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Correction for Absorption and Fluorescence in the Determination of Molecular Weights by Light Scattering[2]

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Application of the light scattering method has been hindered frequently by color or fluorescence, either intrinsic or adventitious, in the scattering solutions. The effects of color or absorption comprise three factors: attenuation of the primary and scattered intensities, an effect due to finite dimensions of the scattering volume, and reflection of a portion of the primary beam at the exit window of the scattering cell. In our apparatus the first factor is compensated. The second and third factors are readily calculated. Fluorescence in a scattering solution is easily detected by use of a sharp cut-off filter to exclude scattered light from the phototube. Equations for the correction of fluorescence are based on measurement of the depolarization of the fluorescent light with this filter, and on measurement of apparent scattering ratios with a Polaroid analyzer oriented vertically and then horizontally. Corrections for absorption and for fluorescence were tested separately by adding increments of absorbing and fluorescent dyes to macromolecular solutions. Turbidities of 0.01 cm.$^{-1}$ were determined with errors of less than 3% even when attentuation by absorption was more than 150 times that by scattering and even when 80% of the total phototube response was due to fluorescence. The absorption corrections presented are applicable also to the determination of fluorescence intensities in the presence of light absorption.

Introduction

Two potential sources of error in the determination of molecular weights by light scattering are light absorption and fluorescence of the macromolecular solutions. The pure solute may in some instances have an inherent absorption, as was the case with lactoperoxidase, a reddish iron-containing protein recently studied in this Laboratory.[3] The presence of light-absorbing impurities in the solute or solvent is more often encountered. Unless compensated or corrected for, such absorption will lead to low estimation of turbidity or molecular weight. Correction for fluorescence is probably more frequently required than correction for absorption. Experience has shown that small amounts of fluorescence are associated with many materials, especially those of biological origin. In some cases, for example in proteinaceous and carbohydrate materials after digestion with acids, the fluorescence may be considerable. The presence of such fluorescence, if not detected and corrected for, will result in an overestimation of the molecular weight. Fluorescence by the solvent is usually small and is not ordinarily a source of error in the determination of molecular weight, since excess turbidity of solution over solvent is obtained unambiguously by the usual subtraction (unless the solute has a quenching action on the fluorescence).

The purpose of the present study is to present and illustrate methods of correcting for the presence of absorption and fluorescence in scattering solutions even when these quantities exceed the turbidity or the scattering intensity considerably.

Absorption

The problem of light scattering measurements in colored solutions has recently been discussed by Doty and Edsall.[4] Alexander and Stacey,[5] in a study of the colloidal behavior of dyes, avoided absorption effects by using a source of near infrared radiation. Equations have been derived by Putzeys and Dory[6] and by Lauer[7] for the correction of light scattering measurements for the presence of absorption in apparatus with either circular or rectangular viewing apertures. Both the primary beam and the scattered light are attenuated in traversing the absorbing solution. The following equation, derived from the analysis of Putzeys and Dory, represents the effect of absorption for a solution of small turbidity, and for apparatus with rectangular apertures and a square scattering cell, using notation modified for the present purpose

$$I_s = A_1 I_0 e^{-2ab}\left(1 + \frac{W^2\alpha^2}{24} + \frac{W^4\alpha^4}{1920} + \cdots\right)\left(1 + \frac{h^2\alpha^2}{24} + \frac{h^4\alpha^4}{1920} + \cdots\right) \quad (1)$$

where I_s is the irradiance (illumination) at the phototube for the transversely scattered light; A_1 is a constant of proportionality; I_0 is the irradiance incident on the scattering cell due to the parallel monochromatic primary beam; b is the distance from the inside face of the square scattering cell to the center of the cell; α is the absorption coefficient of the solution for the primary light, defined by $\alpha = (1/2b) \ln (1/T_a)$, in which T_a is the transmittance of the absorbing solution of depth $2b$ cm.; W is the effective width of scattering volume viewed by the receiver; and h is the width of the primary beam or depth of scattering volume in the direction of transverse viewing. Lauer's equation (1), when $\alpha = k$, and when corrected,[8] leads to the same factors as shown in equation (1) above, derived from the analysis of Putzeys and Dory.

