[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY, UNIVERSITY OF WISCONSIN]

A New Optical Method for Observing Sedimentation Equilibrium

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Introduction

The optical methods successfully employed to observe equilibrium conditions in the Svedberg "equilibrium" ultracentrifuge[2] have been based on absorption[2,3] or refraction[2,4,5,6] of light. The light absorption method leads to a knowledge of the concentration distribution at equilibrium, while the refractive index methods now in use lead to a knowledge of the concentration gradient distribution. However, both the concentration gradient distribution from the meniscus to the cell bottom, and either the concentration distribution or an exact knowledge of the total amount of solute in solution at equilibrium are needed to compute the "weight-average" molecular weight[7] in polydisperse systems. If the refractive index gradients alone are observed, the concentration distribution may be evaluated by integration and the use of additional experimental data to evaluate the integration constant.[2] This should be done to determine most accurately the molecular weights of monodisperse solutes from the observation of sedimentation equilibria.

It is the purpose of this article to outline the theory and experimental method used to observe in a single photograph both the concentration distribution and the concentration gradient distribution at equilibrium. To this end it is proposed to use instead of the conventional cell a double prismatic cell containing the solution in one prismatic chamber and the reference solvent in the other.[8,9]

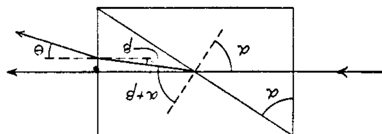

Fig. 1.—The sideward angular deviation at the prismatic cell.

Theory

The Angular Deviation.—An examination of the deviations at the surfaces of such a prismatic cell indicates the possibility of its application to sedimentation equilibrium observations. Reference to Fig. 1 shows that light entering along the normal to the first prism surface undergoes no deviation if solvent of refractive index n_0 fills both chambers. If the first chamber contains solution of refractive index n and the second chamber solvent, the angular deviation of the ray leaving the cell is given by the Snell's law equations

$$n \sin \alpha = n_0 \sin (\alpha + \beta) \quad (1)$$

$$n_0 \sin \beta = \sin \theta \quad (2)$$

For small deviations these simplify to the relation to be used for further practical considerations.

$$\theta = \tan \alpha (n - n_0) \quad (3)$$

If now the refractive index n is a function of the height in the cell (at right angles to the plane of the paper, Fig. 1) the angular deviation θ will be proportional to the refractive index increment at each height. It is noted that θ should be measured with respect to a "reference" angle obtained when both chambers contain solvent. The sensitivity increases rapidly as the prism angle α approaches $\pi/2$, although equation (3) is not valid for large values of θ.[10]

Optical Arrangement.—The relationship (3) for the sideward deviation by the prismatic cell is analogous to the relation given[5] for the downward bending by the gradient dn/dx in a cell of physical thickness a

$$\delta = a(dn/dx) \quad (4)$$

While any of the schlieren methods for recording refractive index gradients[2,4,5,11,12,13,14] could also be used to record this sideward deviation as well, Lamm's scale method,[5] which is already standardized for the equilibrium ultracentrifuge here[6] is most conveniently employed. The application of the scale technique to the prismatic cell involves certain theoretical and practical difficulties. However, by this application both the refractive index gradient and the refractive index increment are recorded as a function of the height in the cell in a single scale photograph. In order to observe

(1) This work was made possible by a grant from the Rockefeller Foundation.

(2) Svedberg and Pedersen "The Ultracentrifuge," Oxford University Press, 1940.

(3) Svedberg and Rinde, THIS JOURNAL, **46**, 2677 (1924).

(4) Wiener, *Ann. Phys. Chem.*, **49**, 105 (1893).

(5) Lamm, *Nova Acta Regiae Soc. Sci. Upsaliensis*, **IV**, **10**, No. 6 (1937).

(6) Bridgman and Williams, *Ann. N. Y. Acad. Sci.*, **43**, 195 (1942).

(7) Lansing and Kraemer, THIS JOURNAL, **57**, 1369 (1935).

(8) The author is indebted to Dr. A. C. Guyton, Georgia Warm Springs Foundation, Warm Springs, Georgia, for originally suggesting the possibility of using prismatic optical systems for direct recording of the concentration function across boundaries, such as in electrophoresis of proteins.

