Anal. Calcd. for $C_{17}H_{16}ON_2$: C, 77.3; H, 6.1; N, 10.6. Found: C, 77.4; H, 5.7; N, 10.7.

2,4-Diamino-5-phenyl-6-(N-methyl-N-phenylaminomethyl)-pyrimidine.—The above nitrile (6.6 g.) in a mixture of ethanol and ether (200 ml., 1:1) was treated with diazomethane (from nitrosomethylurea (5 g.)). After standing overnight the diazomethane and solvent were evaporated. The residue was dissolved in ethanol (50 ml.) and treated with an alcoholic solution of guanidine (from the hydrochloride (2.2 g.) and sodium (0.6 g.) in ethanol (100 ml.). After heating for four hours the alcohol was evaporated and the solution made alkaline with strong sodium hydroxide solution. The residue was dissolved in ether, the ether solution washed with water and dried with sodium sulfate. After removal of the ether the residue was recrystallized from benzene–petroleum ether giving yellow prisms melting at 150–151°.

Anal. Calcd. for $C_{18}H_{19}N_5$: C, 70.8; H, 6.2; N, 23.0. Found: C, 71.2; H, 5.9; N, 22.6.

Condensation of Ethyl N,N-Dimethylaminoacetate with *p*-Chlorophenylacetonitrile.—The ester[39] (26 g.) and *p*-chlorophenylacetonitrile (30 g.) were mixed and added to a solution of sodium ethoxide in ethanol (from sodium (4.6 g.) in ethanol (150 ml.)). The solution was heated for five hours on the steam-bath. After cooling it was poured into water, the insoluble material removed with ether and the aqueous solution was carefully neutralized with 1 *N* sulfuric acid. The separated crystals were washed with water and recrystallized from ethanol. The compound melted with effervescence at 225–231° and melted again sharply with decomposition at 240°.

Anal. Calcd. for $C_{12}H_{13}N_2OCl$: C, 60.9; H, 5.5; N, 11.8. Found: C, 60.7; H, 5.4; N, 12.2.

With phenylacetonitrile in an exactly similar manner a compound was obtained which formed colorless plates melting at 239°.

Anal. Calcd. for $C_{12}H_{14}N_2O$: C, 71.3; H, 6.9; N, 13.9. Found: C, 71.6; H, 7.0; N, 14.2.

Neither of these compounds reacted with diazomethane.

Condensation of Ethyl N-Phenylaminoacetate and *p*-Chlorophenylacetonitrile.—The ester (17.9 g.) and the nitrile (15.1 g.) were added to a solution of sodium ethoxide (from sodium (2.3 g.) in ethanol (100 ml.)). The reaction and isolation of the product were carried out exactly as before. The crystalline product after recrystallization from ethanol melted at 225° (dec.).

Anal. Calcd. for $C_{16}H_{12}ON_2Cl$: C, 67.7; H, 4.2; N, 9.9. Found: C, 68.0; H, 4.5; N, 10.3.

Acknowledgment.—We are indebted to Samuel W. Blackman and N. Martinez, Jr., for the analyses reported here and to Miss Phoebe Lee Graham for assistance in the preparation of many of the compounds and for the measurement of the spectra.

(39) R. Willstätter, *Ber.*, **35**, 584 (1902).

TUCKAHOE 7, N. Y. RECEIVED FEBRUARY 2, 1951

[CONTRIBUTION FROM THE FEDERAL SECURITY AGENCY, PUBLIC HEALTH SERVICE, NATIONAL INSTITUTE OF HEALTH, NATIONAL CANCER INSTITUTE]

The Determination of Sedimentation Constants from Fresnel Diffraction Patterns[1]

BY GERSON KEGELES AND FREDERICK J. GUTTER

A precision method is described for the experimental determination of sedimentation constants, which makes use of micro-comparator measurement of Fresnel diffraction fringes to locate the center of the sedimenting boundary. These fringes, which are symmetrical in pairs about the center of the boundary, originate with the introduction of a bar as diagonal diaphragm into the cylinder lens refractive index gradient recording optical systems. With the aid of this method, which is found to reproduce protein sedimentation constants to considerably better than 1% at concentrations above 0.5%, a study has been made of the concentration dependence of the sedimentation constants of several proteins. Comparison of this observed concentration dependence with available hydrodynamic theories indicates the approximate validity of the theory of Burgers for unhydrated spheres. However, it is believed that residual discrepancies between experiment and theory would justify the extension of the Burgers theory to the actual case of hydrated, elongated, interacting particles.

Introduction

The original application by Svedberg and coworkers of the ultracentrifuge[2] to the study of protein molecules has provided impetus to a continuous development of optical methods for investigating boundary layers in electrophoresis, diffusion and ultracentrifuge studies. These methods have the aim, when applied to sedimentation rate determinations, of determining that level in the boundary where the concentration of sedimenting solute is half that in the constant composition portion of the column below the boundary. For a sedimenting substance showing ideal behavior, it is to a very close approximation the movement of this median concentration level which determines the sedimentation constant. At this level, also, the refractive index gradient passes through a maximum for ideal sedimentation. The refractometric cylinder lens schlieren optical arrangements of Philpot,[3] Svensson,[4] and Andersson[5] possess the marked labor saving advantage over the Lamm refractometric scale method[6] of automatically depicting the refractive index gradient *versus* radius relationship. However, the automatic recording systems do not, within the limit of our experience, provide ultracentrifuge results of satisfactory reproducibility or accuracy, when evaluated by the customary procedure of measurement on enlarged tracings. A very decided improvement has been made by measuring these refractive index gradient diagrams directly on the photographic records with a microcomparator.[7] It is the purpose of this paper to present a method which further facilitates the attainment of high accuracy in the evaluation of sedimentation constants with automatic recording optical systems.

When the diagonal diaphragm or slit of the customary cylinder lens schlieren optical arrangements

(1) Presented at the 117th Meeting of the American Chemical Society in Philadelphia, Pa., April 12, 1950.

(2) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Clarendon Press, Oxford, 1940.

(3) J. St. L. Philpot, *Nature*, **141**, 283 (1938).

(4) H. Svensson, *Kolloid-Z.*, **87**, 181 (1939).

(5) K. J. I. Andersson, *Nature*, **143**, 720 (1939).