Another factor, neglected by the previously mentioned investigators, should appear in equation (1) to account for the fact that a fraction $0.043e^{-3\alpha b}$ of the primary beam is returned to the center of the scattering cell due to reflection at the emergent

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(2) Presented at the 122nd Meeting of the American Chemical Society, Atlantic City, N. J., September 14-19, 1952.

(3) B. D. Polis and H. W. Shmukler, *J. Biol. Chem.*, in press.

(4) M. L. Anson, J. T. Edsall and K. Bailey, ed., "Advances in Protein Chemistry," Vol. VI, Academic Press, Inc., New York, N. Y., 1951, (Doty and Edsall) pp. 115-116; see also A. S. Kenyon and V. K. LaMer, *J. Colloid Sci.*, **4**, 163(1949).

(5) P. Alexander and K. A. Stacey, *Proc. Roy. Soc.* (*London*), **212A**, 274 (1952).

(6) P. Putzeys and E. Dory, *Ann. soc. sci. Bruxelles*, Ser. I, **60**, 37 (1940). See also J. Tonnelat and H. Batsch, *Compt. rend.*, **231**, 960 (1950).

(7) J. L. Lauer, *J. Optical Soc. Am.*, **41**, 482 (1951).

(8) Equation (1) of reference (7) should contain a factor $\sinh(\alpha t/2)$. This has been confirmed by private correspondence with J. L. Lauer.

glass–air surface of the cell relative refractive index 1.52. Equation (1) can be written

$$I_s = A_1 I_0 f_1 f_2 f_3 \qquad (2)$$

where

$$f_1 = e^{-2\alpha b} \qquad (3)$$

$$f_2 = 1 + \frac{W^2 + h^2}{24}\alpha^2 + \left(\frac{W^2h^2}{576} + \frac{W^4 + h^4}{1920}\right)\alpha^4 + \cdots \qquad (4)$$

$$f_3 = (1 + 0.043\, e^{-3\alpha b})/1.043 = (1 + 0.043\, T_s^{3/2})/1.043 \qquad (5)$$

The factor f_1 represents attenuation of the primary and transversely scattered beams by absorption; f_2 is the product of the two factors in parentheses in equation (1), and represents the effect of finite dimensions of the scattering volume viewed; and f_3 represents the decreasing contribution of the reflected primary beam as absorption increases. Putzeys and Dory[6] have emphasized the importance of accurate centering of the scattering cell in applying f_1, and the advantages of choosing small dimensions for the scattering volume in order to minimize f_2.

In the light scattering apparatus developed in this Laboratory,[9] in which rectangular apertures and a square scattering cell are used, compensation for the largest factor, f_1, is inherent. This is because the phototube, in its 0° position, views the transmitted light rather than the incident light. In the "working standard" method of measurement the irradiance at the phototube in this position is

$$I_t = A_2 I_0 e^{-2\alpha b} = A_2 I_0 T_s = A_2 I_0 f_1 \qquad (6)$$

where A_2 is a constant of proportionality. Dividing equation (2) by equation (6) gives the final result

$$I_s/I_t = (A_1/A_2) f_2 f_3 \qquad (7)$$

The correction factor for absorption is thus $1/f_2f_3$. In Fig. 1 the calculated magnitudes of f_2, f_3 and f_2f_3 are shown as a function of absorption coefficient for our apparatus,[9] in which W = 0.9 cm. (approximately), h = 1.2 cm. and b = 2.0 cm. Equation (7), which affords a means of correcting apparent scattering ratios for absorption, was tested experimentally by adding small increments of absorbing dye solutions to macromolecular scattering solutions (see Experimental section).

