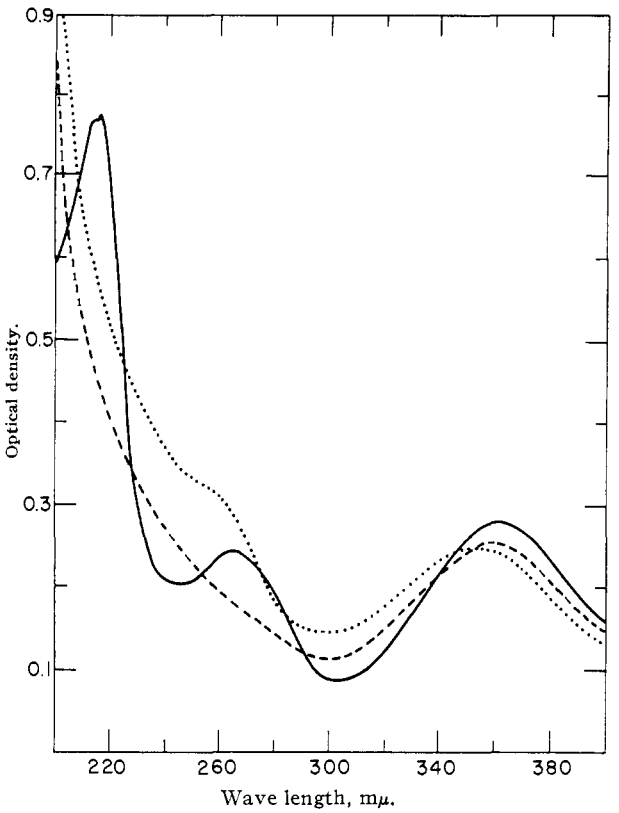
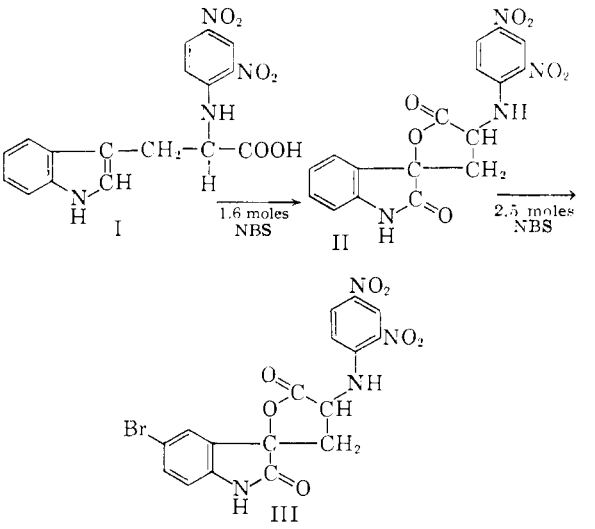


Fig. 2.—0.05 μM DNP-tryptophan in 3.03 ml. water ———; spectrum after addition of 0.08 μM NBS - - - -; spectrum after addition of 0.20 μM NBS ..... Further addition of NBS causes no change in the spectrum.

amount of tryptophan in a sample solution is calculated from the decrease in O.D. 280 mμ obtained and the use of the factor 1.31[6] and an extinction coefficient for tryptophan at 280 mμ of 5,500.

The amount of oxidizing agent taken up to obtain minimum O.D. at 280 mμ varies with the nature of the protein and the medium used. A few examples are as given: Bovine serum albumin takes about 10 moles of NBS per mole of tryptophan present in 10 M urea and human serum albumin (Hg-dimer) in 8.0 M urea 20 moles. The I-peptide in 8.0 M lithium acetate solution at pH 4.15 needs 1.95 moles of NBS, whereas in an aqueous acetate–formate buffer (0.4 M) at pH 4.15 and in the presence of 0.2% SDS 3.3 moles of NBA are required. Lysozyme in aqueous acetate–formate buffer needs 2.3 moles of NBS per mole of tryptophan, 3.0 moles of NBA in 12.0 M lithium acetate and 2.7 moles of NBA in 66% acetic acid. Typical spectrophotometric changes with bovine and human serum albumin (Hg-dimer) are depicted in Fig. 1. Figure 2 shows the changes in the spectrum of DNP-tryptophan on addition of NBS and shows that in such a model system at least two well-defined products result from the use of 1.6 and 4 moles of oxidizing agent per mole of DNP-tryptophan.[32]