(9) Prismatic optical systems have been employed for other refractometric measurements: Thovert, *Ann. chim. phys.*, [9] **2**, 369 (1914); Zuber, *Z. Physik.*, **79**, 280 (1932); Longsworth, *Ind. Eng. Chem., Anal. Ed.*, **18**, 219 (1946); P. P. Debye, *J. Appl. Phys.*, **17**, no. 5, 392 (1946).

(10) To ascertain that equation (3) holds for ordinary boundaries, we might choose $\alpha = \pi/3$ and $n - n_0 = 0.00925$ (for a 5% protein solution), in which case $\sin \theta = 0.01602$. For this value, the difference between θ and $\sin \theta$ is 0.004%, while the difference between 1 and $\cos \theta$ is 0.01%. The error in equation (3) itself amounts to 1% for this extreme concentration.

(11) Philpot, *Nature*, **141**, 283 (1938).

(12) Longsworth, THIS JOURNAL, **61**, 529 (1939).

(13) Andersson, *Nature*, **143**, 720 (1939).

(14) Svensson, *Kolloid-Z.*, **87**, 181 (1939); **90**, 141 (1940).

accurately the sideward deviations, the working scale used has a vertical scale line drawn perpendicular to the ordinary numbered lines.

If the section of the prism through which light from a given numbered line reaches the camera is kept narrow, the thickness a of solution traversed is well defined. Since no sideward deviations occur until the light has passed the solution chamber, this thickness and the resultant recorded gradient are unaffected by the prismatic deviations. This then corresponds, as shown in Fig. 2a, to the use of Lamm's scale method with a conventional cell of thickness a, in so far as the gradient recording is concerned.

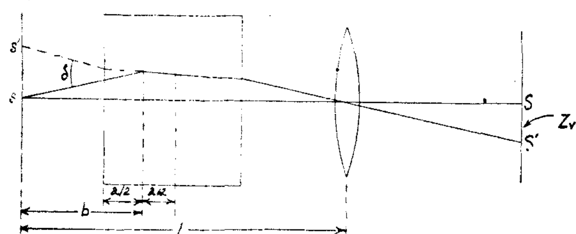

Fig. 2a.—Scale system, side view. Light from the horizontal scale line at s normally comes to a focus at S, but is deviated to S' by the refractive index gradient, appearing to originate at s'.

If the magnification of the scale by the camera lens is G, the vertical deviations of the numbered lines in the photograph through the solution at equilibrium from their corresponding positions[15] in a reference photograph with solvent in both chambers are given by

$$Z_v = Gb\delta \qquad (5)$$

while the positions z of the deviated lines in the photograph are related to the heights x in the cell according to

$$z = Fx = (Gl/((l - b))x \qquad (6)$$

An analogy for the horizontal deviations is obtained from Fig. 2b. If the stop at the camera lens is sufficiently wide, the ray which enters the solution at right angles can proceed along path 1, and the deviation at the plate is given by

$$Z_h = GB\theta \qquad (7)$$

where B is the optical distance from the scale to the solution–glass interface. For the analogy to be complete, however, it is necessary that the same relationship obtains when the camera stop is made indefinitely small. In this case the light must follow path 2, so that the horizontal deviation at the plate is

$$Z'_h = GL\phi \qquad (8)$$

where L is the optical distance from the scale to the lens. Application of Snell's law successively to each interface along path 2 together with the assumption of small angles shows that

$$\phi = \theta - i \qquad (9)$$

(15) The approximation involved here is discussed by Lamm in ref. (2), p. 269.

Also, the distance h can be expressed by

$$h = Bi = (L - B)\phi \qquad (10)$$

Comparison of equations (7), (8), (9) and (10) shows that $Z'_h = Z_h$ for small values of θ.[10] According to equation (3), therefore, the deviated vertical line photograph, except for distortions observed in the reference run, is a direct plot of $GB \tan \alpha(n - n_0)$ against $z = Fx$ where x is the actual height in the cell.[16]

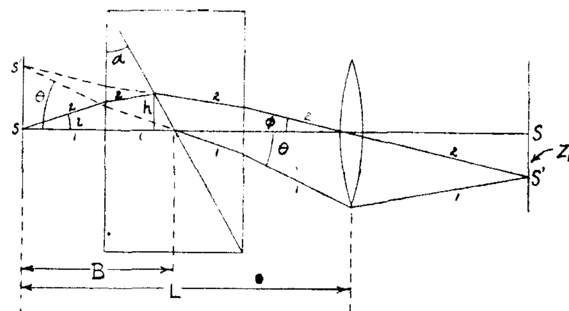

Fig. 2b.—Scale system, top view. Light from the vertical scale line at s normally comes to a focus at S, but is deviated to S' by the refractive index increment at the prism face, appearing to orginate at s'.