Fluorescence

Errors due to the presence of fluorescence in a scattering solution can be minimized by (1) choice of a wave length for the primary light which does not excite the fluorescence; (2) location of the monochromatic filter between the scattering solution and the phototube instead of between the lamp and the solution; and (3) correction of the apparent scattering ratio determined in the usual manner. Method (1) is not always possible since fluorescence may sometimes be excited by both 436 and 546 mμ, the wave lengths commonly available. The use of longer wave lengths involves greatly reduced scattering power. The success of method (2) varies with the system being studied. Method (3) is most satisfactory and will be presented in detail.

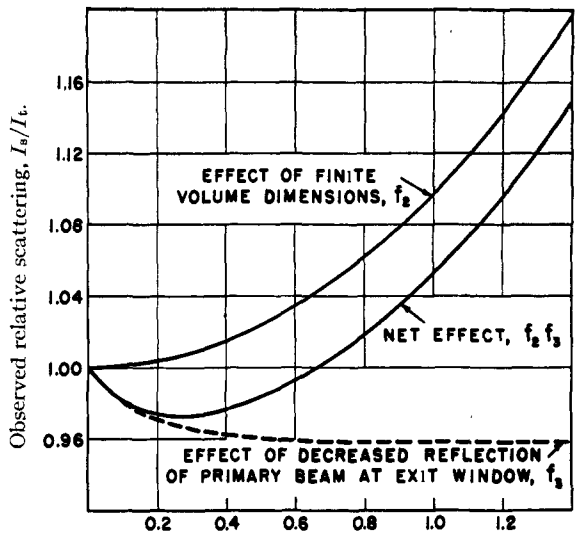

Fig. 1.—Calculated effect of finite scattering volume dimensions (f_2), effect of reflection of primary beam at exit window of 4 cm. square scattering cell (f_3), and the combined effects (f_2f_3) on observed relative scattering as a function of absorption coefficient.

The presence of fluorescence can readily be detected by interposing in the scattered beam a filter having a sharp cut-off to essentially zero transmittance at a wave length slightly longer than that of the primary light. If properly chosen, such "sharp cut-off" filters[10] will have practically zero transmittance for the scattered light and relatively high transmittance for the fluorescent light. In the absence of fluorescence, with such a filter in the 90° scattered beam, an apparent scattering ratio will be obtained which depends on the transmittance of the filter for the primary light. This low transmittance can readily be determined in the photometer. Apparent scattering ratios in excess of this minimum value indicate the presence of fluorescence. In this Laboratory all scattering systems are tested in this manner for fluorescence.

The method presented here for the correction of scattering ratios for fluorescence depends upon the facts that (a) the transversely scattered light is almost completely polarized, (b) the fluorescent light is largely unpolarized, and (c) most of the fluorescent light is of longer wave lengths than the scattered light.

Assume that the scattering cell contains a solution which exhibits both scattering and fluorescence. In the following equations subscripts s, f and t refer to scattering, fluorescence and transmission, respectively; and superscripts V and H refer to vertical and horizontal orientations of the electric vector determined by the position of the analyzer in front of the phototube.[9] With the analyzer vertical, the apparent transverse scattering ratio R^V observed is

$$R^V = (I_s^V + I_f^V)/I_t^V = I_s^V/I_t^V + I_f^V/I_t^V \qquad (8)$$

(9) B. A. Brice, M. Halwer and R. Speiser, *J. Optical Soc. Am.*, **40**, 768 (1950). (The commercial modification of this instrument is known as the Brice-Phoenix Light Scattering Photometer.)

(10) H. P. Gage, *ibid.*, **35**, 276 (1945). See also "Glass Color Filters," Corning Glass Works, Corning, New York.