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

(7) R. Cecil and A. G. Ogston, *Biochem. J.*, **43**, 592 (1948).

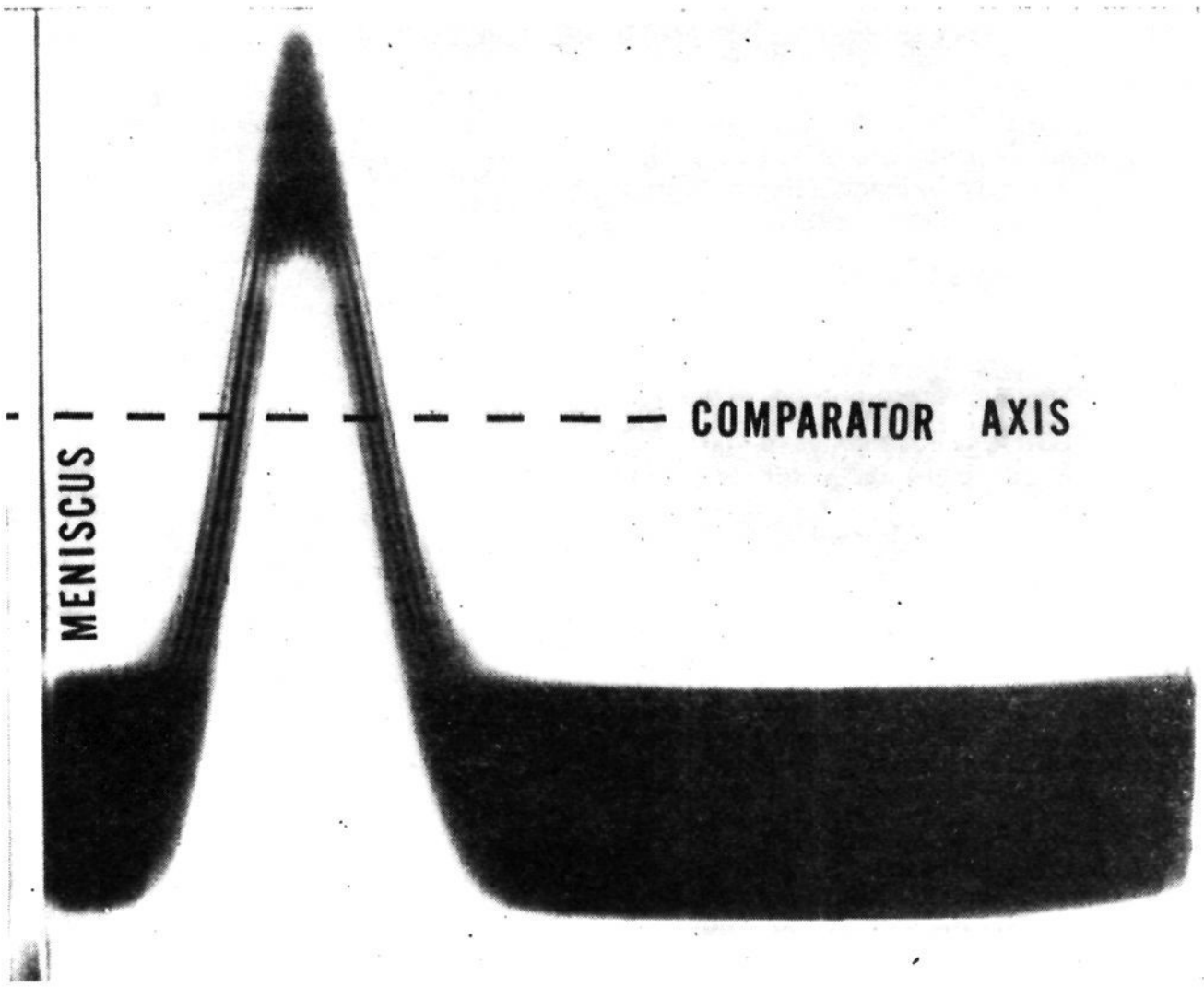

Fig. 1.—Sedimentation diagram of 1.05% solution of four times recrystallized ovalbumin, illustrating Fresnel diffraction fringes. Photograph taken after 56 minutes sedimentation at 996.2 r.p.s.

is replaced by a bar,[8] and the photographs are overexposed, a symmetrical system of fringes results, which is reproduced in Fig. 1. Since these fringes are located symmetrically about the center of a symmetrical peak, that center can be located precisely by means of micro-comparator measurements of fringe positions at a fixed height dn/dx in the diagram, and the peak movement is determined by using the meniscus image as a fixed reference. That these fringes are analogous to those produced within the geometrical shadow of a wire by Fresnel diffraction can be seen on comparison with the photograph of the latter phenomenon made in this Laboratory and reproduced in Fig. 2.[9] The utilization of straight edge Fresnel diffraction fringes for the location of moving boundaries in electrophoresis has already been suggested by Longsworth[10] and by Distèche,[11] who has developed the theory for the fringes originating in the Longsworth schlieren scanning diagrams.[12] The fringes under consideration in the present study are produced by the interaction, within the geometrical shadow of the bar, of the wave fronts from the two edges of the bar. It is readily seen, Fig. 1, that these are no longer obtained beyond the extremes of refractive index gradient where diffraction in the direction of sedimentation can no longer superpose light from the upper diagram upon light from the lower diagram, *i.e.*, in the regions above the peak of the lower diagram and

Fig. 2.—Fresnel diffraction pattern from a wire.

(8) E. G. Pickels, in J. Alexander, "Colloid Chemistry," Vol. V, Reinhold Publ. Co., New York, N. Y., pp. 423–425.

(9) *Cf.* F. A. Jenkins and H. E. White, "Fundamentals of Physical Optics," McGraw-Hill Book Co., Inc., New York, N. Y., 1937, Fig. 8W.

(10) L. G. Longsworth, THIS JOURNAL, **65**, 1755 (1943).

(11) A. Distèche, *Biochim. Biophys. Acta*, **3**, 146 (1949).

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

below the base line of the upper diagram. A practical advantage for following boundary movements offered by the use of the diffraction fringes originating from a bar, as compared with those originating from a straight edge, is that the spacing of the fringes from a bar is nearly independent of the refractive index gradient and, hence, of time.