Oxidation of -SH and -S-S- Groups by NBA.—Table II presents data on the number of sulfur atoms, cysteine or cystine, which are oxidized to cysteic acid when a large excess of oxidant is used under one set of experimental conditions. The oxidations were done in 0.4 M acetate-formate buffer at pH 4.15 for 10 minutes. The oxidized proteins were hydrolyzed for 24 hr. with 6.0 N HCl and the cysteic acid present in the hydrolyzates was estimated as DNP-cysteic acid. The only correction used in the calculations was for a 10% loss in paper chromatography. It is possible that with further study NBA or NBS could be used instead of performic acid[33] for the estimation of cysteine and cystine in proteins as cysteic acid.

(32) Preliminary results (elementary analyses and infrared spectra) on the isolated products (m.p. 196–198° and 248° for II; m.p. 260° (III)) would lead to tentative formulations II and III. The bromine in III is probably present in the five (or six) position.

(33) E. Schram, S. Moore and E. J. Bigwood, *Biochem. J.*, **57**, 33 (1954).

BETHESDA, MD.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, STATE UNIVERSITY OF IOWA]

The Reversible Transformation of β-Lactoglobulin at pH 7.5[1]

BY CHARLES TANFORD, LYLE G. BUNVILLE AND YASUHIKO NOZAKI[2]

RECEIVED JANUARY 31, 1959

The specific optical rotation and the titration curve of β-lactoglobulin both indicate that a reversible change in the configuration of this protein occurs at 25° near pH 7.5. A similar conclusion is reached from sedimentation data obtained by Pedersen in 1936. The configurational change parallels the titration of imidazole groups and is accompanied by the reversible release of two carboxyl groups from the interior of the molecule. The transition is a novel one in that no general unfolding of the molecule occurs: it must be regarded instead as a refolding of part of the polypeptide chains.

Pedersen,[3] in 1936, performed a thorough ultracentrifugal investigation of β-lactoglobulin in aqueous solution. He showed that there is a decrease in the sedimentation coefficient between pH 7 and 8, which, though small, lies outside the limits of

(1) Abstracted, in part, from the Ph.D. thesis of Lyle G. Bunville, State University of Iowa, 1958.

(2) On leave of absence from the National Hygienic Laboratory, Tokyo, Japan.

(3) K. O. Pedersen, *Biochem. J.*, **30**, 961 (1936).

experimental uncertainty (*cf.* Fig. 4). Further evidence for a reaction in this *p*H range comes from Groves, Hipp and McMeekin's study of the alkaline denaturation of β-lactoglobulin,[4] which showed that the specific optical rotation of the native protein, before onset of denaturation, is *p*H-dependent. The present paper will show that both of these observations are reflections of a general configurational change which occurs near *p*H 7.5. The transition is of a novel type, in that no general unfolding of the protein molecule appears to be involved.

Experimental

The β-lactoglobulin used was purchased from the Pentex Corp., Kankakee, Ill. It was used without further purification. Other studies in progress in this Laboratory have indicated that this product is essentially unaffected by deionization and that its titration properties are identical with those of other preparations of the same protein.[5] Stock solutions of the protein were prepared by dissolving the protein in KCl solutions of appropriate concentrations. The concentration of stock solutions was determined by ultraviolet absorption at 280 mμ, the optical density (log I_0/I) of a 1% solution (1 g. dry protein/100 ml.) over a 1 cm. light path having been determined on the basis of dry weight measurements to be 9.4. Solutions for measurement were obtained by dilution of stock solutions with water and addition of appropriate quantities of KCl and KOH solutions.

