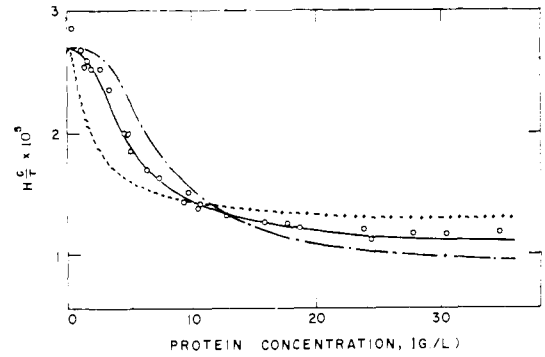


Fig. 6.—Determination of degree of aggregation of pooled β-lactoglobulin: lines calculated: ———, tetramer; — —, trimer; and — · —, pentamer formations. $x = 0.66$; O, experimental points; *p*H 4.65, 4.5°.

reaction, $n\beta_{Ag} \rightleftharpoons (\beta_{Ag})_n$, the equilibrium constant $K_a$ is given by

$$K_a = \frac{\alpha M_m^{n-1}}{n(1-\alpha)^n x^{n-1} C_2^{n-1}} \qquad (5)$$

where $\alpha$ is the fraction of $\beta_{Ag}$ aggregated at concentration $C_2$ and $x$ is the fraction of β-lactoglobulin represented by $\beta_{Ag}$.

The reciprocal of $HC_2/\Delta\tau$ at any given concentration being equal to the weight average molecular weight, the equilibrium constant corresponding to each light scattering point can be calculated for any degree of association using eq. 5 and the relationship

$$\bar{M}_w = M_m [1 + (n-1)x\alpha] \qquad (6)$$

From the average values of $K_a$ obtained in this manner, the smoothed light scattering curves over the entire concentration range studied can be calculated for any values of $n$ and $x$.

From the ultracentrifugal study described in the previous paper,[3] it is known that $x = 0.66$ for pooled β-lactoglobulin (Prep II) and $x = 0.76$ for the "$\beta_2$-enriched" protein.

Using the value of $x$ for the pooled protein, equilibrium constants fitting the data point by point were calculated setting $n = 3, 4$ and 5. The average values of $K_a$ at *p*H 4.65 were found to be $2.0 \times 10^8$ l.$^2$moles$^{-2}$, $2.0 \times 10^{11}$ l.$^3$moles$^{-3}$, and $4.0 \times 10^{14}$ l.$^4$moles$^{-4}$ for trimer, tetramer and pentamer formation, respectively. The smoothed light scattering curves, calculated with these values of $K_a$ are shown in Fig. 6. The dotted line corresponds to the trimer, the dot-dash line to the pentamer and the solid line to the tetramer. It is obvious that of the three, only the curve for tetramer formation fits the data well. The other two curves deviate strongly from the experimental points, the one for trimer being too low in the low concentration range and too high at high concentration, while the situation is reversed in the case of the pentamer. The trimer is further eliminated by the fact that for $x = 0.66$, the experimental data above a concentration of 25 g./l. can be satisfied only by an infinite value of $K_a$. Such a value of $K_a$ would, of course, not permit any observable dissociation at the lower concentrations studied.

The equilibrium constants deduced from the Prep II ($x = 0.66$) results, if correct, should de-

(25) J. G. Kirkwood and J. B. Shumaker, *Proc. Natl. Acad. Sci., U. S.*, **38**, 863 (1952).

(26) S. N. Timasheff, H. M. Dintzis and J. G. Kirkwood, *ibid.*, **41**, 710 (1955).

(27) S. N. Timasheff and I. Tinoco, *Arch. Biochem. Biophys.*, **66**, 427 (1957).

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(29) M. J. Kronman and S. N. Timasheff, *J. Phys. Chem.*, **63**, 629 (1959).

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(32) J. G. Kirkwood and J. B. Shumaker, *Proc. Natl. Acad. Sci. U. S.*, **38**, 855 (1952).