Experimental

It has been found that when the refractive index gradient diagram is symmetrical, the diffraction fringes are symmetrical in pairs about the center of the diagram. To measure the intensity minima, the plate is aligned, Fig. 1, so that the image of the meniscus is perpendicular to the axis of the screw of the comparator. The position of the meniscus image, and the center of each minimum, and its conjugate fringe on the opposite side of the diagram, are estimated, and by means of a cross-axis motion this procedure is repeated at several levels between the baseline and the peak of the diagram. The average from each pair of conjugate fringes provides a value of the distance of the boundary center from the meniscus.

The sedimentation velocity experiments were performed with the Model E geared electric drive ultracentrifuge of the Specialized Instruments Corporation,[13] in which the rotor is operated in high vacuum. This instrument is provided with a parallel beam schlieren optical system, using a cylinder lens, and a 1.5 mm. bar as inclined diaphragm. The spherical lens magnification F of the cell at the plate was determined to be 2.269 by photographing a millimeter scale engraved on a 9.7 mm. thick glass plate, provided with the instrument. The engraved surface was placed in the rotor so that it occupied the position of the center of a 12 mm. cell, and rotated until the scale lines were at right angles to the cylinder lens axis, reaching maximum sharpness. The photograph was taken through the glass plate, which had been designed by the manufacturers to exactly compensate for the parallel plate effect of half a cell-thickness of water and one 5 mm. thick quartz cell window. The same scale is provided with a holder for locating the engraved surface in the plane of the bar, in which case rotation of the lines parallel to the cylinder lens axis, and photographing of the scale, indicated a magnification G of the bar at the plate equal to 3.302. The air spaced schlieren lenses and the cemented camera objective are doublets. The matched schlieren lenses have a specified focal length of 24 inches. The optical lever arm b was found to 59.06 cm., as determined from the relationship $b = d - e(f - d)/f$, where d is the distance from the second schlieren lens to the slit image, e is the distance from the center of the cell to the second schlieren lens and f is the focal length of the lens.

A dilatation of the rotor of 0.36 mm. was measured with a micro-comparator from a photographic plate double exposed at low speed, and at 59,780 r.p.m., to a water-filled cell and balancing cell.

Protein concentrations in g./100 cc. solution corrected to zero time at the meniscus, were obtained from the average areas of the upper and lower diagrams in early photographs according to the equation

$$c = A(x/x_0)^2/kFGab \cot \theta$$

where A is the measured area on the plate, x and x_0 are the distances from the center of rotation to the boundary and the meniscus, respectively, k is the specific refractive index increment for the sedimenting protein, a is the thickness of the cell in the direction of the optic axis, θ is the angle between the slit image and the inclined bar, and F, G and b are the magnification factors and the optical lever arm whose values are given above. In the few experiments where these refractometric measurements could be compared directly with known concentrations by weight, the concentrations measured were in complete agreement with known values, within expected experimental errors of planimetry.

By use of the external "free" thermocouple provided, the temperature of the rotor itself was measured shortly before accelerating, and as soon as possible after decelerating at the end of each experiment. A special measurement was made to determine the temperature rise occurring during the acceleration and deceleration periods alone, and this small rise of 0.4° was subtracted symmetrically from the total temperature rise in each experiment. Assuming the residual temperature rise for a rotor operated in constant pressure high vacuum to be a linear function of the time at constant speed,[14] the temperature at the midpoint of each exposure interval between photographs was interpolated, and an individual temperature correction to 20° for each interval was made by means of the corresponding relative viscosity of water.[15] No attempt was made to regulate the rotor temperature, which rose between 0.5 and 1.5° per hour, and never over 0.4° per 16-minute interval, corresponding to a maximum change of viscosity of 1% between exposures. The relative viscosity of each buffer used was measured in a capillary viscometer. Speed measurement, made two to three times during each experiment with the mechanically geared counter provided with the instrument, indicated constancy of the average speed to about 0.2%. A stroboscopic speed determination, at 7148 r.p.m., made over individual intervals of one minute by counting the beats between the 120 flash per second AH-6 mercury arc lamp and the rotor through the optical system, agreed with the counter over a ten-minute period to within 0.2%.

In order to obtain symmetrical fringe systems on either side of the diagram, considerable care in focusing of the optics is required. In particular, a slight modification of the test of Schardin[16a] and Longsworth[16b] has proved very useful for focusing the camera lens on the center of the cell. A 3% solution of bovine plasma albumin is placed in a 12 mm. cell and rapidly accelerated to 59,780 r.p.m., the cylindrical lens diagram being carefully observed during the first few minutes with the diagonal bar rotated to zero sensitivity. In this case, the gradients are sufficiently great to deflect some light out of the optical system, and a sharp black band perpendicular to the baseline results. As this band leaves the meniscus, it is bordered by a bright band at one edge, when the camera lens is out of focus. Adjustment of the lens position can be made to within about 2 mm. by choosing the setting for which both borders of the dark band are equally bright. Location of the diagonal bar in the focal plane of the schlieren lens system is accomplished by the horizontal adjustment at high sensitivity of the baseline through a water-filled cell rotated at 59,780 r.p.m., after the cylinder lens axis has first been adjusted horizontal. These procedures take into account any lens effect caused by bulging of the cell windows at high speed.[17]

For photographic reproduction of the diffraction fringes, monochromatic light does not appear to be necessary, and sharpness is not visibly increased by its use, presumably because the lens system is color corrected in the visible region, and any residual dispersion and wave length dependence are not important for an interference phenomenon involving a difference of only a few waves. However, the use of the large wave length region registered by unfiltered mercury light from the AH-6 arc on ortho plates was found to be unsatisfactory, because this procedure produced a marked artificial dissymmetry of the fringe systems in the two sides of the diagram. Satisfactory photographs have been obtained with Eastman spectroscopic plates type 103-F (red sensitized), and Wratten filters numbers 16, 77 or 105.

The sedimentation of four proteins was studied as a function of concentration. Crystalline bovine plasma albumin, Armour control No. G4502, was used as obtained from the manufacturer[18] without further purification. This preparation contained about 6% of heavier impurities which readily resolved from the main component. Two prepara-

(13) E. G. Pickels, *Machine Design*, **22**, No. 9, 102 (1950).

(14) The authors are indebted to Dr. E. G. Pickels for information, and to Dr. C. W. Hiatt for data based on direct temperature measurements at high speed, indicating the validity of this assumption.

