[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

# Molecular Weight of Lactoglobulin, Ovalbumin, Lysozyme and Serum Albumin by Light Scattering[2]

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Molecular weights of several proteins were determined from measurements of light scattering at 90°, using an absolute photoelectric turbidimeter. The values obtained were 35,700 for β-lactoglobulin, 45,700 for ovalbumin, 14,800 for lysozyme, a minimum of 73,000 for bovine serum albumin and 79,000 for horse serum albumin. Bovine serum albumin underwent a gradual aggregation when stored near 0°, with a moisture content of 2 to 10%. Satisfactory separation of normal and aggregated molecules was not accomplished by recrystallization, centrifugation or ultrafiltration. Osmotic pressure was a far less sensitive indicator of aggregates than light scattering.

The light scattering method for measuring molecular weights, developed chiefly by Debye,[3,4] was first applied to proteins by Putzeys and Brosteaux,[5] who determined the molecular weights of amandin, excelsin and hemocyanin relative to ovalbumin. Other light scattering studies have been made on the seed globulins of several species of the genus Prunus[6]; on edestin[7]; on influenza virus and tomato bushy stunt virus[8]; on tobacco mosaic virus[9]; on a rabbit antibody protein[10]; on the mercury compound of enolase[11]; on bovine serum albumin[12,13] on serum albumin and human γ-pseudoglobulin[13]; and on ovalbumin.[14,15] The molecular weights of the influenza virus, bushy stunt virus and ovalbumin were evaluated from measurement of the turbidities of solutions by the transmission method. The turbidities of solutions of the other proteins were measured indirectly by comparison of 90° scattering with that of materials of known or assumed turbidity. The turbidities reported here for lactoglobulin, lysozyme, ovalbumin and serum albumin were obtained directly from the scattering at 90° by means of an absolute photoelectric turbidimeter developed in this Laboratory.[16]

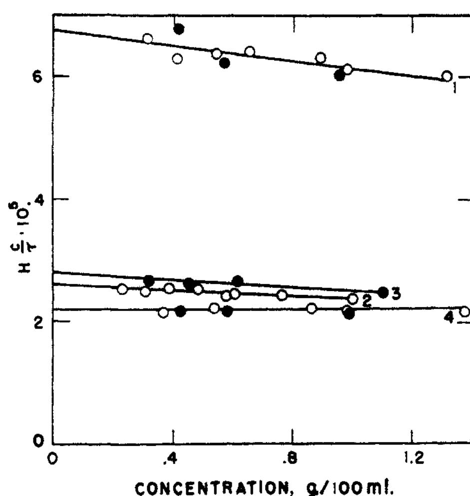


Fig. 1.—Plot of $Hc/\tau$ *vs.* concentration for lysozyme, lactoglobulin and ovalbumin: 1, lysozyme, open circles are before centrifuging, closed circles after centrifuging; 2, lactoglobulin, before centrifuging; 3, lactoglobulin, after centrifuging; 4, ovalbumin.

## Experimental[17]

**Materials.**—β-Lactoglobulin was prepared from fresh milk[18] and was recrystallized three times by dialysis of sodium chloride solutions. The nitrogen content of the purified protein, by the Kjeldahl method[19] was 15.6%.

Ovalbumin was prepared from the whites of fresh eggs.[20] It was recrystallized three times from ammonium sulfate solution, dialyzed against distilled water at 2°, and dried from the frozen state. The nitrogen content was 15.7%.

Lysozyme was obtained from Armour and Co. Analysis showed 2.9% acetic acid and 0.7% hydrochloric acid. The nitrogen content, corrected for these acids, was 19.0%. The sample was used as received.

Three bovine serum albumin samples were obtained from Armour and Co. These are called here samples BSA-46, BSA-49 and BSA-G4502, corresponding to the manufacturer's lot numbers. Sample 46 showed 15.9% nitrogen; sample 49, 16.1%; sample G4502, 16.1%. A fourth bovine serum albumin sample, BSA-4, was obtained from Drs. Edelhoch and Hughes[17] of the Harvard Medical School. The nitrogen content was not determined. A sample of human serum albumin, HSA-1, obtained from Sharp and Dohme, Inc.,[17] contained 16.1% nitrogen. All of these samples were used as received.

Horse serum albumin[17] was prepared from fresh horse serum[21] and was recrystallized four times, freed of electrolytes by dialysis and dried from the frozen state. The ni-

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(2) Presented in part at the 116th Meeting of the American Chemical Society, Atlantic City, N. J., September 18–23, 1949.