(33) A. G. Ogston and J. M. A. Tilley, *Biochem. J.*, **59**, 644 (1955).

(34) H. Klostergaard and R. A. Pasternak, THIS JOURNAL, **79**, 5671 (1957).

(35) The best value for monomer molecular weight seems to be 35,500.[36] The small discrepancy is probably due to the uncertainty in photometer calibration; small amounts of dust would also give an apparent molecular weight which is too high.

(36) F. R. Senti and R. C. Warner, THIS JOURNAL, **70**, 3318 (1948).

scribe also the association of the reactive fraction of β-lactoglobulin in any other preparation.

Theoretical light scattering curves were calculated for the "$\beta_2$-enriched" protein, using the equilibrium constants deduced above, with $x = 0.76$. The theoretical curves for tetramer and pentamer formation at 4.5° are shown by the solid line and dot-dash line, respectively on Fig. 4. The experimental points, which were then obtained, are shown by the open circles. The excellent agreement between the predicted theoretical curve for tetramerization of 76% of the protein and the experimental points is evident. The curve for pentamer formation is again in disagreement with the experimental data. The curve for trimerization (not shown on the figure) was also equally off from the data.

In similar manner equilibrium constants were determined at other temperatures for the Prep II protein at *p*H 4.65. The calculated curves obtained from the average values of $K_a$ at the various temperatures (given in Table I) are shown by the curves of Fig. 3 and are seen to represent the experimental data very well. Theoretical curves were calculated for the "Polis $\beta_2$-enriched" β-lactoglobulin (*p*H 4.65 acetate buffer, $\Gamma/2 = 0.1$) at various temperatures using the tetramerization equilibrium constants of Table I with $x = 0.76$. The agreement with experimental data is found to be excellent as shown in Fig. 4, where the solid line is the calculated curve for 4.5° and the dashed lines are calculated for 8, 15 and 25°.

TABLE I

EQUILIBRIUM CONSTANTS OF THE TETRAMERIZATION OF β-LACTOGLOBULIN

| *p*H 4.65 | | *p*H 4.40 | |
|---|---|---|---|
| *T*, °C. | $K_a$ | *T*, °C. | $K_a$ |
| 4.5 | $(2.0 \pm 0.3) \times 10^{11}$ | 4.5 | $(2.8 \pm 1.4) \times 10^{11}$ |
| 6.5 | $(1.2 \pm 0.2) \times 10^{11}$ | 8.0 | $(9.0 \pm 5.0) \times 10^{10}$ |
| 7.5 | $(9.0 \pm 1.0) \times 10^{10}$ | 12.0 | $(2.1 \pm 1.3) \times 10^{10}$ |
| 8.0 | $(4.5 \pm 1.0) \times 10^{10}$ | 15.0 | $(7.5 \pm 3.0) \times 10^{9}$ |
| 9.0 | $(5.4 \pm 1.0) \times 10^{10}$ | 25.0 | $(3.5 \pm 1.0) \times 10^{8}$ |
| 11.0 | $(3.0 \pm 0.5) \times 10^{10}$ | *p*H 4.90 | |
| 12.0 | $(1.0 \pm 0.3) \times 10^{10}$ | 4.5 | $(1.5 \pm 0.7) \times 10^{10}$ |
| 15.0 | $(6.0 \pm 1.0) \times 10^{9}$ | 12.0 | $(3.0 \pm 2.0) \times 10^{9}$ |
| 25.0 | $(4.5 \pm 2.0) \times 10^{8}$ | 25.0 | $(4.0 \pm 2.0) \times 10^{8}$ |

The concept of tetramerization was further tested on the data obtained at *p*H 4.4 as shown on Fig. 2. The dashed lines shown were calculated in the same manner from average values of the equilibrium constants. The second virial coefficient was again assumed to be zero. The values of the equilibrium constants at various temperatures are given in Table I.

