sulfuric acid. Isolation of the acidic and neutral products in the usual manner afforded 37 mg. of neutral and 174 mg. of acidic split products.

c. **Hypoiodite Oxidation.**—Samples (5 to 10 mg.) of both the neutral and the acidic degradation products from lactobacillic acid were dissolved in dioxane (0.2–0.4 ml.) in a centrifuge tube and 10% sodium hydroxide (0.5 ml.) was added. Iodine–potassium iodide reagent (6.35 g. of potassium iodide plus 3.13 g. of resublimed iodine in 25 ml. of water) was added dropwise until the iodine color remained, and the tube was heated at 60° for 10 minutes. More reagent was added, if necessary, to maintain the iodine color. The mixture was cooled, the excess of iodine was decolorized by addition of 10% sodium hydroxide, and water (1 ml.) was added. The iodoform was collected, washed with water, and dried; m.p. 117–118°. No melting point depression was observed when this material was admixed with an authentic sample of iodoform.

Degradation of Dihydrosterculic Acid.—Treatment with hydrogen bromide in glacial acetic acid of 229 mg. of dihydrosterculic acid (prepared as described previously[5]) afforded 277 mg. of bromo acids; found Br, 20.4%. This material on dehydrobromination gave 205 mg. of a material with an iodine number of 60.5. On oxidation 30 mg. of neutral and 125 mg. of acidic products were obtained.

Degradation of *trans*-DL-9,10-Methyleneoctadecanoic Acid.—Treatment with hydrogen bromide in glacial acetic acid of 184 mg. of the synthetic acid[5] afforded 213 mg. of bromo acids; found Br, 20.9%. This material on dehydrobromination gave 165 mg. of a material with an iodine number of 57.7. On oxidation 28 mg. of neutral and 92 mg. of acidic products were obtained.

Degradation of *trans*-DL-11,12-Methyleneoctadecanoic Acid.—Treatment with hydrogen bromide in glacial acetic acid of 231 mg. of the synthetic acid[5] afforded 285 mg. of bromo acids; found Br, 20.6%. This material on dehydrobromination gave 210 mg. of a material with an iodine number of 76.9. On oxidation 38 mg. of neutral and 120 mg. of acidic products were obtained.

Chromatographic Procedures.—A mixture of silicic acid[14] (12 g.) and a 2 *M* phosphate buffer of *p*H 7.05 (6 ml.) was ground in a glass mortar until uniform. The resulting wet powder was suspended in chloroform containing 1% of 1-butanol v./v. (35 ml.) and the slurry was poured into a chromatogram tube (15 × 350 mm.). Entrapped air was removed by stirring, and the column was subjected to one pound of nitrogen pressure to ensure firm packing The column was then layered with 5 mm. of sand, followed by a filter disk and another 5 mm. layer of sand. The sample to be analyzed (25–50 mg.) was dissolved in chloroform containing 30% of 1-butanol v./v. (1 ml.) and the solution was delivered to the top of the column. The meniscus was allowed to reach the level of the filter paper, and two washings of 1 ml. each of chloroform containing 1% 1-butanol v./v. were used to effect a quantitative transfer of the sample to the column. The liquid level was allowed to reach the filter paper level after each washing. The column was mounted on a Technicon automatic fraction collector set at a drop rate of 50 drops per minute, and the impulse counter was adjusted to collect fractions of 280 drops each (approximately 5 ml.). The column was then eluted with mixtures of chloroform and 1-butanol having a butanol content v./v. of 1, 10 and 30%; the volume of eluent required was 100–130 ml., 100–130 ml. and 200–250 ml., respectively. Each eluting solvent was equilibrated with 2 *M* dipotassium phosphate prior to use. In order to maintain approximately equal drop rates with these various solvents it was necessary to apply positive nitrogen pressure to the top of the column; the pressure was increased with the various eluents from 0.2–0.3 to 0.5–0.7 to 1 pound, respectively. Individual fractions were titrated with 0.01 *N* methanolic potassium hydroxide to the phenolphthalein end-point, mixing being effected by bubbling a stream of nitrogen through the solutions. The meniscus of each eluent was allowed to fall to the top of the column prior to introducing the next solvent. The columns were operated at a temperature of 27 ± 2°. Typical chromatograms are illustrated in Figs. 1 and 2. The material recovery was calculated by comparing the milliequivalents of alkali required to titrate the combined effluents from the column to the milliequivalents of alkali necessary to titrate the total sample introduced; in three experiments recoveries of 98.5, 95.6 and 99.5% were realized.