Optical rotation measurements were made in 20 cm. tubes, using a photoelectric polarimeter. All measurements employed the NaD line. The measurement of *p*H utilized various cells, all incorporating Beckman glass electrodes and satd. KCl salt bridges. Beckman *p*H meters (models G or GS) or a Radiometer autotitrator (model TTT 1a) were used to measure *p*H relative to potassium acid phthalate solutions which were used as *p*H standards. All data were obtained at 25° and ionic strength 0.15.

Results

The clearest manifestation of the transition is shown by the optical rotation data reported in Fig. 1. It is seen that the transition is from an initial $[\alpha]_D$ of −25° to a final value of −48°. Up to about *p*H 9.5 the irreversible denaturation of β-lactoglobulin[4] has a half-life of many hours and it does not interfere with the measurements of Fig. 1. Near *p*H 10, however, the irreversible reaction proceeds to an appreciable extent even during the 10 to 15 min. required for optical rotation measurement, so that the point in Fig. 1 near *p*H 10 had to be obtained by extrapolation of a series of measurements to zero time.

Further evidence for the transition can be obtained from examination of the titration curve of β-lactoglobulin. The neutral region of the curve, at ionic strength 0.015, is shown in Fig. 2,[6] and it indicates a clearly evident steepening of the curve near *p*H 7.5. This portion of the curve is completely reversible, agreement between forward and reverse points at each value of *r* being 0.01 *p*H unit or better. It is not possible to account for this steep portion of the curve in the usual way unless at least eight groups with *pK*'s near 7 to 8 are assumed present per molecule (assumed molecular weight

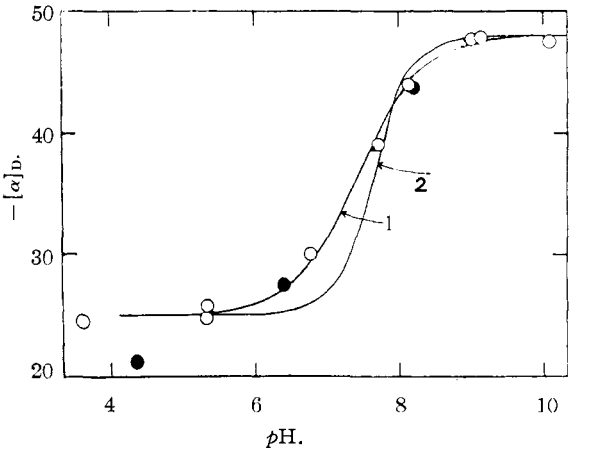


Fig. 1.—Specific rotation (Na-D line) as a function of *p*H. Filled circles represent solutions which were exposed to *p*H 9 before being adjusted to the final *p*H. Curve 1 is simply an experimental curve through the data. Curve 2 is derived from the data of Fig. 2, as described in the text. The equation for curve 1 is $pH - \log x/(1-x) = 7.42$, where x is the degree of conversion to the high *p*H form. No significance is attached to this form of the equation at the present time because of the poor accuracy of the data.

35,000). Analytical data, however, can account for only six such groups, two terminal α-amino groups,[7] and four imidazole groups derived from the molecule's histidine residues.[8] Two of the groups being titrated in the neutral *p*H region must therefore be anomalous. It is possible to identify these two groups as carboxyl groups, for two reasons. (1) Amino acid analysis[7,8] indicates that β-lactoglobulin should possess 51 carboxyl groups, 2 being terminal and 49 derived from aspartyl and glutamyl side chains. Titration curves nearly always show the presence of *more* than the analytical number of carboxyl groups because many procedures used to estimate amide nitrogen (and, hence, the number of carboxyl groups of an amino acid hydrolysate which came from amide side chains) give high results. Thus the titration curve can be expected to show *more* than 51 titratable carboxyl groups. The observed result, however, is that the acid part of the titration curve (Fig. 2) is compatible with a maximum of 50.5 carboxyl groups.[9] (2) If β-lactoglobulin is exposed to *p*H 11, it becomes irreversibly altered. The titration curve of the product of this denaturation, shown in Fig. 3, lacks the steepening of the neutral region of the titration curve and shows that 52.5 rather than 50.5 groups are titrated in the acid range. Thus the two anomalous groups titrated in the imidazole region