With the analyzer horizontal the corresponding ratio is

$$R^H = I_s^H/I_t^H + I_f^H/I_t^H \quad (9)$$

But $I_s^H = \rho_s I_s^V$, $I_f^H = \rho_f I_f^V$, and $I_t^H = I_t^V$, where ρ_s and ρ_f are depolarization factors for unpolarized primary light. Substituting in (9)

$$R^H = \rho_s I_s^V/I_t^V + \rho_f I_f^V/I_t^V \quad (10)$$

The ratio I_f^V/I_t^V can be eliminated between equations (8) and (10). The resulting expression solved for I_s^V/I_t^V is

$$I_s^V/I_t^V = (R^V - R^H/\rho_f)/(1 - \rho_s/\rho_f) \quad (11)$$

The turbidity of the solution is proportional to I_s^V/I_t^V. This equation was tested experimentally by adding small increments of fluorescent solutions to macromolecular scattering solutions (see Experimental section).

By using equations (11) and (8), the transverse fluorescence ratio I_f^V/I_t^V can be calculated if desired

$$I_f^V/I_t^V = R^V - I_s^V/I_t^V \quad (12)$$

So far we have considered only 90° scattering, subscripts 90 having been implied in all numerators of equations (8)–(12). At any angle of observation, θ, the observed apparent scattering ratio $(R^V)_\theta$ can be corrected for fluorescence, since

$$(I_f^V)_\theta/I_t^V = (1/\sin\theta)(I_f^V)_{90}/I_t^V \quad (13)$$

The factor $1/\sin\theta$ arises from the fact that the scattering volume viewed increases as θ departs from 90°. Hence

$$(I_s^V)_\theta/I_t^V = (R^V)_\theta - (1/\sin\theta)(I_f^V)_{90}/I_t^V \quad (14)$$

If dissymmetry of scattering is present, the dissymmetry ratio (for 45° and 135°) corrected for fluorescence is

$$D = (I_s^V)_{45}/(I_s^V)_{135} = \frac{(R^V)_{45} - 1.414\,(I_f^V)_{90}/I_t^V}{(R^V)_{135} - 1.414\,(I_f^V)_{90}/I_t^V} \quad (15)$$

This equation was tested experimentally by adding small increments of dye solutions to a macromolecular solution having substantial dissymmetry of scattering.

Experimental

Test of Correction for Absorption.—Dyes were selected which had minimum fluorescence, high absorption near 436 mμ and low absorption near 546 mμ, or *vice versa*, and only minor selectivity of absorption in the range of wave lengths transmitted by the monochromatic filters[9] of the light scattering photometer. Solutions of cornstarch in water and polystyrene in toluene were satisfactory as macromolecular scattering solutions. They were stable with respect to scattering for several hours; moreover, no evidence was found for interaction between the dyes and the macromolecules since the scattering at the non-absorbing wave length was not influenced by addition of dye.

The starch solution was prepared from a slurry of cornstarch in cold water to which boiling water was added with stirring to give a final concentration of about 0.01%. The solution was centrifuged to remove gel, then filtered through a Corning fine-porosity sintered glass filter into a 4 × 4 cm. scattering cell. The turbidity was approximately 0.008 cm.$^{-1}$ (436 mμ). A 1% solution of polystyrene in toluene was prepared and filtered similarly into a scattering cell; the turbidity was 0.007 cm.$^{-1}$ (436 mμ). Scattering ratios were determined by the working standard method[9] before and after the addition of small increments of dye solution from a micropipet. Corrections were made for scattering by the dye and for apparent solvent scattering by measuring the apparent scattering ratios of the appropriate dye solutions and subtracting them from the ratios for the macromolecular solutions containing dye. Also the transmittance, T_s, of each macromolecular solution to which dye had been added, was measured in the photometer relative to that of the dye-free macromolecular solution, using a ratio of deflections with auxiliary filters of known transmittance to avoid small galvanometer deflections.[9]

Results of testing equation (7) are shown in Table I. It is apparent from these data and from Fig. 1 that the uncompensated combined effects, $f_s f_3$, due to absorption are relatively small unless the absorption coefficient is greater than 1 cm.$^{-1}$. However, the small initial drop and finally the gradual rise in apparent relative scattering as α increases, is completely accounted for, within experimental error, by the calculated effects of equation (7). The corrected relative scattering ratios agree within 2.5%. Similar results were obtained with other dyes (not listed in Table I), with dyes added to casein solutions, and with 3 × 3 cm. scatter-