Isolation and Identification of Acids.—The contents of the titrated tubes from each band were pooled, and the solvents evaporated. The dry residue was dissolved in water (5 ml.) and the solution was acidified to congo red with 50% sulfuric acid. The acids were extracted with ether, and the extracts washed with two 5-ml. portions of water. For separation from phenolphthalein the acids were re-extracted into 5% sodium bicarbonate, and were isolated in the usual manner. Normally 1–2 mg. of the dibasic acids were obtained, although a second recrystallization was often necessary. Identical fractions from several chromatograms were combined and the acids recrystallized from dilute ethanol at −10°. The melting and mixed melting point data are summarized in Table III. Samples of the dibasic acids were powdered in a micro-mortar and placed into glass capillaries of 0.3 mm. diameter and 0.01 mm. wall thickness for the X-ray photographs which were taken on a 57.3 mm. radius camera with nickel filtered CuK radiation. The powder patterns of the unknowns were compared with those obtained under identical conditions with authentic samples.

PITTSBURGH, PA.

(14) $SiO_2 \cdot xH_2O$, 100 mesh, analytical grade, prepared according to L. L. Ramsey and W. I. Patterson, *J. Assoc. Offic. Agric. Chem.*, **31**, 441 (1948), obtained from Mallinckrodt Chemical Works, New York, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF CLARK UNIVERSITY]

Ultracentrifugation of Chemically Reacting Systems

BY GERSON KEGELES AND M. S. NARASINGA RAO

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The validity of the application to chemically reacting systems of Archibald's suggestion for direct ultracentrifuge molecular weight determinations has been examined. It has been shown that the equations for the calculation of the total solute concentrations at either meniscus in the ultracentrifuge cell, applicable both to solutions of monodisperse ideal solutes and to solutions of polydisperse non-ideal solutes are also valid for chemically reacting systems. The possible effects of the centrifugal field on the chemical equilibrium in reacting systems have been discussed. The interpretation of such direct molecular weight ultracentrifuge experiments appears to be much more straightforward than that of the corresponding sedimentation velocity experiments.

Introduction

Recently the study of chemically reacting systems by sedimentation velocity methods has been subjected to a theoretical reinterpretation.[1,2] It has been shown that, neglecting diffusion, even for very fast reactions one should expect serious errors in both the sedimentation rates and the relative concentrations of reacting components as obtained by classical interpretation. In fact, the chemical reaction, even though infinitely rapid, can be ex-

(1) G. A. Gilbert, *Disc. Faraday Soc.*, **20**, 68 (1955).

(2) G. A. Gilbert and R. C. Ll. Jenkins, *Nature*, **177**, 853 (1956).

pected to lead to more than one moving boundary, provided that if a simple polymerization is involved, polymers higher than the dimer are obtained. In the case of isomerization reactions involving an alteration of charge, but not of molecular weight, a more detailed study of electrophoretic separation has been made,[3] including the effects of finite reaction rates and of diffusion. Again, the classical interpretation of the separation of such systems into more than one moving boundary, as implying non-reacting systems, has been indicated to require modification. These theories appear to stand in contradiction to the approach[4-6] which has taken into account similar factors, but has concluded that only a single moving boundary results from the centrifugation or electrophoresis of a reacting system, which is indeed the case for monomer–dimer equilibrium. On the other hand, it is perfectly feasible to measure a constituent sedimentation coefficient in a two component system in which the solute undergoes polymerization.[7,8] This can only be used to determine equilibrium constants for the polymerization reactions if the sedimentation coefficients of the monomer and of each polymer species can be determined separately, and when several polymer species are present together, this will not be generally possible. The method has been used for the determination of polymerization constants when it is reasonable to assume the existence of only a monomer–dimer equilibrium.[8]

With the most recent available theories suggesting that moving boundary experiments apparently need very careful interpretation to justify their application to reacting systems, it has been of interest to us to investigate the applicability to such chemically reacting systems of Archibald's suggestions[9] for direct ultracentrifuge molecular weight determinations. It is the purpose of this paper to indicate under what conditions such application is justified.

According to these suggestions, it is possible, in a polydisperse ideal solution, to determine the weight-average molecular weight $\bar{M}_w$ of the solute species from the total concentration C and concentration gradient dC/dx of the solute at the impervious ends of the fluid column, using an equation identical in form to that applying everywhere in the column to an ideal two-component system at sedimentation equilibrium

$$\bar{M}_w = M = \frac{RT\, dC/dx}{(1 - \bar{V}\rho)\omega^2 x C} \qquad (1)$$

Here R is the molar gas constant, $\bar{V}$ is the solute partial specific volume, ρ is the density of the solution and ω is the angular velocity of the ultracentrifuge.