The data at other *p*H's were analyzed in like manner in terms of a monomer-tetramer equilibrium of 66% of the pooled β-lactoglobulin. Below *p*H 4.40 and above 4.65, the term $2B_0/M_m$ had to be taken into account, since at lower *p*H's the charge–charge repulsion becomes predominant and results in a significant positive slope of light scattering, while at *p*H's closer to the isoelectric point this term becomes quite small and insufficient to counter-balance the negative contributions of the fluctuating charge and ion binding terms. In these cases, the value of $2B_0/M_m$ was taken from the slope of data obtained at 30° and subtracted from the lower temperature data before calculating the equilibrium constant. Details of such calculations are given elsewhere[16] for the data at *p*H 4.14 with the Prep II protein. The excellent agreement between the calculated curves and the experimental points, shown in Fig. 2 of ref. 16, bears out the validity of this procedure.

The shape of the light scattering curves shown on Fig. 2, 3 and 4 reflects the presence of a mixture of aggregating and non-aggregating proteins. The shape at low concentration is due to the presence of non-aggregating protein. As the concentration increases, the aggregation reaction becomes prominent and the weight average molecular weight undergoes a sharp increase. At the highest concentration, when the reaction is close to completion, the increase in weight average molecular weight slows down considerably and the data fall on almost horizontal lines.

Progressive aggregation of β-lactoglobulin to species higher than tetramer seems to be unlikely. In such a case, the weight average molecular weight would continue to increase significantly with increasing protein concentration. The fact that $HC_2/\Delta\tau$ seems to approach asymptotically a limiting value can be seen from Fig. 3 and 4 and is a strong argument for a limit to the degree of association. Values of $HC_2/\Delta\tau$ were also obtained at a concentration of 56 g./l. at *p*H 4.65 and were not significantly different from those at 35 g./l. at the various temperatures studied. Furthermore, in ultracentrifugal experiments, protein concentrations as high as 70 g./l. were used. In these experiments there was observed neither a significant increase of $s_{20,w}$ of the rapid peak over its value at 30 g./l. nor the appearance of any heavier species either as a new leading peak or as a leading shoulder on the rapid peak. It would thus seem that the aggregation of β-lactoglobulin cannot be forced beyond the tetramer stage.

In the preceding analysis of the data, it has been assumed that little or no contribution is made to the weight average molecular weight from species of intermediate degrees of polymerization. This assumption seems to be justifiable on the basis of the following reasoning. First, in the analysis of the ultracentrifugal data, presented in the previous paper,[3] it was shown that the patterns could be resolved into two symmetrical peaks with no residual area between them, the sedimentation constant of the slower peak being always equal to that of β-lactoglobulin monomer. Further, if one assumes that the tetramer is formed by the association of two dimers (formed as intermediates), the equilibrium expressions become

$$2\beta_{Ag} \underset{}{\overset{K_1}{\rightleftharpoons}} \beta_{Ag_2} \qquad 2\beta_{Ag_2} \overset{K_2}{\rightleftharpoons} \beta_{Ag_4}$$

$$K_1 = \frac{[\beta_{Ag_2}]}{[\beta_{Ag}]^2} \qquad K_2 = \frac{[\beta_{Ag_4}]}{[\beta_{Ag_2}]^2} \qquad (7)$$

The over-all equilibrium constant of tetramer formation is

$$K_a = K_2K_1^2 = \frac{[\beta_{Ag_4}]}{[\beta_{Ag}]^4} = \frac{a\sigma M^3_m}{4(1-\alpha)^4 x^3 C_2^3} \qquad (8)$$

where $C_2$ is the total protein concentration, $\alpha$ is the fraction of protein present in aggregated form and $\sigma$ is the fraction of aggregate present in the form of tetramer. Now, if $K_2 >> K_1$ (*e.g.*, if $K_2 = K^2$) $\sigma$ will be close to unity, the dimer will be present only in very small amount and its effect on the light scattering and sedimentation data would be negligible. The assumption that $K_2 \approx K_1^2$ would be quite reasonable for a tetramer in the form of a ring. Such a model seems highly plausible in view of the impossibility of forcing the aggregation beyond the tetramer stage. Furthermore, the consecutive formation of a dimer and a tetramer is equivalent to two consecutive dimerizations since the $(\beta_{Ag})_2$ species could be regarded as the monomer of the second dimerization reaction. In terms of the Gilbert theory,[11] dimer formation produces only a single peak in the ultracentrifuge and consecutive dimerizations would still yield only a single peak should the intermediate product be present in a significant amount. Since the ultracentrifugal patterns obtained have a bimodal area distribution with no residual area between the two peaks, the formation of a significant amount of $(\beta_{Ag})_2$ must be eliminated as a possibility. Similar reasoning eliminates the presence of significant amounts of trimer as an intermediate.