TABLE I

RELATIVE SCATTERING RATIOS, I_s/I_t, OBSERVED AND CORRECTED FOR LIGHT ABSORPTION, IN SCATTERING SOLUTIONS CONTAINING ADDED DYES

I, Calcofast Wool Orange 4RN in cornstarch solution, 436 mμ; II, Orange II (C.I. 151) in cornstarch solution, 436 mμ; III, Acid Violet (C.I. 698) in cornstarch solution, 546 mμ; IV, Sudan I (C.I. 24) in polystyrene solution, 436 mμ.

T_s[a]	α, cm.$^{-1}$[b]	$f_s f_3$[c]	I_s/I_t Obsd.	I_s/I_t Cor.	Error, %
		System I			
1.000	0.000	1.000	1.000	1.000	...
0.844	.043	0.991	0.986	0.997	−0.3
.630	.116	.980	.972	.992	− .8
.465	.191	.975	.972	.997	− .3
.217	.382	.976	.984	1.008	.8
.0984	.582	.991	.980	0.989	−1.1
.0459	.769	1.014	1.000	.986	−1.4
.0114	1.117	1.077	1.070	.994	−0.6
		System II			
1.000	0.000	1.000	1.000	1.000	...
0.713	0.085	0.984	0.976	0.992	−0.8
.508	.169	.976	.972	.996	−0.4
.372	.247	.973	.955	.981	−1.9
.265	.332	.974	.961	.987	−1.3
.144	.484	.982	.956	.974	−2.6
.0428	.787	1.016	1.002	.986	−1.4
.0140	1.066	1.066	1.053	.988	−1.2
		System III			
1.000	0.000	1.000	1.000	1.000	...
0.749	0.073	0.986	0.986	1.000	0.0
.579	.137	.979	.955	.976	−2.4
.362	.255	.973	.950	.976	−2.4
.216	.383	.976	.950	.973	−2.7
.126	.518	.985	.960	.975	−2.5
.0477	.760	1.012	.992	.981	−1.9
		System IV			
1.000	0.000	1.000	1.000	1.000	...
0.728	.079	0.985	0.982	0.997	−0.3
.466	.191	.975	.956	.981	−1.9
.296	.304	.974	.956	.982	−1.8
.185	.421	.978	.992	1.014	1.4
.121	.528	.986	.972	0.986	−1.4
.0723	.656	.999	1.008	1.009	0.9
.0494	.752	1.011	1.030	1.019	1.9
.0143	1.061	1.065	1.088	1.022	2.2

[a] Relative transmittance in 4-cm. scattering cell. [b] Absorption coefficient, $(1/4)\ln(1/T_s)$. [c] Absorption effects; see equations (4), (5), (7) and Fig. 1.

TABLE II

OBSERVED APPARENT SCATTERING RATIOS R^V AND R^H, AND TRUE SCATTERING RATIOS I_s^V/I_t^V CORRECTED FOR FLUORESCENCE, FOR SCATTERING SOLUTIONS CONTAINING FLUORESCENT SUBSTANCES