(15) Ref. 2, pp. 273–274, and Appendix I.

(16) (a) H. Schardin, "Das Toeplersche Schlierenverfahren," B, Vol. 5, VDI-Verlag, Berlin, 1934; (b) L. G. Longsworth, *Ind. Eng. Chem., Anal. Ed.*, **18**, 219 (1946).

(17) The authors are indebted to Dr. E. G. Pickels for correspondence clarifying this point. Since the distortion of the windows is apparently quite reproducible for a given cell and window assembly, we find that taking this lens effect into account in focusing effectively eliminates the major part of the baseline distortion discussed by Cecil and Ogston (ref. 7).

(18) Armour Laboratories, Chicago, Ill.

tions of ovalbumin, recrystallized four times according to the method of Sørensen and Høyrup,[19] were kept at 1 to 7° as a paste under saturated ammonium sulfate, and samples were dialyzed against buffer just before use. One of these, over a year old, gave skew boundaries and a small resolvable heavier component in the ultracentrifuge. The other, freshly prepared for this research, gave nearly symmetrical boundaries in the ultracentrifuge, but additional storage of the dialyzed solution for as little as half a day sometimes produced skew boundaries. However, the sedimentation constants obtained from near the tops of the peaks were essentially the same for both preparations. Human carbon monoxide hemoglobin from the blood of a single donor was prepared by ammonium sulfate precipitation of material from saline-washed and lysed red cells. Electrophoretic analysis indicated that the cell stroma were almost completely removed by a process consisting of ammonium sulfate precipitation and sedimentation of the lysed cells followed by leaching of hemoglobin with water and resedimentation of stroma. The carbon monoxide hemoglobin used gave symmetrical ultracentrifuge and electrophoresis diagrams. It was stored as a suspension under saturated ammonium sulfate. The authors are indebted to Dr. Alton Meister of this institute for providing a highly active preparation of lactic dehydrogenase prepared from hog heart.[20] He found that this material had lost none of its activity at the conclusion of our series of ultracentrifuge experiments.

The buffer used for dialysis of the ovalbumin and bovine plasma albumin solutions consisted of 0.15 molar sodium chloride, 0.02 molar sodium acetate and 0.03 molar acetic acid, with a *p*H from 4.38 to 4.40. Both proteins are found electrophoretically to be nearly isoelectric in this buffer. Human carbon monoxide hemoglobin was studied in 0.1 ionic strength potassium phosphate buffers of the following compositions: 0.0143 molar potassium dihydrogen phosphate and 0.0286 molar dipotassium hydrogen phosphate at *p*H 7.07, and 0.0226 molar potassium dihydrogen phosphate and 0.0258 molar dipotassium hydrogen phosphate at *p*H 6.87. The isoelectric point of this protein lies between these limits.[21a,b] We have observed no electrophoretic migration of carbon monoxide hemoglobin at 1° in 0.1 ionic strength *p*H 7.02 potassium phosphate buffer. No significant secondary charge effect[22] could be detected in changing from *p*H 6.87 buffer to *p*H 7.07 buffer. The buffer used for studying the lactic dehydrogenase sample consisted of 0.0143 molar potassium dihydrogen phosphate and 0.0286 molar dipotassium hydrogen phosphate.

TABLE I

FOUR TIMES RECRYSTALLIZED OVALBUMIN

2.17 g./100 cc. in 0.15 *M* NaCl–0.02 *M* NaAc–0.03 *M* HAc; *p*H 4.40, 996 r.p.s., 16 minutes between exposures

Movement in cell $x_2 - x_1$, cm., Fresnel	Sedimentation constant, Svedberg units, at 20° in water $\hat{s}_{20}$, Fresnel	$\hat{s}_{20}$, enlarger
0.0814	3.09	3.08
.0828	3.08	3.24
.0855	3.13	2.98
.0866	3.12	2.93
$\hat{s}$	3.10	3.07
Std. error ϵ	±0.006	±0.033

Fresnel diffraction micro-comparator method. Sedimentation constants s were computed from the formula

$$s = \frac{\Delta x/\Delta t}{\omega^2 x} = \frac{2(x_2 - x_1)/\Delta t}{\omega^2(x_2 + x_1)} \qquad (1)$$

where x represents distance from the axis of rotation, t the time, and ω the angular velocity of the rotor.

Correction to water at 20° was made with the formula

$$s_{20} = s\,(\eta^t_w/\eta^{20}_w)\cdot(\eta^t/\eta^t_w)\cdot\left(\frac{1 - V_{20}\rho^{20}_w}{1 - V_{t}\rho^{t}_w}\right) \qquad (2)$$

where V represents partial specific volume of the protein, ρ the liquid density, η^t/η^t_w the relative viscosity of the buffer at the mean temperature of the experiment, and the viscosity ratio η^t_w/η^{20}_w represents the viscosity, relative to water at 20°, of water at the temperature prevailing during the middle of the interval between photographic exposures.[15]

As pointed out by Oncley,[23] the averaging of such sedimentation constants results in the elimination from the average of all data except those taken from the initial and final photographs. In order to take advantage of the intermediate data, a procedure was worked out equivalent to least-squaring the temperature corrected values of ln x as a linear function of time. In a series consisting of five equally spaced photographs, it is readily shown,[24] by arbitrarily choosing the time at the third photograph as equal to zero, that the slope of this least square formulation is expressible by

$$\hat{s}\omega^2 = (1/10)(2s_1 + 3s_2 + 3s_3 + 2s_4)\omega^2$$

where $\hat{s}\omega^2$ is the value of the slope which makes the sum of the residuals in ln x a minimum, and s_1, s_2, s_3 and s_4 are the values of the sedimentation constant computed from successive pairs of photographs as outlined above. The standard error in the sedimentation constant $\hat{s}$ can then be computed by means of the formula

$$\epsilon = \pm\sqrt{\frac{1}{30}\left[(s_1 + s_2)^2 + s_2^2 + s_3^2 + (s_3 + s_4)^2 - \frac{1}{5}(-s_1 - 2s_2 + 2s_3 + s_4)^2 - \frac{1}{10}(2s_1 + 3s_2 + 3s_3 + 2s_4)^2\right]}$$

Results.—Comparison is made in Table I of the results from a typical experiment, evaluated with both the enlarger tracing method and the Fresnel diffraction micro-comparator method. Sedimentation constants s were computed from the formula

and the standard error in ln x can be obtained from this by multiplication by $\sqrt{10}\,\omega^2\,\Delta t$. In Table I, and the tables to follow, the value represented by $\hat{s}$ is the least square value computed as just outlined,

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

(20) A. Meister, *J. Biol. Chem.*, **184**, 117 (1950).