One of the suggestions of Archibald[9] was that when the centrifugal field causes separation of species during analysis, a correct analysis of the original sample might be made by extrapolation to zero time. The procedure adopted in this Laboratory for the determination of such weight-average molecular weights therefore was based on the use of early photographs taken when a considerable region of the centrifuge cell (a so-called "plateau" region) showed no variation of refractive index with radius of rotation. For monodisperse ideal solutes[10] and also for polydisperse non-ideal solutes,[11] it was shown that equations (2) and (3) apply

$$C_{x_0} = C_0 - \frac{1}{x_0^2}\int_{x_0}^{X} x^2 \left(\frac{dC}{dx}\right) dx \qquad (2)$$

$$C_{x_b} = C_0 + \frac{1}{x_b^2}\int_{X}^{x_b} x^2 \left(\frac{dC}{dx}\right) dx \qquad (3)$$

Here C_0 is the original uniform solute concentration, C_{x_0} and C_{x_b} are at any time the total solute concentrations at the upper and lower boundaries x_0 and x_b of the solution being centrifuged, dC/dx is the total concentration gradient at any radius x of rotation and X represents any radius of rotation in the "plateau" region where $dC/dx = 0$. The following section will therefore be devoted to a re-examination of the validity of equations 2 and 3 for a chemically reacting system.

Calculation of the Total Concentrations at the Menisci.—For the sake of simplicity a polymerization $n\mathrm{M} \rightleftarrows M_n$ will be considered, although the same arguments might be extended to any reacting system. The concentration of monomer, M, will be taken as c_1, and the concentration of polymer, M_n, as c_2. For convenience, although not of necessity, it will be assumed that the kinetics, as well as the equilibrium, are represented by this reaction (which could only be true, of course, for reactions of low order). In general, assuming convection-free sedimentation, the equations of continuity for the ultracentrifuge may be written[3,12]

$$\frac{\partial c_1}{\partial t} = -\frac{1}{x}\frac{\partial}{\partial x}\left(-D_1 x \frac{\partial c_1}{\partial x} + S_1\omega^2 x^2 c_1\right) + k_2 c_2 - k_1 c_1^n \qquad (4)$$

$$\frac{\partial c_2}{\partial t} = -\frac{1}{x}\frac{\partial}{\partial x}\left(-D_2 x \frac{\partial c_2}{\partial x} + S_2\omega^2 x^2 c_2\right) - k_2 c_2 + k_1 c_1^n \qquad (5)$$

Here k_1 and k_2 are the specific reaction rates for disappearance of monomer and polymer, respectively. Addition of equations 4 and 5 results in

$$\frac{\partial C}{\partial t} = \frac{\partial c_1}{\partial t} + \frac{\partial c_2}{\partial t} = -\frac{1}{x}\frac{\partial}{\partial x}\left\{\left(-D_1 x \frac{\partial c_1}{\partial x} + S_1\omega^2 x^2 c_1\right) + \left(-D_2 x \frac{\partial c_2}{\partial x} + S_2\omega^2 x^2 c_2\right)\right\} \qquad (6)$$

where $C = c_1 + c_2$ is the total solute concentration. It is of interest to note that kinetic terms have already disappeared from equation 6. Now for the plateau region

$$\frac{\partial C}{\partial x} = \frac{\partial c_1}{\partial x} + \frac{\partial c_2}{\partial x} = 0 \qquad (7)$$

which will in general be possible only if

$$\frac{\partial c_1}{\partial x} = \frac{\partial c_2}{\partial x} = 0 \qquad (8)$$

If it is further assumed that sedimentation and diffusion coefficients are independent of pressure (but *not* of concentration) then application of equation 8

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(12) O. Lamm, *Archiv. Math. Astron. Fysik*, **2**, 21B (1929).

to equation 6 leads to

$$\left(\frac{\partial C}{\partial t}\right)_X = -2 S_1\omega^2 c_1 - 2 S_2\omega^2 c_2 \qquad (9)$$

where the subscript X at the left of equation 9 indicates that this equation is limited to the "plateau" region of the ultracentrifuge cell. Now it is possible to proceed to the calculation of the total solute flow past any surface of radius X in the "plateau" region

$$\int_{x_0}^{X} (C_0 - C)\, x\, dx = \int_0^t (c_1 S_1\omega^2 X\cdot X + c_2 S_2\omega^2 X\cdot X)dt \qquad (10)$$