Fluor., %[a] of total	R^V[b]	R^H[b]	ρ_f	I_s^V/I_t^V[c]	Error, %	Fluor., %[a] of total	R^V[b]	R^H[b]	ρ_f	I_s^V/I_t^V[c]	Error, %
Fluorescein[d] (λ 436 mμ)						Neutral acriflavine[d] (λ 436 mμ)					
0	0.249	0.0050	...	0.249	...	0	0.252	0.0050	...	0.252	...
9.4	.277	.0355	0.969	.246	−1.2	18.6	.310	.0408	0.658	.255	1.2
19.7	.311	.0678	.964	.246	−1.2	28.2	.351	.0694	.671	.255	1.2
34.6	.381	.1392	.966	.242	−2.8	40.5	.424	.1228	.695	.254	0.8
46.6	.465	.214	.972	.250	0.4	55.1	.562	.228	.719	.252	0.0
72.0	.878	.620	.972	.246	−1.2	63.4	.688	.326	.748	.258	2.4
Rhodamine[d] (λ 546 mμ)						Eosin[d] (λ 546 mμ)					
0	0.0446	0.00089	...	0.0446	...	0	0.0447	0.00089	...	0.0447	...
10.0	.0496	.00512	0.752	.0440	−1.3	10.0	.0496	.00517	0.758	.0439	−1.8
21.7	.0569	.01094	.800	.0443	−0.7	25.6	.0602	.0159	.875	.0430	−3.8
39.2	.0733	.0251	.828	.0441	−1.1	38.5	.0734	.0279	.896	.0432	−3.4
58.8	.1105	.0777	.845	.0459	2.9	59.6	.1118	.0635	.916	.0435	−2.7
80.5	.228	.160	.866	.0438	−1.8	67.3	.138	.0880	.921	.0436	−2.5
Motor oil[e] (λ 436 mμ)						Hydrolyzed casein[f] (λ 436 mμ)					
0	0.130	0.0026	...	0.130	...	0	0.0736	0.0015	..	0.0736	...
11.3	.147	.0209	0.942	.127	−2.3	16.4	.0857	.0133	0.896	.0724	−1.6
22.1	.167	.0383	.949	.130	0.0	26.3	.0998	.0258	.902	.0728	−0.7
42.3	.225	.0915	.945	.131	0.8	49.1	.145	.0636	.905	.0747[g]	1.5
64.0	.360	.224	.950	.127	−2.3	69.5	.224	.128	.901	.0755[g]	2.6

[a] Per cent. of total phototube response due to fluorescence. [b] Observed apparent scattering ratio with analyzer vertical, FG^V/G_w^V, or horizontal, FG_s^H/G_w^H. (F = transmittance of neutral filter). See reference 9. [c] Calculated from equation (11). [d] Dye added to Ludox solution. [e] Added to solution of polystyrene in toluene. [f] Added to solution of casein in 0.1 M K_2HPO_4. [g] Corrected for a small contribution of the hydrolyzed casein to the turbidity of the solution, determined by measurements at 546 mμ.

ing cells. Retention of the fourth power terms of equation (4) is necessary with our apparatus only if α is 1 cm.$^{-1}$ or greater. It is obvious that the dimensions W and h need not be known with high accuracy.

Larger deviations than those shown in Table I were found for values of α from 1 to 1.5 cm.$^{-1}$ with dyes having sharp absorption maxima between 432 and 403 mμ. The low relative scattering ratios observed with these highly selective dyes (for example, with Xylene Yellow, C.I. 639, I_s/I_t corrected was 0.884 for α = 1.0 cm.$^{-1}$) are readily explained on the basis of substantial alteration, by the dye, of the spectral distribution of the primary light originating from the Type AH-3 mercury lamp[11] and transmitted by the monochromatic filters.[9] With such dyes, for high values of α, a large part of the scattered light would arise from continuous radiation of wave lengths longer than 436 mμ, which would have reduced scattering power. This complication was avoided in obtaining the data of Table I by use of dyes having only minor selectivity of absorption in the range of wave lengths transmitted by the monochromatic filters, a characteristic more likely to be encountered in practice.

Larger errors than those corrected for in Table I can be made by improper centering of the scattering cell. The attenuation factor $f_1 = e^{-2\alpha b}$ of equations (1) and (3) is eliminated in equation (7) only if the centering is accurate. Centering errors will not affect equation (6). If an error $\pm\Delta$ is made in lateral or longitudinal centering of the scattering cell, the factor f_1 becomes $e^{-2\alpha b}\ e^{\mp\alpha\Delta}$, and the uncompensated factor $e^{\mp\Delta}$ gives rise to an error in the scattering ratio of approximately $\pm 10\%$ for $\Delta = \pm$ 0.1 cm. If an error of this size is made in both lateral and longitudinal centering, the maximum error in the scattering ratio will be doubled.