(21) (a) Ref. 2, Table 48; (b) L. Pauling, H. A. Itano, S. J. Singer and I. C. Wells, *Science*, **110**, 543 (1949).

(22) Ref. 2, p. 27.

(23) J. L. Oncley, *Ann. N. Y. Acad. Sci.*, **41**, 121 (1941), footnote 12.

(24) We are indebted to Nathan Mantel of the Statistical Section, National Institutes of Health, for this formulation and for some further help in the statistical treatment of the data.

and the values denoted by ± represent the standard error ϵ within the individual experiment.

In Table I, it may be noted that, while the least square values of the sedimentation constants from the two experimental techniques agree, the fluctuations are much greater in the case of the standard enlarger technique. The standard error ϵ of ±0.033 Svedberg unit, using the enlarger technique, corresponds to an average uncertainty of about 25 microns in measuring the boundary displacement in the cell, or approximately 0.22 mm. in measuring the 8.6 times magnified boundary displacement on the enlarger tracing. On the other hand, the standard error in $\hat{s}$ of ±0.006 Svedberg unit, using the Fresnel diffraction fringes, corresponds to an average uncertainty of about 4.6 microns in measuring the boundary displacement in the cell, or about 9 microns in measuring the 2.269 times magnified boundary displacement on the photographic plate with the comparator. A further large increase in the accuracy of fringe measurement, such as might be the goal in the observation of interference fringes, has not generally been realizable, probably due to the fact that the contrast between the light and dark fringes is inherently low in the diffraction phenomenon being utilized.[9]

In Table II are shown all the results obtained

TABLE II

SEDIMENTATION CONSTANTS OF CRYSTALLINE BOVINE PLASMA ALBUMIN

Svedberg Units, Corrected to Water at 20°

Buffer: 0.15 *M* NaCl–0.02 *M* NaAc–0.03 *M* HAc; *p*H 4.40

$k^{\lambda 5461\text{ Å.}}_{27.5^\circ}$ = 0.001907 (Perlmann and Longsworth)[a]

Initial total protein concn., g./100 cc.	$\left(\frac{x_0}{x}\right)^{2b} - \left(\frac{x_0}{x}\right)^{2c}$	s_{20}, Fresnel	s_{20}, enlarger
2.45	0.878 – 0.775	3.86 ± 0.006	
1.63	.894 – .773	4.00 ± .003	3.97 ± 0.024
	.893 – .767	3.99 ± .009	3.93 ± .015
1.49	.922 – .805	4.03 ± .005	4.06 ± .024
1.15	.949 – .820	4.10 ± .003	4.07 ± .019
0.83	.939 – .808	4.11 ± .004	4.11 ± .028
.76	.955 – .830	4.10 ± .010	4.05 ± .033
.58	.947 – .882	4.15 ± .011	4.25 ± .071
.53	.951 – .887	4.08 ± .022	4.17 ± .113
.34	.960 – .893	4.21 ± .014	4.22 ± .039
.20	.978 – .951	4.43 ± .029	4.29 ± .138
	.978 – .951	4.44 ± .028	4.48 ± .058
.10	.967 – .951	4.33 ± .043	
	.978 – .958	4.61 ± .036	
	.976 – .945	4.44 ± .055	

[a] G. Perlmann and L. G. Longsworth, THIS JOURNAL, **70**, 2719 (1948). [b] Value of dilution factor at time of first photograph. [c] Value of dilution factor at time of last photograph.

with the diffraction method for the sedimentation constant of bovine plasma albumin, corrected to water at 20°. The first column gives the total protein concentration, estimated from the areas under the schlieren diagrams (corrected to the meniscus with the dilution factor (x^2/x_0^2)) with the aid of the specific refractive index increment equal to 0.001907 at 27.5° and λ 5461 Å. interpolated from the data of Perlmann and Longsworth.[25] The concentrations at 2.45 and 0.1% were determined directly by weighing albumin into the buffer, taking into account the 16% moisture content measured by desiccation over phosphorus pentoxide. The second column indicates the dilution at the time of the first and last photographs. In the third column are given the sedimentation constants as determined by measurement of Fresnel fringes with the microcomparator, with the values following ± indicating the standard error of the least square sedimentation constant within the individual experiment. Repeated in the fourth column are similar results for the identical experiments, but measured from the movement of the peak center on enlarger tracings of the schlieren diagrams, without reference to the fringes. Values at 1.63, 0.20 and 0.10% derive from repeated experiments performed, at each concentration, on the identical solution. It should be noted that the decrease in precision at lower concentrations is partly due to the requirement that, as the concentration is decreased, the interval between exposures must be shortened, from 16 minutes at 1% and above to only 2 minutes at the lowest concentrations.

Table III presents in similar fashion the results obtained for ovalbumin. It is observed that no significant difference has been found between the sedimentation constants of a preparation stored for a year under saturated ammonium sulfate and those of a fresh preparation.

TABLE III

SEDIMENTATION CONSTANTS OF 4 × RECRYSTALLIZED OVALBUMIN

Svedberg Units, Corrected to Water at 20°

Buffer: 0.15 *M* NaCl–0.02 *M* NaAc–0.03 *M* HAc; *p*H 4.40

$k^{\lambda 5461\text{ Å.}}_{27.5^\circ}$ = 0.001857 (Perlmann and Longsworth)[c]