Experimental[17]

Methods.—The double prismatic cell was constructed with a conventional steel barrel to fit the standard rotor. Figure 3 shows the essentials of the cell construction in perspective. The cylindrical insert was machined of duraluminum, with a diagonal cut of 41° forming the faces to support the circular glass prism window. These faces were bored out sufficiently to hold this window, made of thin photographic plate, together with several thin rubber packing rings on either side. Rotation and sliding of the two halves of the insert over each other during assembly were prevented by two dowel pins parallel to the axis of the cylinder. As usual, the outer glass windows were recessed into the insert, and were packed with thin rubber and tightened with guard rings threaded into the barrel. The chambers themselves were made rectangular rather

(16) The analogy with the gradient recording is imperfect. Lamm has shown that the optical distance b for vertical deviations should be measured from the scale to the middle of the solution chamber, while Fig. 2b shows that the optical distance B for the horizontal deviations should be measured to the solution-glass interface. Hence $B = b + a/2n$. In addition, photography through very steep gradients gives incorrect values for the refractive index increment because of the downward curvature of the light path in the solution chamber. Due to the imperfect analogy in optical distances the photograph of the scale through the gradient assigns to a refractive index increment characteristic of the end of the curved light path in the solution chamber a height x which is characterisitc of this path in the neighborhood of the $a/2$ plane in this chamber. The error in x can be shown to be $-(3a^2/8n)(dn/dx)$ to a first approximation, which is three times that given by Wiener[4] and Lamm[5] for the gradient determination. When the gradients are sufficiently low to be correctly recorded, this error is always negligible. The magnitude of such errors has been discussed in detail by Bridgman and Williams.[6] For long focal length systems and cells of the thickness employed here, the additional error in the magnification factor F caused by use of the optical distances l and b in its calculation is negligible.

(17) The greater part of the cell design, as well as its actual construction, were performed by Mr. Edwin M. Hanson of this Laboratory, without whose coöperation much of the experimental work would not have been accomplished. His assistance is very gratefully acknowledged.

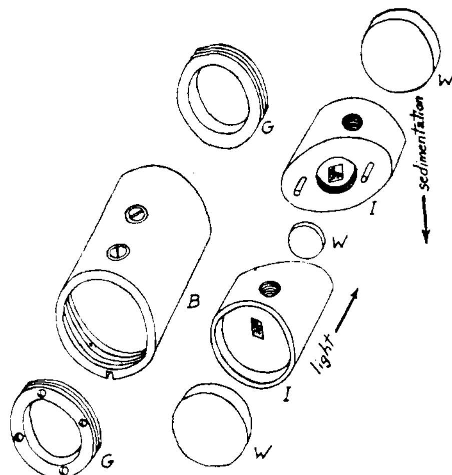

Fig. 3.—Prismatic cell in perspective (not to scale). B, barrel; G, guard rings; W, windows; I, inserts. Packings not shown.

than sectorial in cross section, so that the cross-sectional area was constant rather than a complicated function of the height in the cell. Each chamber was supplied with a filling hole sealed by a steel screw and rubber gasket, making the use of a paraffin oil layer to prevent evaporation of water unnecessary.

A mask parallel to the direction of sedimentation was provided in the slide which covers the cell in the rotor, allowing only a narrow section of the prism face to pass light from the scale during the rotation of all possible cell thicknesses past the scale. In this way blurring of the numbered scale lines due to a lack of definition of the cell thickness was avoided. Experience showed that while a thinner mask was theoretically desirable, 1 mm. was the smallest size practicable, due to diffraction of the vertical scale line by smaller mask openings, especially at the higher scale distances. With the prism angle of 41° used, this mask size corresponds to a maximum variation of 0.87 mm. in the solution chamber thickness. The thickness itself was determined by focusing a Gaertner slide comparator, equipped with an M304 adapter for micrometer focusing, successively on the inner surface of the outer window of the empty solution chamber, and on the nearer surface of the prism window. The microscope viewed the cell in each case through the mask, with the mask and cell assembled in the rotor. The mean visible solution chamber thickness was found in this way to be 9.2$_3$ mm., with an uncertainty of slightly less than 0.1 mm. As the actual variation in the thickness from one side of the mask to the other merely results in a corresponding 10% increase in the line thickness, no serious error is produced. The lines while approximately 50 microns thick in the photographs could be measured to their centers with satisfactory reproducibility.