Test of Correction for Fluorescence.—The scattering solution selected for most of the tests was a 0.07% solution of Ludox (du Pont colloidal silica) in water, passed through a fine sintered glass filter into a 3 × 3 cm. scattering cell. The turbidity was approximately 0.01 cm.$^{-1}$ for 436 mμ and 0.004 cm.$^{-1}$ for 546 mμ; the depolarization ρ_s was 0.02 for both wave lengths. With the analyzer in place, scattering ratios R^V and R^H were determined by the working standard method, before and after adding very small increments of water solutions of fluorescent dyes. All ratios were corrected for apparent solvent scattering. The depolarization for the fluorescent light, ρ_f, was determined by a ratio of deflections for horizontal and vertical orientations of the analyzer, with a sharp cut-off filter mounted at the entrance aperture of the receiver between the scattering cell and the phototube. These filters were Corning No. 3384 (C.S. 3-70), having an apparent transmittance of 0.0030% for the blue primary light, and Corning No. 2424 (C.S. 2-63) having an apparent transmittance of 0.025% for the green primary light.

A non-aqueous system was included in the tests. A 1% solution of polystyrene in toluene was filtered through a fine sintered glass filter into the scattering cell. The turbidity was 0.007 cm.$^{-1}$ for 436 mμ; the depolarization ρ_s was 0.02. Small increments of a 10% solution of motor oil in toluene, filtered through an ultrafine sintered glass filter, were added to the macromolecular solution to impart fluorescence. Readings were made as for the aqueous systems.

A system approximating one likely to be encountered in practice was studied by adding a solution of acid-hydrolyzed casein to one of casein. The acid-hydrolyzed protein solution is excited to fluorescence by both 436 and 546 mμ, although much more strongly by the former wave length. The hydrolyzed solution was prepared by refluxing 1 g. of casein with 50 ml. of 6 M hydrochloric acid for 16 hours, evaporating to dryness, heating several hours at 110° to remove residual acid, dissolving in 0.1 M K_2HPO_4, raising the pH to 9 by addition of ammonia, diluting to a concentration of approximately 10%, and clarifying by passing the solution through an ultrafine sintered glass filter. Small increments of this solution were added to a 0.05% solution of casein in 0.1 M K_2HPO_4, which had been clarified by ultrafine filtration. The initial turbidity for 436 mμ was about 0.003 cm.$^{-1}$ and the depolarization ρ_s was 0.02. The quantities R^V, R^H and ρ_f were determined as described above.

Results are shown in Table II, illustrating the correction of observed apparent scattering ratios for the presence of fluorescence in accordance with equation (11). In general the corrected scattering ratios agree with the initial scattering ratio within 3%. The errors are further reduced, par-

(11) B. T. Barnes and W. E. Forsythe, *J. Optical Soc. Am.*, **27**, 83 (1937).

ticularly for the data involving 546 mμ excitation, if the values obtained for ρ_f are corrected for the very small component of scattered light transmitted by the sharp cut-off filter. This can be done by determining an apparent ρ_f value on a solution having roughly the same turbidity as the one under examination, but free from fluorescence.

Test of Correction of Dissymmetry Ratios for Fluorescence.—A 0.01% solution of cornstarch in water was filtered through a fine sintered glass filter into the 45°/135° dissymmetry cell. The following quantities were measured before and after adding increments of a solution of fluorescein: $(R^V)_{90}$, $(R^H)_{90}$, $(R^V)_{45}$, $(R^V)_{135}$ and ρ_f, using the sharp cut-off filter for the latter quantity, and using blue light (436 mμ) for all measurements. The scattering ratios were corrected for apparent scattering of solvent. Results are presented in Table III, illustrating determination of dissymmetry ratios, corrected for fluorescence, with errors of 2.3% or less. These dissymmetry ratios are subject to a further correction for reflection of the primary beam at the exit window of the cell.[12]