Applying equation 9 to the terms under the integral in equation 10, we find

$$\int_{x_0}^{X} (C_0 - C)\, x\, dx = -\int_0^t \frac{1}{2} X^2 \left(\frac{\partial C}{\partial t}\right)_X dt \qquad (11)$$

The left-hand side of this equation may be integrated by parts.[13] For the right-hand side, it is recalled that $C = C_0$ when $t = 0$ and $C = C_X$ when $t = t$, X being independent of t. After rearrangement, equation 2 follows directly from (11). A similar development for the bottom of the liquid column yields equation 3. Consequently, even in the case of a reacting system, these equations 2 and 3 are applicable for the calculation of total concentrations at the top and bottom of the column of sample being centrifuged. It is noted that it was unnecessary to neglect diffusion or to assume anything about rates of reaction, this experimental procedure being thus much easier to treat theoretically than is the comparable moving boundary experimental method.

Effect of the Centrifugal Field on the Chemical Reaction.—The chemical reaction $n\mathrm{M} \rightleftarrows M_n$ proceeds at different parts of the centrifuge cell, and hence under various centrifugal fields. The method under consideration for the determination of the average molecular weight of the system requires that the effect of the centrifugal field be evaluated at the boundaries of the liquid column which are impervious to flow and through which the total mass transport of each solute species is consequently zero.

One effect of the centrifugal field is to deplete the total solute concentration at the upper meniscus and to augment the total solute concentration at the lower meniscus, by transport of solute in the neighborhood of, but not through, these menisci. Such changes of total concentration will clearly disturb the chemical equilibrium, since more molecules appear on one side of the chemical equation than on the other. In order to reduce this effect, it is in principle necessary to work under conditions where the reaction rate constants for polymerization and dissociation are large (infinite, if possible), or under conditions where if the reaction rates are not large, the centrifugal field is small, and times of centrifugation are so short that the original equilibrium mixture remains practically undisturbed. Experience alone will serve to indicate whether these conditions are attainable for a given reacting system. However, it is in principle possible to extrapolate measurements to zero time and zero field, which is in practice more difficult in the case of sedimentation velocity experiments.

Another effect to be considered is the possibility that the centrifugal field itself will have an effect on the reaction. The direct effect of the field is that the reacting particles, having mass, will change their potential energy as they move from one point to another in the cell. As has been pointed out very clearly,[14,15] however, the conversion from one species to another involves no change in mass and consequently involves no change of free energy due to a superimposed gravitational field. Therefore there is no direct effect of the field on the chemical reaction, provided that the reaction is fast enough so that chemical equilibrium is maintained. An indirect effect of the field is that the reacting system finds itself in a pressure gradient. This implies the possibility of a shift in the position of equilibrium at each point in the cell, if the chemical reaction is accompanied by a change in volume. The volumes under consideration are the partial specific volumes, in solution, of monomer and polymer, which might be expected to be nearly identical. If the measurements are also performed at low centrifugal fields, this effect might be expected to be unimportant. In any event, one measurement is always made at the top surface of the liquid column, where the pressure is essentially at one atmosphere, and comparison between this measurement and that made at the bottom of the liquid column at different centrifugal fields might indicate any pronounced effect of pressure. Any effects on the reacting system resulting from the pressure gradient will have no bearing on the validity of equations 2 and 3 above, as these equations did not in the first place require for their validity any statement concerning chemical equilibrium.

If the chemical reaction is sufficiently rapid so that chemical equilibrium is always maintained, independent of small shifts of total solute concentration, then $\bar{M}_w$ from equation 1 represents the weight-average molecular weight at that concentration C which is characterized by a knowledge of the equilibrium constant for the reaction. If the system is sufficiently disturbed so that it is observed under conditions no longer corresponding to chemical equilibrium, $\bar{M}_w$ from equation 1 will still be the true weight-average molecular weight for the species existing at the meniscus, but it will be incorrect to calculate the equilibrium constant for the chemical reaction from a knowledge of $\bar{M}_w$ and C. It is therefore important in any application of this technique to reacting systems that in each case some experimental estimate be made of the extent of disturbance of the chemical equilibrium by virtue of the solute concentration shifts produced through centrifugation.

When it can be demonstrated that such shifts of concentration have not disturbed the chemical equilibrium appreciably, it is a straightforward matter to employ the ultracentrifuge as indicated, for the purpose of investigating chemical equilibria in macromolecular systems.

Some independent results of the application of

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this technique to reacting systems already have been reported,[8,16] and the companion paper[17] describes a detailed study of such an application.