(4) M. L. Groves, N. J. Hipp and T. L. McMeekin, THIS JOURNAL, **73**, 2790 (1951).

(5) *E.g.*, the phenolic ionization studies reported by C. Tanford and S. A. Swanson, *ibid.*, **79**, 3297 (1957), which were performed on a sample supplied by Dr. T. L. McMeekin, gave identical results when performed with Pentex preparations.

(6) A complete study of the titration has been performed by us, and a detailed report will be presented in the near future.

(7) It would appear to be completely established that β-lactoglobulin molecules consist of two identical or close to identical polypeptide chains. See R. Townend and S. N. Timasheff, THIS JOURNAL, **79**, 3613 (1957)

(8) W. H. Stein and S. Moore, *J. Biol. Chem.*, **178**, 79 (1949). The raw experimental figure is 3.6 histidine residues per 35,000 g. of protein. It is inconceivable that this analysis can be sufficiently in error to allow the presence of six imidazole groups per molecule.

(9) A non-integral number of carboxyl groups per molecule is acceptable here because the protein is known to be a mixture of two forms, β-lactoglobulin A and β-lactoglobulin B. Work in progress indicates that these two forms differ in the number of carboxyl groups which they possess but that their titration properties are otherwise close to identical.

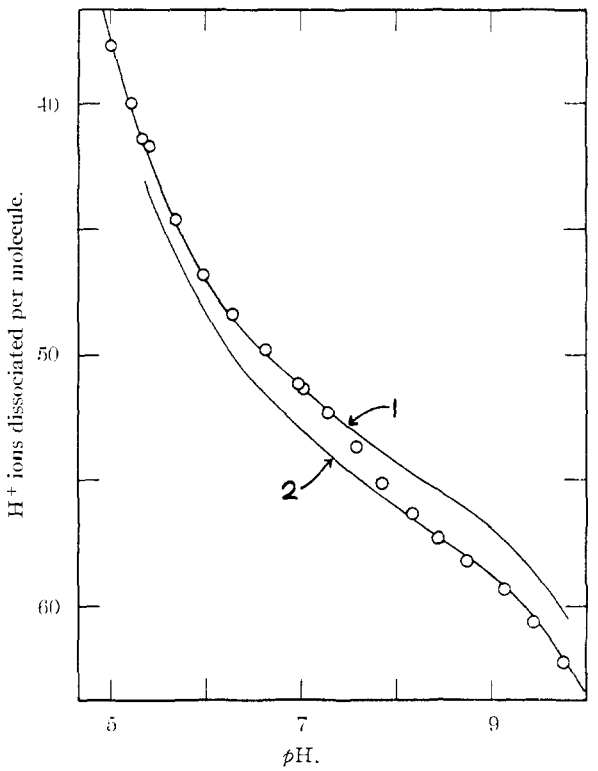


Fig. 2.—The neutral region of the titration curve at ionic strength 0.15. Curves 1 and 2 are calculated curves, differing only in the number of carboxyl groups assumed available for titration (see text). Almost all of the experimental points are superpositions of points from forward and reverse titration curves, at identical pH values. The agreement between them was always 0.1 group titrated or better.