Since a high precision two-coördinate comparator was not available, a technique was developed which permitted the simultaneous viewing of the deviated vertical line and a precisely defined reference line under the 5.5 mm. field of the comparator microscope. The solution and solvent columns were reduced to 3.6 mm. and masked just above the meniscus level. A plate was made for the reference cell with 1 mm. holes spaced immediately before the meniscus level and past the cell bottom. The photograph obtained during rotation then showed two reference areas immediately surrounding the solution column, with both numbered and vertical scale lines visible in each reference area. With the photographic plate placed under the comparator so that the numbered lines ran parallel to the screw, the comparator hair-line could be set to coincide with the two vertical reference lines, and horizontal deviations from this reference line at heights in the cell corresponding to numbered lines could be directly measured. After similar measurements from the reference run, the horizontal deviations were computed by difference. Measurement of the vertical deviations presented no unusual difficulties.

Results

Recrystallized ovalbumin[18] prepared by the method of Kekwick and Cannan[19] was examined at 0.62 and 0.43% in the prismatic cell. Photographs taken during the first run at 14, 39, 60 and 118 hours after the start demonstrated that the equilibrium condition was essentially established in 60 hours.

Combination of equations (3), (4), (5) and (7) with the standard sedimentation equilibrium equation in differential form[2] gives for the molecular weight

$$M = \{RT/(1 - V\rho)\omega^2\}\{(\tan \alpha)/a\}\{B/b\}\{Z_v/Z_h x\} \quad (11)$$

This would also be the "weight–average" molecular weight M_{wx} for an ideal polydisperse system[7,20] as a function of height x in the cell.

Details of the runs, and the average molecular weights calculated by equation (11) together with their mean deviations from the mean for the vari-

TABLE I

SEDIMENTATION EQUILIBRIUM EXPERIMENTS WITH OVALBUMIN

Buffer 0.15 M sodium chloride + 0.001 M sodium acetate at pH 4.65. Cell prism angle 41°. Cell thickness 0.92$_3$ cm. Optical scale distance $B = b + 0.34$ cm. Scale magnification G: 1.130 throughout. Optical distance l: 279.5$_2$ cm. throughout. Camera diaphragm stop $F/70$. Plates Kodagraph C.T.C. Filter Wratten 77(λ = 5461 Å). Temperature 25.0°. Partial specific volume V = 0.751.[a] Solution density ρ = 1.007.

	Run no. 1	Run no. 2
Condition of sample	Undried	Lyophilized[b]
Ovalbumin concn.[c]	0.62%	0.43%
Date run started	2300 h. 14 Aug.	1830 h. 7 Oct.
Number of plates	4	1
Speed, R. P. S.	179.6	179.4
Time of final photographs after start, hr.	118	113

Values of the Mean Molecular Weight M_{wx}, and Mean Deviation

Scale distance b, cm.	Run no. 1	Run no. 2
6.67	40,200 ± 1400	44,300 ± 3600
8.67	43,100 ± 1200	43,800 ± 2500
10.67	44,200 ± 900	45,800 ± 3000
12.67	42,900 ± 800	

[a] Svedberg and Nichols, THIS JOURNAL, **48**, 3081 (1926). [b] M_{wx} values for the lyophilized sample increased toward the cell bottom, probably indicating polydispersity (see Bull, THIS JOURNAL, **66**, 1499 (1944)). [c] Ovalbumin concentrations estimated from refractive index increment deviations only.

(18) The author is indebted to Dr. Alfred G. Polson, of the Veterinary Research Laboratories, Onderstepoort, South Africa, for furnishing a large quantity of his ovalbumin preparation made during a recent visit to this Laboratory. He had recrystallized this material six times, and it was homogeneous in the velocity ultracentrifuge.