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WORCESTER, MASS.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF CLARK UNIVERSITY]

An Ultracentrifuge Study of the Polymerization of α-Chymotrypsin

BY M. S. NARASINGA RAO[1] AND GERSON KEGELES

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The molecular weight of α-chymotrypsin has been determined as a function of protein concentration in phosphate buffer solution of *p*H 6.2 and ionic strength 0.2, by the Archibald ultracentrifuge method. From molecular weight measurements alone, the data can be interpreted within experimental error either by assuming monomers and trimers to be present or by assuming the simultaneous presence of monomers, dimers and trimers. The equilibrium constants and corresponding free energies of depolymerization have been calculated for both assumptions. In spite of the presence of polymers larger than the dimer, only one peak is observed in velocity ultracentrifugation. In conjunction with the recent theory of Gilbert for velocity ultracentrifugation of reacting systems, this fact would lend preference to the assumption that monomers, dimers and trimers are present together at equilibrium. A straightforward extension has been made of Gilbert's theory to the case where monomers, dimers and trimers are present simultaneously, and this predicts, moreover, only a single peak for velocity ultracentrifugation of such a system. It is therefore possible to deduce that under the conditions of these experiments, chymotrypsin is present as an equilibrium mixture containing monomers, dimers and trimers.

In a separate communication[2] we have justified the extension to the case of chemically reacting systems of the suggestions of Archibald[3] for the determination of molecular weights with the ultracentrifuge. The conditions under which such determinations may be expected to be strictly valid for reacting systems also have been indicated. This communication reports the results obtained by the application of these suggestions to a system containing α-chymotrypsin, which has been reported to undergo a concentration-dependent polymerization reaction.[3-7]

Experimental

The α-chymotrypsin was a Worthington Biochemical Corporation product, lot no. 577-82.

Electrophoretic mobility measurements were made with a Tiselius Electrophoresis Apparatus (Perkin–Elmer Model 38) fitted with a Longsworth scanning camera.[8] The isoelectric point of a 1% protein solution in phosphate buffers of ionic strength 0.2 was found to be *p*H 6.2. This value differs markedly from the reported values of 8.1[9] and 8.3.[10] The cited investigations were made in buffer solutions of univalent salts and of 0.1 ionic strength. The discrepancy can be explained on the basis that this protein binds phosphate ions strongly whereas it binds, perhaps, very little of the univalent buffer anions used by these investigators. That the phosphate ions are strongly bound by this protein is further suggested by the fact that the isoelectric point in phosphate buffer shifted to *p*H 6.9 when the ionic strength was decreased to 0.1; also when the experiments were repeated in acetate and glycine–sodium hydroxide buffer systems (ionic strength 0.1) the isoelectric *p*H was found to be 8.4 in good agreement with the reported values.[9,10] Molecular weight determinations were made with a Spinco Model E Ultracentrifuge, in a phosphate buffer solution of *p*H 6.2 and ionic strength 0.20 (0.029 *M* disodium hydrogen phosphate + 0.114 *M* sodium dihydrogen phosphate). The protein solutions were dialyzed in the cold, overnight against the buffer solution. At high protein concentrations it was observed that slight *p*H changes took place when the protein was dissolved in the buffer solution. Dialysis against a large volume of buffer solution is essential to restore the *p*H back to the original value.

The experimental technique and the method of molecular weight calculation described by Klainer and Kegeles[11,12] were followed, all experiments being performed with isoelectric protein to obviate the effects of possible non-ideality of the solutions. Previous work[11,12] had demonstrated no observable concentration-dependence of the molecular weight of simple proteins at their isoelectric points. The original protein concentrations, C_0 in refractive index units were measured directly by using the boundary-forming cell.[13] At low and high protein concentrations, extrapolated values for C_0 were used, extrapolation being done with a calibration curve of area *versus* concentration. For concentrations below 5 g. per liter, a cell with 30 mm. optical path was used and appropriate corrections to constant optical sensitivity were made in such cases. All other measurements were made with a standard 12 mm. cell. Carbon tetrachloride was used for the false bottom.

The individual experiments were done at various temperatures between 20 and 25°.

For the calculation of molecular weight a value of 0.73_6 was used for the partial specific volume of α-chymotrypsin.[5]

Absolute protein concentrations were determined by measuring the ultraviolet absorption at 282 mμ. The specific absorption coefficient determined with a portion dried to constant weight over phosphorus pentoxide was 2.07, in excellent agreement with the reported value[5] of 2.07_5.

Buffer salts were of reagent quality. *p*H measurements were made at 25° with a Beckman *p*H-meter, model G.

Results and Calculations

Figure 1 gives a plot of the weight-average molecular weight of α-chymotrypsin as a function of protein concentration. In this plot we have used the time-dependent values of C at the two

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