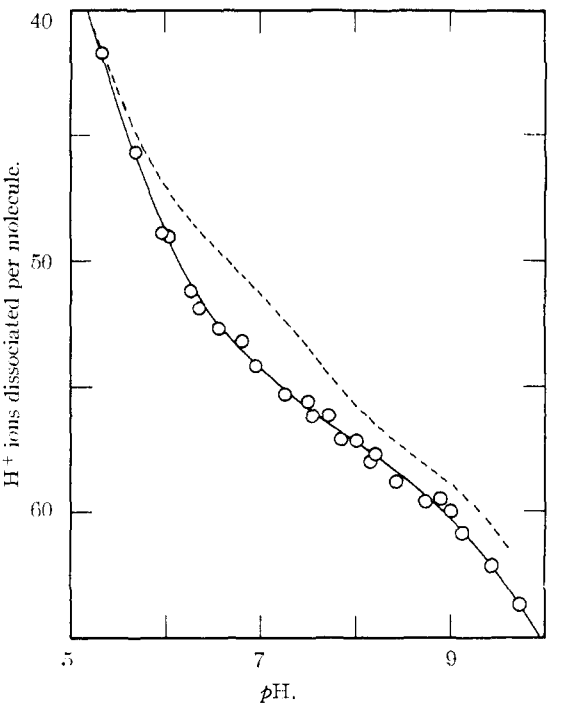


Fig. 3.—Titration curve of alkali denatured β-lactoglobulin. The dashed curve represents the titration curve of native protein, *i.e.*, the best curve through the experimental points of Fig. 2. The difference between the two curves at high pH is to be ascribed to a reduction in electrostatic interaction in the denatured protein (its viscosity being nearly twice that of the native form). This difference, in the absence of other effects, would become progressively less and disappear at pH 5.2. The observed result is clearly a shift in pK of two groups, on which the progressively changing electrostatic difference is superimposed.

in Fig. 2 appear after denaturation in the pH region characteristic of the titration of carboxyl groups.

Since the titration of the two anomalous groups occurs at essentially the same pH as the transition observed by means of optical rotation, it is likely to be associated with this transition. One can then obtain a measure of the progress of the transition from the titration curve by use of two calculated curves shown in Fig. 2. Curve 1 of this figure is a calculated curve based on the presence of 2 α-COOH groups (pK_{int} = 3.75), 48.5 side chain COOH groups (pK_{int} 4.7), 4 imidazole groups (pK_{int} = 7.0), 2α-NH_2 groups (pK_{int} = 7.8), 28 ε-NH_2 groups (pK_{int} = 9.9) and 6 phenolic groups (pK_{int} = 10.2), with a value of w equal to 0.039. It is seen to agree closely with the experimental titration data below pH 6.5, and it is assumed that it would continue to represent the titration curve of the low pH configuration if this could be kept intact at higher pH. Curve 2 of Fig. 2, on the other hand, is based on the same parameters as curve 1, but with the inclusion of 50.5 side chain COOH groups (instead of 48.5). It fits the experimental data above pH 8.5 and is assumed to represent the hypothetical titration curve of the high pH configuration at lower pH values. The experimental points of Fig. 2 may now be used as a measure of the extent of configurational transition at any pH simply by setting the observed value of r at any pH equal to x times the value of r predicted by curve 2 plus $(1 - x)$ times the value predicted by curve 1, x being again the fraction of protein molecules in the high pH configuration. The resulting plot of x *versus* pH is shown as curve 2 of Fig. 1, and it is clearly in as good agreement with the optical rotation data as can be expected.[10]

Finally, Pedersen's sedimentation data[3] may be used as a measure of the transition. His experimental data, shown in Fig. 4, show considerable scttaer but, within the limits of experimental error, are adequately described by either of the two curves of Fig. 1.