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(6) P. Putzeys and M. L. Beeckmans, *Bull. soc. chim. biol.*, **28**, 503 (1947).

(7) M. L. Beeckmans and R. Lontie, *ibid.*, **28**, 509 (1947).

(8) G. Oster, *Science*, **103**, 306 (1946).

(9) G. Oster, P. M. Doty and B. H. Zimm, THIS JOURNAL, **69**, 1193 (1947).

(10) D. H. Campbell, R. H. Blaker and A. B. Pardee, *ibid.*, **70**, 2496 (1948).

(11) T. Bücher, *Biochim. et Biophys. Acta*, **1**, 467 (1947).

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(13) J. T. Edsall, H. Edelhoch, R. Lontie and P. R. Morrison, THIS JOURNAL, **72**, 4641 (1950).

(14) M. Bier and F. F. Nord, *Proc. Natl. Acad. Sci. U. S.*, **35**, 17 (1949).

(15) W. Heller and H. B. Klevens, *Phys. Rev.*, **67**, 61 (1945) (Abstract).

(16) R. Speiser and B. A. Brice, *J. Optical Soc. Am.*, **36**, 364 (1946) (Abstract); B. A. Brice, *Phys. Rev.*, **75**, 1307 (1949) (Abstract). An improved form of this apparatus is described by B. A. Brice, M. Halver and R. Speiser, *J. Optical Soc. Am.*, in press.

(17) The authors express their thanks to H. Edelhoch and W. L. Hughes, Jr., of Harvard Medical School for a sample of bovine serum albumin; to P. Masucci and R. B. Pennell of Sharp and Dohme, Inc., for samples of human serum albumin and horse serum, respectively; to W. H. Stahl of the Philadelphia Quartermaster Depot for the use of a high speed centrifuge; to B. D. Polis of this Laboratory for lactoglobulin samples and for the electrophoretic examination of serum albumin; and to T. L. McMeekin of this Laboratory for the preparation of horse serum albumin.

(18) A. H. Palmer, *J. Biol. Chem.*, **104**, 359 (1934).

(19) A. Hiller, J. Plazin and D. D. Van Slyke, *ibid.*, **176**, 1401 (1948).

(20) S. P. L. Sørensen and M. Høyrup, *Compt. rend. trav. lab. Carlsberg. Ser. chim.*, **12**, 12 (1917).

(21) T. L. McMeekin, THIS JOURNAL, **61**, 2884 (1939).

TABLE I

SPECIFIC REFRACTIVE INCREMENTS OF THE PROTEINS AT 25°

| Protein | Solvent and *p*H | $(n - n_0)/c$, ml./g. 436 mμ | 546 mμ |
|---|---|---|---|
| β-Lactoglobulin | 0.1 *M* NaCl, *p*H 5.2 | 0.1890 ± 0.3%[a] | 0.1822 ± 0.4%[a] |
| Ovalbumin | 0.1 *M* NaCl, *p*H 4.8 | .1883 ± 0.5% | .1820 ± 0.5% |
| Lysozyme | 0.1 *M* NaCl, *p*H 6.2 | .1955 | .1888 |
| Bovine serum albumin | 0.1 *M* NaCl, *p*H 5.2 | .1924 ± 0.6% | .1854 ± 0.4% |
| Horse serum albumin | 0.1 *M* sodium acetate–acetic acid buffer, *p*H 4.8 | .1912 ± 0.4% | .1844 ± 0.5% |

[a] Each of these is 1.9% below the values 0.1926 at 436 mμ and 0.1856 at 546 mμ calculated from the data of G. E. Perlmann and L. G. Longsworth, THIS JOURNAL, **70**, 2719 (1948). Similar discrepancies exist between our results and theirs for ovalbumin and bovine serum albumin. The calibration of the differential refractometer used by us was frequently checked by means of a dipping refractometer, using potassium chloride solutions. It was also calibrated directly with sucrose solutions of known refractive index (see C. A. Browne and F. W. Zerban, "Sugar Analysis," 3rd Ed., John Wiley and Son, Inc., New York, N. Y., 1941, p. 1206).

Results showed that the determination of $n - n_0$ is probably not more accurate than ±0.5% but almost certainly more accurate than ±1%. We determined protein concentrations by drying aliquots of solution to constant weight and subtracting the non-protein solids in the cases of lactoglobulin and ovalbumin, and by direct weighing of protein samples of known moisture content, in the cases of lysozyme and the serum albumins. Perlmann and Longsworth used Kjeldahl determinations. We found for lactoglobulin and ovalbumin that the two methods gave the same result for $c$ within 0.1%. Dialysis of the protein solution against the solvent had no effect. We did not confirm the increase in specific refractive increment of serum albumin on adding sodium chloride, observed by Perlmann and Longsworth.