The possibility of various types of mixed aggregates must also be considered. It has been shown in the previous paper[3] that *ca.* 30% of β-B enters into the aggregation if β-A is present. If the bond energies are identical for the two genetic species the case becomes trivial, the two proteins being indistinguishable from the point of view of this reaction. In the case of a small difference in bond energies, any difference in extent of aggregation would be small and not detectable by the experimental techniques used in this study. From the good agreement between the ultracentrifugal results obtained on preparation of various compositions[3] and between the light scattering data on β-lactoglobulins of two different compositions, it seems unlikely that any major difference exists between the bonds formed. It becomes possible, then, as a first approximation, to treat this system as a mixture of a reactive protein ($\beta_{Ag}$, composed of 90% of the β-A and 30% of the β-B) and an unreactive one. The unreactive protein may reflect either the presence of structurally different proteins or an isomerization which must precede the aggregation ($\beta \rightleftarrows \beta^*$, with only $\beta^*$ aggregating). For the β-A protein, the first case seems to be true in view of Tombs' finding[37] of a minor (10%) component which behaves differently in electrophoresis from the bulk of β-A. The situation in the case of β-B is not clear at the present time, although its electrophoretic behavior[4] points to some type of molecular rearrangement.

From the equilibrium constants of the tetramerization reaction, thermodynamic parameters have been calculated as a function of *p*H. In Fig. 7, the values of log $K_a$ obtained at *p*H 4.65 are plotted as a function of $1/T$. Values were obtained from light scattering measurements at nine different temperatures (between 4.5 and 25°),

(37) M. P. Tombs, *Biochem. J.*, **67**, 517 (1957).

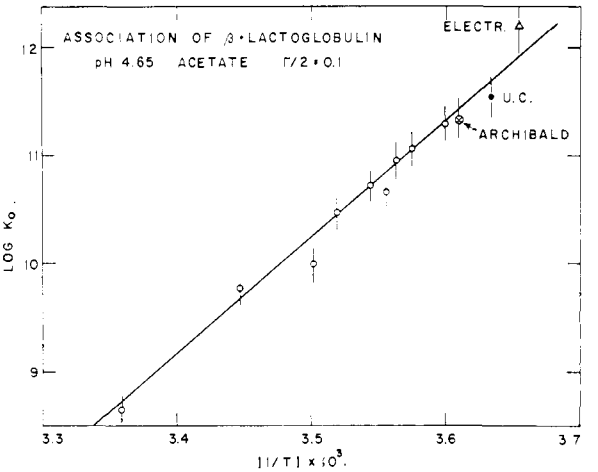


Fig. 7.—$\Delta H^\circ$ plot of data on β-lactoglobulin at *p*H 4.65: O, light scattering; ●, sedimentation (Gilbert analysis); Δ, electrophoresis (Gilbert analysis); ⊗, Archibald sedimentation analysis.

deduced from an Archibald type sedimentation experiment[38-41] at 4.1°, Gilbert analysis of sedimentation data[3] at 2° and electrophoretic data at 0.5°. It can be seen that they fall on a single straight line. The agreement obtained between the four types of measurements must be taken as further strong evidence that the reaction is adequately described by a monomer–tetramer equilibrium. From the slope of the plot in Fig. 7, $\Delta H^\circ$ of tetramerization is found to be − 52.7 kcal./mole, $\Delta F^\circ$ at 4.5° = −14.4 kcal./mole and $\Delta S^\circ$ = −138 e.u.