An important aspect of all three of these sets of data is the invariance of all pertinent properties between the end of the transition and the onset of irreversible denaturation (pH 9.5 to pH 10, depending on the speed with which measurements are made). Both the specific rotation and the sedimentation coefficients are pH-independent in this region. The titration curve is as flat after the

(10) The value of w used is derived from analysis of the titration of carboxyl groups. Exactly the same value of w will not necessarily apply to the neutral region of the curve. More important, however, is the fact that if titration of one or more imidazole groups triggers the transition (as is suggested below), then the pK_{int} of these groups must be different before and after the transition has occurred. Thus curve 1 of Fig. 2 should correspond to a higher pK_{int} for imidazole groups. This would have the effect of raising this curve above its present location, and this would make curve 2 of Fig. 1 considerably flatter than it is at present.

transition as before, unequivocally excluding the possibility that the electrostatic interaction factor w can be pH-dependent, or the possibility that its value after the transition can be appreciably different from that applicable to the more acid portions of the titration curve. These results clearly indicate that the product of the transition is still a rigid compact molecule.[11] This conclusion stands in sharp contrast to that reached in the study of the configurational transition of serum albumin (near pH 4), the product of which is a flexible, loosely coiled molecule which expands continuously with increasing charge, this expansion being manifested by continuous variation with pH of the optical rotation,[12] the sedimentation coefficient[13] and the electrostatic interaction factor.[14]

Discussion

Whereas most configurational changes which have been observed in aqueous protein solutions involve a general unfolding to a structure which is at least partially randomly coiled,[15] the transition here studied is clearly a reaction which involves a new way of folding rather than an unfolding. It is probable that only part of the molecule participates in the reaction, for β-lactoglobulin undergoes a more general denaturation at higher pH which is slow and irreversible.[4] The latter result indicates that much of the secondary structure of the molecule is tenaciously maintained, difficult to alter, and, when altered, difficult or impossible to return to its original form. The reaction here considered, on the other hand, is instantaneous and reversible.

In view of the current interest in the detailed folding of the polypeptide chains of proteins, it is in order to suggest a speculative picture of the observed transition, although a definitive explanation must await experimental studies of greater precision than those reported here, as well as studies of the effect of temperature, ionic strength, etc.

The simplest possible explanation would seem to be that a good part of the stability of the native compact configuration of β-lactoglobulin is due to hydrophobic bonding, *i.e.*, to intramolecular micelle formation brought about by the fact that non-polar side chains of the protein cannot be accommodated by water without interference with the normal hydrogen-bonded structure of this solvent. Intramolecular hydrogen bonds (especially peptide hydrogen bonds) undoubtedly play an important part in this process in permitting uncharged polar groups to be incorporated within the non-polar regions, hydrogen bonding from one group to another replacing the hydrogen bonds to water which one would normally expect if these groups were in contact with the solvent.

An important aspect of this kind of a model is that the intramolecular micellar region need not

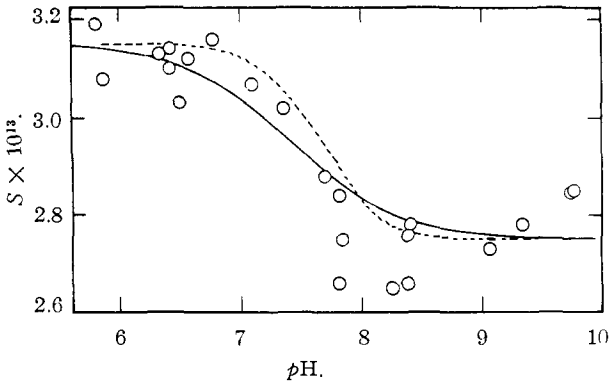


Fig. 4.—Sedimentation coefficients determined by Pedersen.[3] The temperature was 20°, the ionic strength varied from 0.11 to 0.64, the protein concentration from 0.1 to 0.34%. The solid line represents the predicted variation in sedimentation coefficient according to curve 1 of Fig. 1, the dashed line according to curve 2 of Fig. 1, initial and final values of s × 10^{13} being taken as 3.15 and 2.75.

be continuous but could consist of several more or less independent parts. Each such independent region could have alternative configurations differing relatively little in free energy, so that titration of even a single group could shift the equilibrium from one configuration to another. The present reaction could be explained as such a shift. The fact that the transition occurs near pH 7.5 suggests that the particular side chain group whose titration sets off the reaction might well be an imidazole group.