The figures for lactoglobulin are averages of six determinations on several different preparations, ranging from 0.1877 to 0.1902 at 436 mμ and from 0.1804 to 0.1828 at 546 mμ; figures for ovalbumin are averages of six determinations on two preparations, ranging from 0.1872 to 0.1903 at 436 mμ and from 0.1800 to 0.1835 at 546 mμ; the lysozyme figures represent one determination; the bovine serum albumin figures are averages of eight determinations on three different preparations, ranging from 0.1906 to 0.1940 at 436 mμ and from 0.1842 to 0.1869 at 546 mμ; figures for horse serum albumin are averages of two determinations. Average deviations from the mean are listed for each protein. We estimate the accuracy of our specific refractive increments to be ±1% or better.

trogen content was 15.8%. Carbohydrate determination by the orcinol method[22] showed 0.05%.

**Lactoglobulin.**—A solution of the freshly recrystallized protein in 0.1 *M* potassium phosphate buffer of *p*H 5.2 was filtered through an ultrafine sintered glass filter, pore diameter approximately one micron, into a light scattering cell. The filtrate was led down a glass rod, since it is important to avoid dropping. Turbidities were measured at 436 and 546 mμ. This procedure was repeated at a number of protein concentrations, the latter being determined by drying an aliquot to constant weight *in vacuo* at 100° and subtracting the known weight of buffer solids. The apparent turbidity of the solvent was also determined and subtracted from that of each solution. To obtain the molecular weight, the function $Hc/\tau$ was calculated for each protein concentration.[4] Here, $\tau$ is the turbidity of the solution in excess of that of the solvent, is cm.$^{-1}$; $c$ is the protein concentration, g./ml.; $H = 32\pi^3 n_0^2[(n - n_0)/c]^2/3N\lambda^4$, where $n_0$ is the refractive index of the buffer solution, $n$ that of the protein solution, $\lambda$ is the wave length, and $N$ is Avogadro's number.

The specific refractive increments, $(n - n_0)/c$, were determined with a differential refractometer.[23] The values found for lactoglobulin and the other proteins used in this study are collected in Table I. The values for human serum albumin were not determined but were assumed to be the same as for bovine serum albumin.

Values of $Hc/\tau$ at the two wave lengths agreed to within about 2%, the higher figures being obtained at 546 mμ. Average values of $Hc/\tau$ for the two wave lengths were plotted against $c$ (Fig. 1) and the best straight line drawn by the method of least squares. The intercept of this line at zero concentration is the reciprocal of the molecular weight, in accordance with Debye's equation,[4] $Hc/\tau = 1/M + 2BC$, where $B$ is a constant. A small correction is necessary for the depolarization of the scattered light.[4] This was measured by interposing a Polaroid analyzer in the scattered beam, and found to be 0.021 at 436 mμ. The same value can safely be applied at 546 mμ as well. The molecular weight is therefore to be multiplied by the factor $(6\text{-}7 \times 0.021)/(6 + 3 \times 0.021) = 0.965$. The molecular weight found[24] was 38,300. This is about 9% higher than the average of recent values by other methods, namely, 35,020 by osmotic pressure[25]; 35,500 by X-ray diffraction[26]; and 35,400 by sedimentation and diffusion.[27] Acting on the possibility that aggregates might have been present in the protein solution which passed through the filter, new solutions were centrifuged[17] at about 125,000 gravity for one hour at a rotor temperature of about 20°. There was no visible sediment. However, the supernatants were drawn off with a pipet, filtered through the ultrafine filter to remove a small amount of floating material and turbidity and concentration determined as above. The four points thus obtained are plotted in Fig. 1. The best straight line through the points extrapolates to molecular weight 35,700, in good agreement with the results obtained by the other methods.

**Ovalbumin.**—The sample was approximately two months old at the time the determinations were made and had been stored at −25°. The solvent was 0.1 *M* sodium chloride at *p*H 4.6. The procedure was the same as described above. In Fig. 1, average values of $Hc/\tau$ for the two wave lengths are plotted against concentration The values have been corrected for a depolarization of 0.024. Centrifuging for one-half hour at 140,000 gravity had no significant effect, as Fig. 1 shows. The best straight line leads to molecular weight 45,700. Recent values by other methods are: 41,500–44,900,[28] 45,000[29] and 46,000[30] by osmotic pressure, 37,500 by X-ray scattering[31] and 49,000[32] by sedimentation and diffusion. Cohn and Edsall,[33] summarizing older data, give the reliable range as 40,000–46,000. The present value is thus within the rather wide range of values given by other methods and agrees well with recent osmotic pressure determinations. The value of 37,000 obtained by Bier and Nord,[14] by light scattering measurements, is considerably lower. However, we have reason to believe that the true turbidity of the standard solution they used for calibration purposes is 0.00350 cm.$^{-1}$ at 436 mμ, rather than the value of 0.00270 found in Debye's laboratory.[16] This would raise their value to 48,000. Moreover, there is some question about their value for specific refractive increment, which

(22) M. Sørensen and G. Haugaard, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **19**, 1 (1933).