(19) Kekwick and Cannan, *Biochem. J.*, **30**, 227 (1936).

(20) Wales, Bender, Williams and Ewart, *J. Chem. Phys.*, **14**, 353 (1946).

ous scale distances are given in Table I. While there appear to be small systematic shifts with changing scale distance, these averages compare satisfactorily with the accepted values for the molecular weight[2,21] of ovalbumin. Table II shows the horizontal and the vertical deviations as directly measured with the comparator for the scale distance $b = 12.67$ cm. in the first run. The molecular weights calculated with equation (11) directly from these deviations without graphical smoothing are also given in this table. The fluctuations in the unsmoothed molecular weights calculated for this case are only a little larger than those obtained in calculating "weight-average" molecular weights by the customary integration procedure, after smoothing the data by curve plotting.[2,22]

TABLE II

MOLECULAR WEIGHTS OF OVALBUMIN

Date run started 2300 h., 14 August
Time of photograph after start, hr. 118
Scale distance b, cm. 12.67
Ovalbumin concentration[a] 0.62%

Distance from center of rotation, x, cm.	Vertical deviation Z_v, microns	Horizontal deviation Z_h, microns	Gram molecular weight M_{wx}
5.078	265	90	44,700
5.093	277	102	41,100
5.108	290	106	41,300
5.123	304	106	43,100
5.138	315	114	41,400
5.152	333	117	42,500
5.167	348	127	40,800
5.182	363	132	40,900
5.196	378	133	42,100
5.210	404	141	42,400
5.225	425	146	42,900
5.239	444	152	43,000
5.253	465	161	42,400
5.267	488	166	43,000
5.282	514	171	43,800
5.296	535	178	43,700
5.310	564	194	42,200
5.323	590	202	42,300
5.337	613	207	42,700
5.351	639	214	43,000
5.364	(658)	(218)	(43,400)
5.377	(692)	(228)	(43,400)
5.389	(736)	(235)	(44,700)
(5.060)	meniscus		
(5.427)	cell bottom		

[a] Concentration estimated from refractive index increment deviations only.

Discussion

The fluctuations in molecular weight shown in Table II may be taken as a measure of the expected non-systematic errors in the individual unsmoothed weight average molecular weights M_{wx} at each height in the cell for a polydisperse system. Smoothing the observed horizontal and vertical deviations graphically will materially decrease the magnitude of such errors. On the basis of the data obtained for ovalbumin, therefore, the prismatic cell also offers considerable promise for the accurate evaluation of "weight–average" molecular weights in polydisperse systems. Precise values for such systems are difficult to obtain from the standard cells because of the necessity for accurately integrating the refractive index gradient function from the meniscus to the bottom of the cell. At the limits, where a precise knowledge of the gradient function is most important, it is most difficult to obtain. In addition, independent measurements of specific refractive index increments must be performed, and it must be assumed that no part of the sample either settles to the bottom of the cell or floats to the top, an error in the integration constant automatically resulting if such be the case.[23]

Greater precision may be expected by improving the accuracy of the working scale and by detailed investigation of the distortions of all windows. The packing of additional cells for work with non-aqueous solvents is projected, and higher prism angles will be tried.

Acknowledgments.—In addition to acknowledgments made in the footnotes, the author wishes to express his appreciation to Dr. Kai O. Pedersen of the University of Uppsala for his kindness in discussing in detail operational problems in the equilibrium ultracentrifuge during his recent visit here. He is indebted to Messrs. Louis J. Gosting, Robert A. Alberty and Michael Wales for helpful discussions. He wishes especially to thank Professor J. W. Williams for placing the facilities of this Laboratory at his disposal and for his kind support and encouragement of these studies.

Summary

The theory for the use of a double prismatic cell for the simultaneous observation of the concentration distribution and concentration gradient distribution at sedimentation equilibrium has been presented. A cell and the experimental details connected with its use have been described. Results obtained in two experiments with purified ovalbumin have been encouraging, and the method offers promise for accurate determinations of "weight–average" molecular weights in polydisperse systems.

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(21) Bull, *J. Biol. Chem.*, **137**, 143 (1941).

(22) Pedersen, *Biochem. J.*, **30**, 961 (1936).

(23) For possible experimental methods of minimizing such errors see, however, Pedersen, ref. 22.