Assuming then that an imidazole group is indeed the crucial one, we may suppose that, before it is titrated, the more stable configuration of each of the two polypeptide chains[7] is one in which the coiling near this group leads to a high helix content (*i.e.*, relatively positive specific rotation) and a buried COOH group. After titration of the imidazole group another configuration, with a lower helix content and with an exposed carboxyl group, could become the stable one. All exposed carboxyl groups are ionized at the pH of the transition, so that release of two protons (one for each polypeptide chain) accompanies the transition.[16]

An alternative possibility is that the occurrence of the transition in the titration region of the imidazole groups is just accidental, and that the driving force for the transition is simply the desire of the buried carboxyl groups to become ionized. They cannot do so while buried because of the prohibitive electrostatic work required to create a charge in the interior of the molecule, so that ionization is necessarily accompanied by configurational change. The pK for the ionization is the normal pK for a carboxyl group plus the $\Delta F^\circ/2.303RT$ for the configurational change.

It is worth noting, finally, that Timasheff and Townend[17] have measured the molecular weight of β-lactoglobulin by the Archibald method and have found that no change occurs in the pH region here considered.

(11) The change in sedimentation coefficient, of course, indicates that a change in over-all shape has occurred. In terms of an equivalent sphere the transition represents an expansion from a radius of 24.5 Å to one of 28 Å.

(12) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954).

(13) W. F. Harrington, P. Johnson and R. H. Ottewill, *Biochem. J.*, **62**, 569 (1956).

(14) C. Tanford, J. G. Buzzell, D. G. Rands and S. A. Swanson, THIS JOURNAL, **77**, 6421 (1955).

(15) C. Tanford, in "Symposium on Protein Structure" (A. Neuberger, ed.), Methuen and Co., London, 1958.

(16) This discussion assumes that each of the two polypeptide chains of β-lactoglobulin[7] possess one of the two anomalous COOH groups. There is nothing in our experiments, of course, which bears on this question.

(17) S. N. Timasheff and R. Townend, personal communication.

Acknowledgments.—This work was supported by research grant RG-2350 from the National Institutes of Health, U. S. Public Health Service; and by grant G-1805 from the National Science Foundation.

IOWA CITY, IOWA

[CONTRIBUTION FROM THE ROCKEFELLER INSTITUTE]

Application of the Archibald Principle for the Ultracentrifugal Determination of the Molecular Weight in Urea Solutions of Histone Fractions from Calf Thymus

BY RODES TRAUTMAN[1] AND CHARLES F. CRAMPTON

RECEIVED JANUARY 12, 1959

The Archibald principle has been employed for the determination of molecular weights by a method in which knowledge of the initial concentration of the solute is not essential. A least squares method is given by which data from various exposures in Archibald measurements may be combined so that a single value for the molecular weight may be obtained from a series of experiments. Analysis of the data for the known reference protein ribonuclease, at about 1% concentration in acetate buffer, *p*H 5.5, verified both the linearity of the function plotted as the boundary separated from the meniscus and the applicability of the method. Ribonuclease was also centrifuged in *p*H 5.5 acetate buffer containing 6 *M* urea. The data indicated that the non-ideality activity coefficient correction term for ribonuclease in urea is reasonably small. Two possibly heterogeneous, aggregating proteins were analyzed to determine the molecular weight *M*. Data for calf thymus histone Fraction A indicated that it did not dissociate in 6 *M* urea and that $M = 10{,}000 \pm 2000$. Data for calf thymus histone Fraction B indicated that it dissociated in half in 6 *M* urea and that for the monomer $M = 16{,}000 \pm 1600$. The minimal molecular weights calculated from amino acid analyses were higher than these values, especially for histone fraction A, and the most probable explanation is that histone Fractions A and B both contain mixtures of proteins of different composition.