(23) B. A. Brice and R. Speiser, *J. Optical Soc. Am.*, **36**, 363 (1946) (Abstract).

(24) The value of 33,700 previously reported (M. Halwer and B. A. Brice, *J. Colloid Sci.*, **4**, 439 (1949)) is too low, since the turbidity equations used at the time did not take proper account of refraction corrections. The value should be 38,300.

(25) H. B. Bull and B. T. Currie, THIS JOURNAL, **68**, 742 (1946).

(26) F. R. Senti and R. C. Warner, *ibid.*, **70**, 3318 (1948).

(27) R. Cecil and A. G. Ogston, *Biochem. J.*, **44**, 33 (1949).

(28) A. V. Guntelberg and K. Linderstrøm-Lang, *Compt. rend. trav. lab. Carlsberg. Ser. chim.*, **27**, 1 (1949).

(29) H. B. Bull, *J. Biol. Chem.*, **137**, 143 (1941).

(30) H. Gutfreund, *Nature*, **53**, 406 (1944).

(31) D. P. Riley and D. Herbert, *Biochem. et Biophys. Acta*, **4**, 374 (1950).

(32) F. Eirich and E. K. Rideal, *Nature*, **146**, 541 (1940).

(33) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 390.

they took from the data of Barker[34] and apparently used without modification. However, Barker's specific refractive increment value applies to the 589 mμ line of sodium, whereas the light scattering measurements were made at 436 mμ. Using the value for specific refractive increment given in Table I leads to a molecular weight of 46,000, in very good agreement with our own value.

Heller and Klevens[15] found 47,000 by transmission measurements, a result in good agreement with our value.

Another sample of ovalbumin, which had been prepared from fresh eggs, crystallized three times and dried from the frozen state, was two and one-half years old at the time of testing, and had been stored at 5° during this time. Solutions were clarified by filtration through an ultrafine sintered glass filter. This sample gave a molecular weight of 48,600, possibly indicating a very slow aggregation on storage under these conditions

**Lysozyme.**—Solutions in 0.1 *M* sodium chloride at *p*H 6.2 were treated as already described for lactoglobulin. Concentrations were determined from the amount of protein weighed out, moisture content, as determined by drying to constant weight at 100°, and the acetic acid and hydrochloric acid contents. The points obtained are plotted in Fig. 1. The best straight line through the points leads to a molecular weight of 14,800. This includes a correction for a depolarization of 0.030. Centrifuging at 125,000 gravity for one hour had no definite effect, as shown by Fig. 1. Other values for this protein are 14,900[35] and 14,700[36] by amino acid analysis, 13,900 by X-ray diffraction,[37] 14,700 by sedimentation and diffusion measurements[38] and 17,500[38] and 16,600[39] by osmotic pressure.

**Serum Albumin.**—Sample BSA-46, dissolved in 0.1 *M* sodium chloride at *p*H 5.2 and filtered through ultrafine sintered glass, gave the lowest curve in Fig. 2. Protein contentrations were determined from the amount of protein weighed out and the moisture content as determined by drying at 100°. Correction has been made for a depolarization of 0.027. These data lead to a molecular weight of 99,000. It is generally accepted that the molecular weight of both bovine and human serum albumin is close to 70,000. A recent determination[40] by the osmotic pressure method gave a value of 69,000. Oncley, Scatchard and Brown[41] found 69,000 by sedimentation and diffusion. Others, using the osmotic pressure method, have reported results ranging from 69,000 to 76,000.[42] The value found for Sample BSA-46 is thus about 42% higher than the accepted value. Sample BSA-49, dissolved in 0.1 *M* sodium acetate buffer of *p*H 4.8, gave a molecular weight of 98,000. The sample of human serum albumin, dissolved in 0.1 *M* sodium chloride at *p*H 5.2, also gave 98,000. Thus, all three samples on hand at the beginning of these experiments gave values that were too high by more than 40%.