Initial concn., g./100 cc.	$\left(\frac{x_0}{x}\right)^{2d} - \left(\frac{x_0}{x}\right)^{2e}$	s_{20}, Fresnel	s_{20}, enlarger	Preparation No.
2.17	0.867 – 0.781	3.10_5 ± 0.006	3.07 ± 0.033	2[a]
2.01	.859 – .764	3.14 ± .009	3.13 ± .040	1[b]
1.39	.941 – .843	3.25 ± .003	3.20 ± .031	2
1.10	.941 – .835	3.31 ± .007	3.26 ± .044	1
	.931 – .823	3.31_5 ± .010	3.33 ± .050	
1.05	.955 – .924	3.30 ± .008	3.28 ± .025	2
	.956 – .850	3.32 ± .025	3.38 ± .056	
0.91	.939 – .830	3.37 ± .005	3.49 ± .036	1
.77	.958 – .880	3.44 ± .007	3.42 ± .034	2
.65	.956 – .904	3.50 ± .017	3.58 ± .025	2
.58	.966 – .910	3.47 ± .018	3.36 ± .039	1
.35	.970 – .941	3.59 ± .011	3.58 ± .050	1
.33	.982 – .975	3.66 ± .033	3.61 ± .065	1
.33	.966 – .951	3.62 ± .059	3.91 ± .063	2
	.966 – .953	3.60 ± .031	3.67 ± .047	
.25	.978 – .962	3.58 ± .023	3.55 ± .358	1
.13	.978 – .962	3.51 ± .019		2
	.976 – .962	3.57 ± .036		

[a] Freshly prepared. [b] Stored one year under saturated ammonium sulfate. [c] Perlmann and Longsworth, THIS JOURNAL, **70**, 2719 (1948). [d] Value of dilution factor at time of first photograph. [e] Value of dilution factor at time of last photograph.

In Table IV are presented the corresponding data obtained for human carbon monoxide hemoglobin. The reproducibility, using the Fresnel fringes, of the large number of duplicate experiments, generally made at temperatures differing by several degrees betwen duplicates, bears out the contention, also made by Cecil and Ogston in their study of the

(25) G. E. Perlmann and L. G. Longsworth, THIS JOURNAL, **70**, 2719 (1948).

TABLE IV

SEDIMENTATION CONSTANTS OF HUMAN CARBON MONOXIDE HEMOGLOBIN

Svedberg Units, Corrected to Water at 20°.
Buffer: 0.0286 *M* K_2HPO_4–0.0143 *M* KH_2PO_4; *p*H 7.07;
$k^{27.5°}$ = 0.00181 (Howard)[a]

Initial concn., g./100 cc.	$\left(\frac{x_0}{x}\right)^{2b} - \left(\frac{x_0}{x}\right)^{2c}$	s_{20}, Fresnel	s_{20}, enlarger	Remarks
2.01	0.865 – 0.757	3.92 ± 0.012	3.92 ± 0.009	
	.857 – .748	3.96 ± .003	3.99 ± .009	
1.56	.912 – .796	4.05 ± .003	4.00 ± .026	
	.910 – .790	4.05 ± .002	4.02 ± .023	
1.13	.943 – .813	4.15 ± .006	4.13 ± .050	
1.00	.968 – .843	4.19 ± .010		Lyophilized sample; concn. by dry weight. Buffer: 0.0258 *M* K_2HPO_4–0.0226 *M* KH_2PO_4; *p*H 6.87
1.00	.949 – .825	4.17 ± .002	4.13 ± .026	
	.975 – .824	4.16 ± .004	4.14 ± .016	
0.97	.957 – .830	4.17 ± .003		Buffer: 0.0258 *M* K_2HPO_4–0.0226 *M* KH_2PO_4
	.953 – .824	4.16 ± .007		
.68	.945 – .880	4.19 ± .013	4.13 ± .047	
	.941 – .872	4.21 ± .011	4.23 ± .025	
.51	.953 – .920	4.22 ± .011	4.18 ± .050	
	.957 – .924	4.21 ± .011	4.22 ± .074	
.28	.970 – .951	4.18 ± .024	4.57 ± .192	
	.983 – .949	4.17 ± .004	4.98 ± .189	
.13	.974 – .925	4.22 ± .030		Buffer: 0.0258 *M* K_2HPO_4–0.0226 *M* KH_2PO_4
.13	.973 – .924	4.33 ± .033		
.12	.978 – .960	4.35 ± .034		

[a] Howard, *J. Biol. Chem.*, 41, 537 (1920). [b] Value of dilution factor at time of first photograph. [c] Value of dilution factor at time of last photograph.

accuracy of the Svedberg oil-turbine ultracentrifuge,[7] that the fluctuations of the sedimentation constant within an individual experiment represent a close measure of the reproducibility and precision of the measurement of the sedimentation constant.

Table V contains the sedimentation constants for hog heart lactic dehydrogenase, as determined from measurement of the Fresnel diffraction fringes.

TABLE V

SEDIMENTATION CONSTANTS OF HOG HEART LACTIC DEHYDROGENASE

Svedberg Units, Corrected to Water at 20°
Buffer: 0.0286 *M* K_2HPO_4–0.0143 *M* KH_2PO_4; *p*H 7.11

Initial concn. g./100 cc. (assuming 16% N)[20]	Initial concn. g./100 cc. (assuming k = 0.00185)	$\left(\frac{x_0}{x}\right)^{2a} - \left(\frac{x_0}{x}\right)^{2b}$	s_{20}, Fresnel
1.30	0.92	0.908 – 0.774	6.66 ± 0.009
0.85	.65	.958 – .753	6.88 ± .009
.50	.39	.970 – .861	7.08 ± .016
.25	.25	.974 – .943	7.29 ± .014

[a] Value of dilution factor at time of first photograph. [b] Value of dilution factor at time of last photograph.

Discussion.—Comparison with existing data for the sedimentation constants of the proteins studied reveals much larger discrepancies than might be expected. In the case of bovine plasma albumin, early measurements at 1% made in this country[26,27] gave results appreciably lower than those of Pedersen.[28] More recently, Koenig and Pedersen[29] have carried out a series of measurements on crystalline bovine plasma albumin which show much higher sedimentation constants than those reported here.[30]

Of considerable interest is the comparison of data for the concentration dependence of the sedimentation constant[28] with values predicted by existing theories. The theoretical treatment of Powell and Eyring[31] predicts a linear dependence of the sedimentation constant on the viscosity of the solution. The theoretical problem has been studied in great detail by Kermack, M'Kendrick and Ponder,[32] and by Burgers.[33] These latter two studies agree in the requirement for taking into account the backflow of solvent replacing the sedimenting particles, as well as the steric reduction of the solution viscosity in the neighborhood of a particle. Burgers' study applied only to a suspension of non-interacting spheres, but Kermack, M'Kendrick and Ponder obtained identical solvent backflow for spheres and

(26) E. J. Cohn, J. A. Luetscher, J. L. Oncley, S. H. Armstrong and B. D. Davis, THIS JOURNAL, **62**, 3396 (1940).