The investigations reported here were undertaken to determine the molecular weight in solutions of the chromatographically purified histone Fractions A and B from calf thymus.[3] These fractions have reproducible amino acid compositions, and, if they are assumed to be homogeneous, the analytical data would require minimal molecular weights of 20,600 and 19,700, respectively. In order to determine the molecular weights an ultracentrifugal method that utilizes an extension of the Archibald principle[4] was employed. The method was verified by the use of ribonuclease, the molecular weight of which is accurately known.[5,6] During the course of this work there appeared a complete review by Luck, *et al.*,[7] in which were described the molecular weights and association properties of histones very similar to some of those reported here.

The original exposition of the Archibald principle was in terms of the sedimentation and diffusion coefficients s and D, respectively, and the concentration at the limits of the solution column. The principle states that since no dissolved molecules can flow out of the cell through either end, the processes of outward settling and inward diffusion balance exactly. Klainer and Kegeles[8] were first able to shift the analysis from one requiring c to one requiring the initial concentration c^0, when measurements of the concentration gradient in a sector cell were made. Then, recently, Kegeles, Klainer and Salem[9] have gone one step further and apparently have succeeded in removing any explicit expression of s or D, so that only the anhydrous molecular weight M and c^0 appear, in addition to the usual partial specific volume, density and activity coefficient terms.

Since a molecular weight itself is independent of the concentration and since the velocity ultracentrifuge yields sedimentation and concentration information almost independently, it would seem that c^0 also could be removed from the concept and application of the Archibald principle. One proposal along this line, made by Ginsburg, Appel and Schachman,[10] combines the data for the top and bottom limits of the solution column in any one exposure. The method to be described here, in a way, is an extension of this notion, but instead utilizes the data at different *times* at the same limit to determine both M and c^0. Looked at another way, this analysis indicates how all the data from one run or a series of runs can be averaged to yield a single value of the molecular weight, either with or without using the initial concentration.

Theory

Prior to complete separation of the boundary region from the meniscus, the Archibald principle, as presented by Kegeles, Klainer and Salem[9] states that at the axial limit of the solution, r_a, the concentration gradient will adjust itself as the concentration falls so that

$$M(1 - \bar{V}\rho)\omega^2 r_a - (\partial\mu/\partial r)_{T,P,r_a} = 0 \qquad (1)$$

where M, $\bar{V}$ and μ are the (anhydrous) molecular weight, partial specific volume and molar chemical

(1) U. S. Dept. of Agriculture, Plum Island Animal Disease Laboratory, Greenport, New York. A preliminary report was presented before the Division of Biological Chemistry at the 130th National Meeting of the American Chemical Society, Atlantic City, September, 1956. Data on the modifications of the Archibald method proposed elsewhere[2] have not yet appeared, and hence the control data on ribonuclease serve to substantiate the technique involved.

(2) R. Trautman, *J. Phys. Chem.*, **60**, 1211 (1956).

(3) C. F. Crampton, W. H. Stein and S. Moore, *J. Biol. Chem.*, **225**, 363 (1957).

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

(5) C. H. W. Hirs, W. H. Stein and S. Moore, *J. Biol. Chem.*, **221**, 151 (1956).

(6) S. M. Klainer and G. Kegeles, *Archiv. Biochem. Biophys.*, **63**, 247 (1956).

(7) J. M. Luck, H. A. Cook, N. T. Eldredge, M. I. Haley, D. W. Kupke and P. S. Rasmussen, *Arch. Bicchem. Biophys.*, **65**, 449 (1956).

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

(9) G. Kegeles, S. M. Klainer and W. J. Salem, *ibid.*, **61**, 1286 (1957).

(10) A. Ginsburg, P. Appel and H. K. Schachman, *Archiv. Biochem. Biophys.*, **65**, 545 (1956).