Two additional bovine serum albumin samples were obtained. Sample BSA-G4502 from Armour and Co., dissolved in 0.1 *M* acetate buffer at *p*H 4.8, gave 73,000, which is within 5% of the accepted value. Sample BSA-4, from Harvard Medical School, showed a molecular weight of 75,000 in 0.1 *M* acetate buffer.

Sample BSA-46 was examined electrophoretically[17] for the possible presence of a high molecular weight protein impurity, but none was found. The high molecular weights obtained for the first three samples seemed most likely to be due to aggregates not removed by the filtration technique used. This hypothesis was strongly supported by the observation, made a few months later, that samples BSA-G4502 and BSA-4 had increased in apparent molecular weight. Subsequent investigation showed a progressive increase in molecular weight with time for all samples (Table II). In this table, the second column refers to the length of storage after the initial determination of molecular weight. For the first three samples in Table II, the initial determinations were not made until long after the samples were received. Sample BSA-46 was received about 16 months before the first determination was made; BSA-49 was received about 10 months before the initial determination; HSA-1 was received nearly four years before the initial determination was made. The dates of original preparation are not precisely known. The two bovine samples had been crystallized by the manufacturer four times by the low temperature alcohol method of Cohn, Hughes and Weare[43] and had been stored at, or below, −5° prior to shipment. The human serum albumin sample had been prepared by the low temperature alcohol crystallization method of Cohn, *et al.*,[44] and had been crystallized only once. These samples were stored at about 5° after receipt. They were warmed to room temperature from time to time and opened for removal of samples. BSA-4 was an Armour and Co. product which was recrystallized several times further at Harvard Medical School, where it was stored continuously at 0°. After receipt at this Laboratory, it was kept at room temperature.

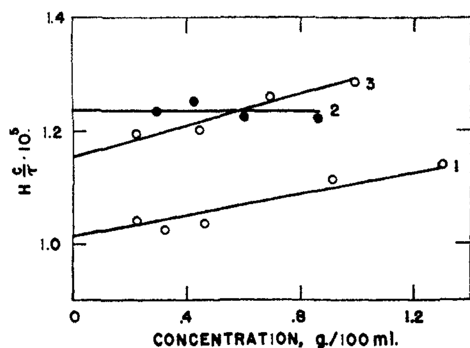


Fig. 2.—Plot of $Hc/\tau$ *vs.* concentration for bovine serum albumin: 1, sample 46 in 0.1 *M* NaCl, *p*H 5.2; 2, sample G4502-B in 0.1 *M* sodium acetate buffer, *p*H 4.8; 3, sample G4502-B in 0.1 *M* NaCl, *p*H 5.2.

TABLE II

CHANGES IN APPARENT MOLECULAR WEIGHT OF SERUM ALBUMIN SAMPLES WITH DURATION OF STORAGE

| Sample No. | Storage period[a] | Moisture, % | Molecular weight |
|---|---|---|---|
| BSA-46[b] | 0 | 6.8 | 99,000 |
| | 3 months | 7.0 | 101,000 |
| | 18 months | 9.4 | 237,000 |
| BSA-49 | 0 | 8.0 | 98,000 |
| | 9 months | 8.8 | 103,000 |
| HSA-1[b] | 0 | 7.8 | 98,000 |
| | 5 months | 8.3 | 107,000 |
| BSA-4 | 0 | 9.2 | 75,000 |
| | 11 months | 8.2 | 84,000 |
| BSA-G4502-A | 0 | 2.5 | 73,000 |
| | 8 months | 5.2 | 83,000 |
| | 11 months | 6.5 | 95,000 |
| BSA-G4502-B | 0 | (2.5)[c] | (73,000)[c] |
| | 8 months | 2.2 | 77,000 |
| | 11 months | 3.0 | 81,000 |
| | 17 months | 6.2 | 81,000 |

[a] After the initial molecular weight measurement. [b] In 0.1 *M* sodium chloride at *p*H 5.2. All other measurements in 0.1 *M* sodium acetate at *p*H 4.8. [c] Assumed the same as sample G4502-A.

(34) H. A. Barker, *J. Biol. Chem.*, **104**, 667 (1934).

(35) J. C. Lewis, N. S. Snell, D. J. Hirschmann and H. Fraenkel-Conrat, *ibid.*, **186**, 23 (1950).

(36) C. Fromageot and M. P. de Garilhe, *Biochem. et Biophys. Acta*, **4**, 509 (1950).

(37) K. J. Palmer, M. Ballantyne and J. A. Galvin, THIS JOURNAL, **70**, 906 (1948).