The values of the thermodynamic parameters as a function of *p*H are presented in Table II. It can be seen that while $\Delta F^\circ$ passes through a maximum at *p*H 4.40 − 4.65, $\Delta H^\circ$ and consequently $\Delta S^\circ$ seem to assume progressively more negative values as the *p*H decreases.

TABLE II

THERMODYNAMIC PARAMETERS OF THE TETRAMERIZATION OF β-LACTOGLOBULIN

| *p*H | $\Delta F^1$ (4.5°) kcal./mole | $\Delta H^\circ$, kcal./mole | $\Delta S^\circ$, e.u. |
|---|---|---|---|
| 3.90 | −11.1 ± 1 | −75 ± 15 | −230 ± 60 |
| 4.14 | −13.8 ± 0.4 | −59 ± 6 | −163 ± 23 |
| 4.40 | −14.6 ± .4 | −54 ± 2 | −143 ± 8 |
| 4.65 | −14.4 ± .4 | −53 ± 1 | −138 ± 6 |
| 4.90 | −12.8 ± .5 | −35 ± 5 | − 76 ± 25 |
| 5.10 | −10 7 ± 1 | −20 ± 4 | − 34 ± 18 |

In order to check further the extent of agreement between the light scattering and ultracentrifugal results, the area distributions under the reaction boundaries of the ultracentrifugal patterns were calculated from the $\Delta F^\circ$ and $\Delta H^\circ$ values listed in Table II. The calculations were done with the

(38) W. J. Archibald, *J. Phys. Colloid Chem.*, **51**, 1204 (1947).

(39) R. A. Brown, D. Kritchevsky and M. Davies, THIS JOURNAL, **76**, 3342 (1954).

(40) S. M. Kleiner and G. Kegeles, *J. Phys. Chem.*, **59**, 952 (1955).

(41) Archibald runs were carried out as described before.[42] That the Archibald technique can be applied to systems in rapid equilibrium has been shown previously.[43]

(42) R. Townend and S. N. Timasheff, THIS JOURNAL, **79**, 3613 (1957).

(43) G. Kegeles and M. S. Narasinga Rao, *ibid.*, **80**, 5721 (1958).

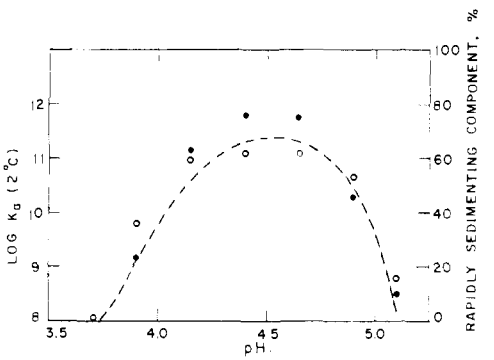


Fig. 8.—Comparison of experimental and calculated ultracentrifugal area distributions on pooled β-lactoglobulin at 2°, 30 g./l. protein in 0.1 ionic strength acetate buffer: ●, values of $K_a$ for tetramerization at 2°, calculated from light scattering data; O, area distributions calculated from light scattering data using the Gilbert theory ($x$ = 0.66); — —, curve drawn through experimentally determined area distribution under ultracentrifugal reaction boundaries.

use of eq. 3 of Paper II of this series, remembering that the two equilibrium constants are related by

$$K_G = \frac{M_m{}^{n-1}}{nK_a} \qquad (9)$$

Setting $M_m$ = 37,000 and $n$ = 4, the values of log $K_a$ and of the per cent of protein under the rapidly sedimenting "component" were calculated. These are shown on Fig. 8, together with the experimentally obtained[3] area distributions at the same conditions. The agreement obtained in this comparison of light scattering and ultracentrifugal results is quite satisfactory and bears out once again the treatment of the data in terms of tetramer formation, as well as the participation of the fractions of β-A and β-B deduced from the ultracentrifugal studies.[3] One should further consider that the comparison involves both the errors in the analysis of light scattering data and the uncertainty inherent in the "component" analysis of reaction boundaries.[44,45] This calculation of ultracentrifugal area distributions from light scattering data appears to be the first of its kind and can be considered as a further verification of the Gilbert theory of sedimentation in aggregating systems.