(27) F. W. Putnam, J. O. Erickson, E. Volkin and H. Neurath, *J. Gen. Physiol.*, **26**, 513 (1943).

(28) K. O. Pedersen, Dissertation, University of Uppsala, 1945.

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

(30) Dr. Pedersen has very kindly sent us a sample of the Armour lot 46 crystalline bovine albumin studied in reference 29. In 0.2 *M* sodium chloride the corrected sedimentation constant for 2% protein was 4.26 Svedberg units (ref. 29). Dr. Pedersen's redetermination for 40.5 mg. protein dissolved in 2.00 ml. of a buffer consisting of 0.2 *M* sodium chloride–0.019 *M* disodium hydrogen phosphate–0.043 *M* sodium dihydrogen phosphate gave a sedimentation constant of 4.20 Svedberg units (private communication). Our corrected results for the same sample at 1.80 g. protein per 100 cc. solution are 3.82 and 3.81 Svedberg units in 0.2 *M* sodium chloride, 3.79 and 3.77 Svedberg units in the 0.2 *M* sodium chloride–sodium phosphate buffer, and 3.91 and 3.92 Svedberg units in the 0.15 *M* sodium chloride–sodium acetate buffer used in this research. From our results on control No. G4502 albumin we interpolate for s_{20} 3.96 Svedberg units in the chloride–acetate buffer. These discrepancies still remain to be resolved.

(31) R. E. Powell and H. Eyring, in E. O. Kraemer, "Advances in Colloid Science," Vol. I, Interscience Publishing Co., New York, N. Y., 1942, p. 183.

(32) W. O. Kermack, A. G. M'Kendrick and E. Ponder, *Proc. Roy. Soc. Edinburgh*, XLIX Part II, No. 15, 170 (1929).

(33) J. M. Burgers, *Proc. Acad. Sci. Amsterdam*, **44**, 1045, 1177 (1941); *ibid.*, **45**, 9, 126 (1942).

for highly asymmetrical discs. They obtained approximate experimental verification of their theory by studying the gravity sedimentation of red blood cells. Enoksson[34] has recently interpreted the problem in terms of a solvent backflow, but has not taken the viscosity effect into account. Lauffer[35] has demonstrated the applicability of the Powell–Eyring theory in the case of the elongated tobacco mosaic virus particle. However, the generality of the relative viscosity relationship has been found to break down in the case of synthetic high polymers by Jullander.[36] In the case of proteins, the multiplication of the observed sedimentation constant by the relative viscosity of the solution has been found to result in an over-correction by Sanigar, Krejci and Kraemer,[37] and by Pedersen.[28] Indeed, the recent paper on tobacco mosaic virus by Schachman and Kauzmann[38] emphasizes the inapplicability of a pure relative viscosity correction except as a fortuitous circumstance.

The necessary protein viscosity values to test the Powell–Eyring theory have been determined in an Ostwald–Fenske capillary viscometer with a water flow time of 168 seconds. Kinetic energy corrections were found to be negligible. The data were least squared (using appropriate weights for the probable percentage error in timing) according to $(\eta/\eta_0 - 1)/\phi = \nu + b\phi$, where ν is the intrinsic viscosity determined from the least square solution and ϕ is the volume fraction of anhydrous protein. The values of ν found in this study, 6.41 for human carbon monoxide hemoglobin at 21.6°, 5.79 for bovine plasma albumin at 25° and 5.33 for ovalbumin at 25°, compare reasonably well with those in the literature.[27,39,40,41] The ovalbumin studied in the viscometer had been stored in the refrigerator as a paste under saturated ammonium sulfate for approximately nine months since its preparation. However, the protein showed no change in either the symmetry of its sedimentation diagram or the

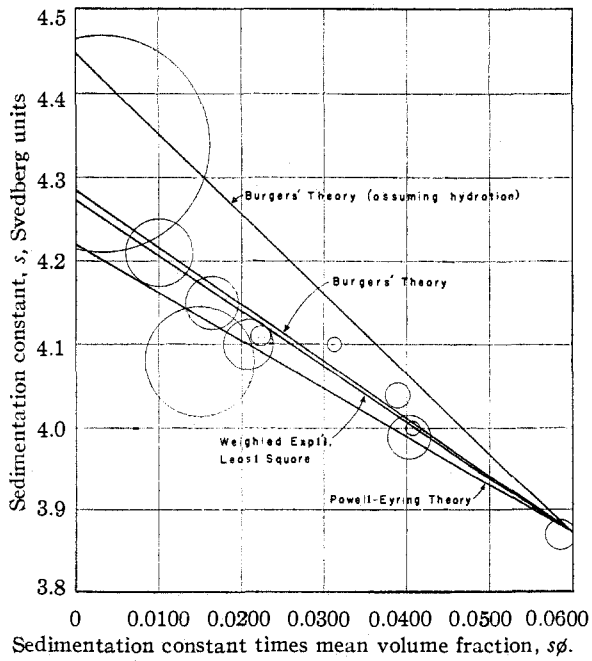

Fig. 3.—Sedimentation constants of bovine plasma albumin

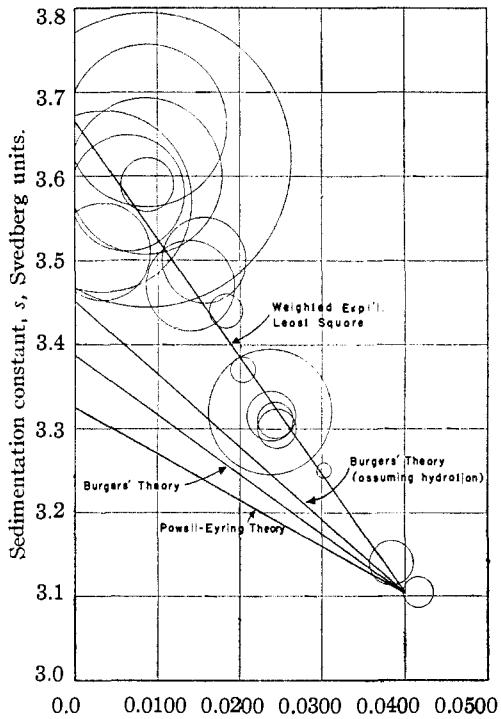

Fig. 4.—Sedimentation constants of ovalbumin.