(38) G. Alderton, W. H. Ward and H. L. Fevold, *J. Biol. Chem.*, **157**, 43 (1945). The sedimentation and diffusion value is based on a partial specific volume of 0.720, as calculated by Lewis, *et al.* (35).

(39) A. Mohammed, unpublished experiments, quoted by Lewis, *et al.*[35]

(40) G. Scatchard, A. C. Batchelder and A. Brown, THIS JOURNAL, **68**, 2320 (1946).

(41) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 184 (1947).

(42) E. J. Cohn and J. T. Edsall, ref. 33, pp. 390, 392.

(43) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(44) E. J. Cohn, J. A. Luetscher, Jr., J. L. Oncley, S. H. Armstrong and B. D. Davis, *ibid.*, **62**, 3396 (1940).

Sample BSA-G4502 was presumably made in the same way as BSA-46 and BSA-49. Its molecular weight was measured immediately after receipt, and the sample was then stored at 5°.

The molecular weight of all samples increased with time. For BSA-46, the increase was very large. BSA-G4502 was received in two bottles, designated here BSA-G4502-A and BSA-G4502-B. Of these, bottle A was frequently warmed to room temperature and opened for removal of samples. It shows a rapid increase in moisture content and molecular weight. Bottle B was never opened or removed from the refrigerator until eight months after it was received. It is assumed that the initial values for bottle B were the same as those found for Bottle A. Table II shows that G4502-B, despite its low moisture content and continuous low temperature storage, underwent a small but definite increase in molecular weight.

Several methods were tried in the hope of freeing the samples of aggregates or at least of reducing their number. One was recrystallization. Sample 49 was recrystallized twice by the low temperature alcohol method of Cohn, Hughes and Weare.[43] Specifically, procedure 2 on p. 1757 was followed. Well-formed needle crystals were obtained. These were dissolved in water at 0–3°, the solution frozen in a Dry Ice-bath and dried from the frozen state. The molecular weight of the resulting material was 97,000, as compared with 103,000 before recrystallization. A second recrystallization gave 100,000.

It was hoped that the aggregates might disperse at *p*H values considerably removed from the isoelectric point. However, the molecular weights of sample 49 at *p*H 9.0 (0.05 *M* borate buffer) and at *p*H 4.0 (0.1 *M* NaCl, *p*H adjusted with HCl) were only slightly different from the molecular weight at *p*H 4.7.

Partial removal of aggregates was accomplished by differential ultrafiltration through swollen cellophane membranes. These were prepared by swelling discs of No. 300 cellophane in zinc chloride solutions of various concentrations.[45] The swollen membrane was clamped in a Seitz filter and a 1% solution of the protein filtered through by pressure. Best results were obtained by using a membrane swelled for 15 minutes at 20° in a solution containing 104 g. of zinc chloride per 100 ml. This membrane had an average pore diameter of 68Å., calculated by means of the equation given in Ref. 45. Sample 46, after filtration through this membrane, had a molecular weight of 85,000, as compared with 102,000 before filtration. Membranes of lower porosity than this retained all but traces of the protein.

High speed centrifuging also was partially effective in removing aggregates. An extensively aggregated sample (the protein in the supernatant from the recrystallization of sample 49) showing a molecular weight of 137,000, was dissolved in 0.1 *M* sodium chloride and centrifuged one hour at 125,000 gravity. There was no visible sedimentation. The protein in the supernatant had a molecular weight of 102,000.

Comparison of light scattering and osmotic pressure molecular weights was made for some of the bovine serum albumin samples. Osmotic pressures were determined at 25° with a Bull and Currie osmometer,[25] using collodion membranes. The solvent was 0.5 *M* sodium chloride. The levels were adjusted until they were close to the equilibrium positions, a procedure which generally required 3–4 hours, then 24 hours was allowed for final equilibration. Protein concentrations were determined after equilibrium had been established by drying an aliquot of the solution to constant weight at 100° and subtracting the known weight of sodium chloride. The results are given in Table III.

TABLE III

COMPARISON OF MOLECULAR WEIGHTS BY LIGHT SCATTERING AND BY OSMOTIC PRESSURE FOR SAMPLES OF BOVINE SERUM ALBUMIN

| Protein samples | Molecular weight Light scattering | Molecular weight Osmotic pressure | Ratio |
|---|---|---|---|
| BSA-G4502-B | 80,000 | 70,000 | 1.14 |
| BSA-G4502-A | 95,000 | 72,000 | 1.32 |
| BSA-49 | 103,000 | 78,000 | 1.32 |
| BSA-46 | 237,000 | 104,000 | 2.28 |

(45) W. B. Seymour, *J. Biol. Chem.*, **134**, 701 (1940).