The agreement obtained between the light scattering, ultracentrifugal and electrophoretic results proves that a portion of β-lactoglobulin can undergo a reversible tetramerization between the *p*H's of 3.7 and 5.2. Between *p*H 4.65 and 4.40, in the range of maximal association, the charge per molecule of 37,000 varies from +7 to +10.[16] In the region where the association is no longer detectable (*p*H 3.7 − 3.5) the charge per 37,000 molecular weight is found to be +20 to +22. The observed association takes place then in a *p*H region at which there is still a strong non-specific electrostatic repulsion between the individual molecules. Using the Verwey and Overbeek potential,[46] one can calculate a repulsive energy (between the two molecules at closest approach) of 0.7 and 14.0 kcal./mole at *p*H 4.5 and 3.5, respectively. This calculation is only approximate and is based on the assumption that β-lactoglobulin is a spherical molecule with an even charge distribution. It is assumed also that all charges are located 1 Å. beneath the surface of the molecule.[47] The value of the dielectric constant used was, therefore, 20. The actual intramolecular energy pattern may be quite different from this idealized situation. Tanford has shown that the pattern of charge distribution on a molecule can play a primary role in determining the value of the electrostatic energy of a molecule and of its variation with *p*H. Thus, although an over-all repulsive force might be expected to exist between molecules such as are present in this system, molecular conformation and charge configuration may bring strong local attractive forces into play at close approach. The existence of proper constellations of ionizable groups at appropriate specific sites on the molecule could thus lead to intermolecular bond formation. While it would appear, therefore, that the interaction between molecules of β-lactoglobulin is of a specific nature, it seems that a detailed discussion of the mechanism of association and the types of forces involved must await more information on the nature of the participation of the two genetic species[48,49] of β-lactoglobulin in this reaction. Work to that effect is presently in progress.

The *p*H dependence of the thermodynamic parameters of this reaction presents an interesting pattern. The dependence of $\Delta F°$ on *p*H falls on a curve which is essentially bell-shaped, while no such effect is present in $\Delta H°$ or $\Delta S°$ While the values of the enthalpy at the extreme *p*H's carry a large uncertainty, there seems to be definitely a trend to less negative values of the enthalpy with increasing *p*H. The resulting values of the entropy reflect the uncertainty of the $\Delta H°$ and $\Delta F°$ values. Nevertheless, a trend to larger values with increasing *p*H seems to be present in this case also. Using the ideal gas equations, the calculated value of changes in entropies of rotation and translation for the tetramerization of β-lactoglobulin is *ca.* −250 e.u. The experimental value is −140 e.u. This latter seems to be accountable completely in terms of the change in size of the kinetic unit. The difference between −250 and −140 e.u., if considered real, may be ascribed to a molecular rearrangement which precedes aggregation and which has a positive entropy, a change in proton binding of the units of the aggregate or a loss of water of hydration in the course of the reaction. If it is considered that each water molecule lost contributes a positive entropy increment of 5 e.u., there would be required a loss of only five to six water molecules per site in a β-lactoglobulin monomer. Loss of such an amount of water of hydration in the interacting site is not unreasonable.

(44) L. G. Longsworth, in M. Bier "Electrophoresis," Academic Press, Inc., New York, N. Y., 1959, p. 91.

(45) R. A. Brown and S. W. Timasheff, *ibid.*, p. 317.

(46) E. J. W. Verwey and J. Th. G. Overbeek, "Theory of the Stability of Lyophobic Colloids," Elsevier Publishing Co., Inc., Amsterdam, 1948.

(47) C. Tanford, THIS JOURNAL, **79**, 5340, 5348 (1957).

(48) R. Aschaffenburg and J. Drewry, *Nature*, **176**, 218 (1955).

(49) S. N. Timasheff and R. Townend, THIS JOURNAL, **80**, 4433 (1958).