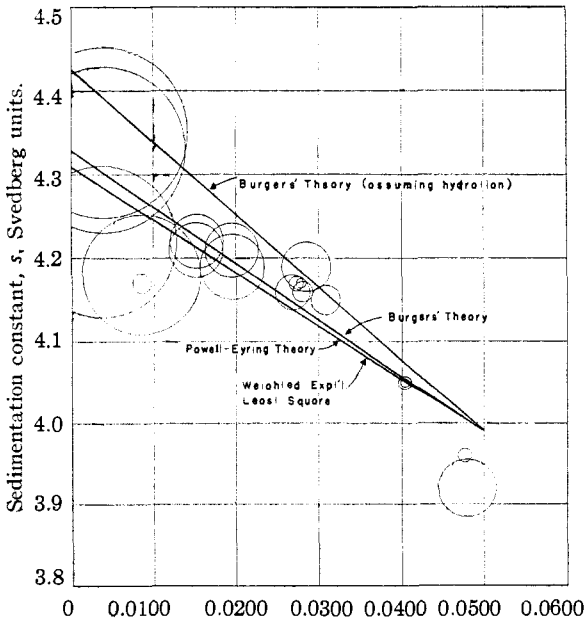

Fig. 5.—Sedimentation constants of human carbon monoxide hemoglobin.

(34) B. Enoksson, *Nature*, **161**, 943 (1948).
(35) M. A. Lauffer, THIS JOURNAL, **66**, 1195 (1944).
(36) I. Jullander, *Arkiv Kemi, Mineral. Geol.*, **A21**, No. 8 (1945); *J. Polymer Sci.*, **2**, 329 (1947); *ibid.*, **3**, 631 (1948).
(37) E. B. Sanigar, L. E. Krejci and E. O. Kraemer, THIS JOURNAL, **60**, 757 (1938).
(38) H. K. Schachman and W. J. Kauzmann, *J. Phys. Colloid Chem.*, **53**, 150 (1949).
(39) A. Polson, *Kolloid-Z.*, **88**, 51 (1939).
(40) J. W. Mehl, J. L. Oncley and R. Simha, *Science*, **92**, 132 (1940).
(41) J. L. Oncley, *Ann. N. Y. Acad. Sci.*, **41**, 121 (1941).

value of its sedimentation constant at the time of the viscosity measurements.

In the Burgers theory, the structural relationship of the sedimenting particle to the same material in bulk is not considered, *i.e.*, the particle is not assumed to bind tightly any of the solvent. However, in the case of proteins, it is generally considered that some water is an integral part of the molecule as it exists in solution. The Burgers theory is therefore applicable with the volume fraction calculated either from the anhydrous partial specific volumes[2] V or from the partial specific volume V_h, in cc. hydrated solute per gram of anhydrous solute.

The hydrate partial specific volumes have been estimated on the basis of the probable values given by Oncley[41,42] for the hydration of dissolved proteins (0.3 g. water per g. serum albumin, 0.4 g. water per g. hemoglobin, and 0.2 g. water per g. ovalbumin).

In the graphs of Figs. 3, 4 and 5, the circles are experimental, with radii equal to three times the standard error of the least square sedimentation constant value in the individual experiment. The abscissa for these graphs is the product of the sedimentation constant by the *mean* volume fraction of protein in the middle of each experiment (Tables II, III and IV). The values for bovine plasma albumin at 0.2% protein concentration and below have been largely omitted, because in the experiments omitted, observation of the boundaries took place at a time when the heavy impurity was still resolving from the main peak, thus artificially elevating the sedimentation constant. The experimental data were then weighted inversely as the standard error and least squared according to the equation $s = s_0 - k(s\phi)$. The lines calculated from the coefficients s_0 and k are shown in Figs. 3, 4 and 5.

The theoretical lines are made to fit the weighted experimental least square line in the neighborhood of the highest concentration measured for each protein, and their slopes are calculated according to the Powell–Eyring theory from

$$s = s_0 - \nu(s\phi) \qquad (3)$$

or from the Burgers equation

$$s = s_0 - 6.875\,(s\phi) \qquad (4)$$

assuming no "fixed" hydration, or from the Burgers equation

$$s = s_0 - 6.875\,(V_h/V)(s\phi) \qquad (5)$$

assuming hydrated particles in solution.

In the case of bovine plasma albumin, the Burgers theory slope of 6.875 and the Kermack, M'Kendrick and Ponder theory slope of 7.1 fit the experimental least square slope of 6.67 somewhat better than the Powell-Eyring theory, with an intrinsic viscosity slope of 5.79. Introduction of a correction for hydration makes the Burgers theory fit very poorly. In the case of human carbon monoxide hemoglobin, a straight line appears to fit the data rather poorly compared with a curve drawn concave downward. This cannot be explained in terms of solute–solute interaction, and may, possibly, indicate increasing dissociation at low protein concentrations.[2] However, if a straight line is assumed, the experimental least square slope, 6.46, coincides with the intrinsic viscosity, 6.41, of the Powell–Eyring theory, and agrees fairly well with the Burgers and the Kermack, M'Kendrick and Ponder theories. Here again, if a straight line is assumed, the introduction of a correction for hydration makes the Burgers theory fit poorly. In the case of ovalbumin, the experimental least square gives a straight line of slope 13.85, which represents all the data very well. None of the theoretical lines has a sufficiently large slope to fit the data, however. A logical explanation could be made in the case of the Burgers theory by noting that the data presented here are consistent with a much higher value for the hydration than the one proposed on the basis of other evidence.[40,41] However, whatever the effect of hydration, it is already included in the experimental determination of intrinsic viscosity for testing the Powell–Eyring theory, and no logical explanation is offered here except that in the case of ovalbumin, this theory does not hold.

Thus it is necessary to conclude that additional factors need to be taken into account in order to explain in more general terms the sedimentation behavior of proteins in dilute solutions. In view of the attainability of protein sedimentation data of high precision, further extension of the hydrodynamic theory to take more general account of hydration, molecular dissymmetry and solute–solute interaction might be justified.

(42) J. L. Oncley, *Chem. Revs.*, **30**, 433 (1942).

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