The table illustrates the much greater sensitivity to the presence of aggregates of the light scattering method, which gives a weight-average molecular weight, than the osmotic pressure method, which gives a number-average molecular weight.

It is of interest that Koenig and Pedersen[46] in a recent ultracentrifuge study of bovine serum albumin prepared by the alcohol method found a small amount of high molecular weight material.

In view of the effect of storage time on the molecular weight of serum albumin, and of the fact that for none of these samples was the precise age and storage conditions known, a fresh sample of serum albumin was prepared. It was also desired to avoid the use of alcohol in the preparation, since it is possible that the gradual aggregation noted in Table II is caused by traces of alcohol not removed in the preparation of the samples. For this reason, it was decided to prepare horse serum albumin rather than bovine or human, since the former is readily crystallized by the ammonium sulfate technique, in contrast to the other two. The purified horse serum albumin, after drying from the frozen state, was stored at −25°. The experiments were completed within three weeks after the preparation of the sample. Measurements were confined to wave length 546 m$\mu$, since 436 m$\mu$ excited a fluorescence which could not be readily removed. The protein was dissolved in 0.1 *M* sodium acetate buffer of *p*H 4.8. As was the case with the other serum albumins, the plot of $Hc/\tau$ *vs.* $c$ showed a zero slope under these conditions. Haze was removed by filtering through an asbestos pad in a Seitz filter. A final filtration was made through ultrafine sintered glass. The molecular weight found under these conditions was 79,000. This value was not significantly changed either by prolonged high speed centrifuging (5 hours at 144,000 gravity, rotor temperature 26°) or by repeated centrifugings of one-half hour each. The value found is 14% higher than the generally accepted value of 69,000[47] and indicates that the sample was aggregated, in spite of its freshness and the fact that no alcohol was used in the preparation.[48]

Consistently higher molecular weights were found in 0.1 *M* sodium chloride at *p*H 5.2 (the "natural" *p*H of these samples) than in 0.1 *M* sodium acetate buffer at *p*H 4.8. A typical comparison is shown in Fig. 2 for sample G4502-B. The molecular weight in 0.1 *M* acetate buffer was 81,000. In 0.1 *M* sodium chloride, it was 87,000, or 7% higher. The effect is not traceable to differences in specific refractive increments, for these are the same in acetate buffer and in sodium chloride. The reason for the difference is not clear. Except where otherwise stated, the values in Table II are for acetate buffer.

## Discussion

The light scattering method is capable of giving satisfactory values for the molecular weights of proteins, provided that a sufficiently pure and unaltered sample is available for the determination. For a sample that meets these requirements, the light scattering method has a number of advantages over the osmotic pressure method. One is speed: a complete determination can be made in a few hours. Moreover, it has a greater range. For small molecules, like lysozyme, it is difficult to find an osmotic membrane which will retain the protein without being so sluggish as to be impractical; for large molecules like the viruses, the osmotic rise is too small for accurate measurement. The light scattering method dispenses with membranes and becomes more sensitive the higher the molecular

(46) V. L. Koenig and K. O. Pedersen, *Arch. Biochem.*, **25**, 97 (1950).

(47) E. J. Cohn and J. T. Edsall, *op. cit.*, p. 392.

(48) The stability of serum albumin may depend in some unknown manner on the way the sample was prepared or stored, or both. J. T. Edsall, in a private communication to the authors, states, concerning the experience of both P. M. Doty and himself, "We have both used different preparations of bovine albumin and have kept them for periods up to two years in the dry state without detecting any of the aggregation phenomena reported by the present authors."

weight. Furthermore, the light scattering method, with its instantaneous response, is nearly ideal for following systems which change with time.

The principal problem in the light scattering method is the preparation of the solutions. The sensitivity to the presence of aggregates is great, and exceptional care in clarifying the solutions is a necessity. Further investigation of techniques for doing this is desirable. High speed centrifuging seems to offer the most attractive possibilities.

The sensitivity to the presence of large particles makes the method useful in detecting aggregates in seemingly normal samples. Thus, in Table III, two of the samples have nearly normal osmotic pressure molecular weights, but the light scattering values show them to be partially aggregated. Ideally, both methods should be used on an unknown sample. Agreement would indicate strongly that the sample is pure and unaggregated.

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[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY][1]

# Effect of *p*H on the Denaturation of β-Lactoglobulin and its Dodecyl Sulfate Derivative

BY M. L. GROVES, N. J. HIPP AND T. L. MCMEEKIN

Factors that affect the stability of β-lactoglobulin are evaluated. It denatures unimolecularly in buffer solutions more alkaline than *p*H 8. As indicated by increase in optical rotation, the rate of denaturation of β-lactoglobulin is inversely proportional to the 1.1 power of the hydrogen ion concentration at 3 and 25° in both veronal and borate buffers. Under similar conditions, β-lactoglobulin that contains two equivalents of dodecyl sulfate denatures more slowly than does normal β-lactoglobulin and shows a positive temperature coefficient of denaturation. The effects of *p*H on denaturation of β-lactoglobulin are only partially consistent with the idea that denaturation is associated with ionization of amino groups. Denaturation of β-lactoglobulin by *p*H, heat, urea, guanidine hydrochloride and a detergent is briefly compared.

The stability of β-lactoglobulin solutions is of importance in its preparation and characterization. Bull[2] noted that when it is dissolved with dilute sodium hydroxide during recrystallization, its sensitivity to local excess of alkali causes a formation of a heterogeneous product. Its denaturation, particularly by urea, has been extensively studied.[3,4,5,6] Also it has been found[6] that denaturation of β-lactoglobulin is accompanied by a large change in optical rotation and that the rate is slow at *p*H 8.25, but increases at higher *p*H values.

Our previous work[7] on the heat stability of β-lactoglobulin and its dodecyl sulfate derivative, in dilute salt solutions at *p*H 5.2, demonstrated that the presence of two molecules of dodecyl sulfate attached to the β-lactoglobulin molecule increases its stability. The present communication describes the rate of denaturation of solutions of β-lactoglobulin and its dodecyl sulfate derivative as a function of *p*H at 3 and 25°.

## Materials and Methods

**β-Lactoglobulin and β-Lactoglobulin Dodecyl Sulfate.**—These substances were prepared and purified as previously described.[7] For denaturation studies, approximately 2% solutions were prepared in veronal or borate buffers of 0.1 ionic strength and of different *p*H. Protein concentrations were determined by dry weight at 105° as well as by the Nesslerization method for determining nitrogen before diluting to 2% in the buffers.

Denaturation was measured by optical rotation and by insolubility at the isoelectric point in the presence of salt. In earlier experiments, toluene was used as a preservative. It was found, however, that turbidity, caused by toluene complicated determination of optical rotation and that essentially the same values were obtained without a preservative. Consequently most of the measurements were made on solutions without preservatives. The protein solutions were kept in polarimeter tubes, and all readings of optical rotation were made at 25°. The solutions kept at 3° were warmed rapidly by immersing the polarimeter tube in water at 25°. After the readings were taken, the solutions were immediately placed in the water-bath at 3°. The specific rotation of undenatured and denatured protein varies with *p*H, and consequently it was necessary to know the specific rotation of the undenatured and denatured protein as a function of *p*H. Since the extent of denaturation is a function of time, a method was needed for determining the specific rotation of native proteins at zero time for each *p*H value. The value for specific rotation at zero time has been estimated by taking readings immediately after adjusting the protein solutions to the desired *p*H, as well as by plotting log $(\alpha_t - \alpha_\infty)$ against time and extrapolating to zero time.[8] The results obtained by the two methods generally were in agreement. The specific rotation of denatured protein was calculated from the maximum specific rotation values in the higher *p*H ranges, where denaturation went to completion. Specific rotation values as a function of *p*H were also determined on isolated completely denatured protein in the presence of veronal buffer of 0.1 ionic strength. Figure 1 summarizes the effect of *p*H on the specific rotation of undenatured and denatured β-lactoglobulin and its dodecyl sulfate derivative.

In determining denaturation by insolubility at the isoelectric point, the denatured protein was precipitated by adding 0.1 *N* acetic acid to *p*H 5.0. The insoluble protein was removed by centrifugation and washed with 0.05 *M* sodium chloride. The protein was estimated from the nitrogen content of the insoluble material as determined by the Nesslerization method, the conversion factor of 6.40 was used.

The accuracy of the two methods was evaluated on known mixtures of native and denatured β-lactoglobulin. These mixtures were made to a total concentration of 2% protein at *p*H 8.44 in veronal buffer. The results obtained for the amount of denatured protein in the mixture by the two methods were in good agreement with the known composition. The values obtained by insolubility at the isoelectric point were slightly more precise than those found by the optical rotation method on the unseparated mixture. The

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

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(6) L. K. Christensen, *ibid.*, **163**, 1003 (1949).

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(8) M. A. Rosanoff, R. H. Clark and R. L. Sibley, *ibid.*, **33**, 1911 (1911).