TABLE III

OBSERVED APPARENT SCATTERING RATIOS AT 90°, 45° AND 135°, AND DISSYMMETRY RATIO CORRECTED FOR FLUORESCENCE

Fluor., % of total[a]	$(RV)_{90}$	$(RH)_{90}$	$(RV)_{45}$	$(RV)_{135}$	ρf	Dissymmetry ratio Uncor.[b]	Cor.[c]	Error, %
0	....	....	...	...	...	3.04	3.04	...
14.6	0.2095	0.0336	0.666	0.250	0.976	2.67	3.02	−0.7
25.9	.245	.0660	.672	.284	.987	2.37	3.00	−1.3
42.5	.303	.1286	.750	.365	.973	2.06	3.11	2.3
58.9	.436	.257	.928	.546	.989	1.70	3.09	1.6

[a] Per cent. of total phototube response at 90° due to fluorescence (fluorescein added to cornstarch solution). [b] $(R^V)_{45}/(R^V)_{135}$. [c] Calculated using equations (15), (12) and (11).

Effect of Location of Monochromatic Filters on Reducing Fluorescence Errors.—The blue monochromatic filter was removed from the lamp housing and mounted between the scattering cell and the phototube. Transverse scattering ratios, without use of the analyzer, were determined for a Ludox solution before and after addition of fluorescein. Relative scattering ratios were constant within 3% up to a point where the phototube response due to fluorescence was 72% of the total response, indicating that fluorescence errors were successfully eliminated by this procedure.

The green monochromatic filter was then similarly located, and the system Ludox plus eosin tested. When the phototube response due to fluorescence was only 15% of the total response, the apparent scattering ratio was 1.64 times that of the Ludox solution alone. This factor was reduced to 1.12 when similar green filters were placed in both the incident and scattered beams. Hence, in this case, fluorescence errors were not satisfactorily reduced by relocation of monochromatic filters or by auxiliary filters.

Discussion and Conclusions

It is concluded that scattering ratios can be determined with satisfactory accuracy (within 2.5%), in scattering solutions exhibiting light absorption, by compensating for the attenuation and by applying simple corrections. This conclusion holds for absorption coefficients up to at least 1.2 cm.$^{-1}$. In the systems tested the maximum attenuation by absorption (1.2 cm.$^{-1}$) was at least 150 times the attenuation by scattering (0.008 cm.$^{-1}$). Accurate centering of the scattering cell is essential. Effects due to absorption will be minimized by use of a small scattering volume and a small scattering cell. The presence of absorption should not significantly affect determination of dissymmetry ratios unless they exceed about 1.2. For higher ratios, when the reflection correction (12) becomes significant, absorption has to be considered.

Correction for the absorption effects f_1, f_2, and f_3 should also to the determination of fluorescence intensities in the presence of light absorption; a fourth factor e^{-kb} will, however, be necessary to represent attenuation of the transverse fluorescence by absorption,[7] where k is an effective absorption coefficient of the solution for the fluorescence radiation.

It is also concluded that scattering ratios and dissymmetry ratios can be determined by correction with satisfactory accuracy (within 3%) in scattering solutions showing solute fluorescence, even when the fluorescence intensity exceeds substantially that due to scattering. Attempts to reduce fluorescence errors by locating monochromatic filters in the scattering beam were not wholly successful.

Scattering ratios corrected for absorption, calculated from equation (7), are proportional to turbidity and can be substituted directly in equation (22) of reference (9) since I_s/I_t is equivalent to I_s/I_w. Scattering ratios corrected for fluorescence, calculated from equation (11), can be substituted directly in the turbidity equation (22) after multiplication by the theoretical factor 1/2; no correction of the turbidity for depolarization is necessary in this case. Simultaneous correction of scattering ratios for both absorption and fluorescence should present no problem since the correction factor $1/f_2f_3$ indicated by equation (7) could just as well be applied to the scattering ratio I_s^V/I_t^V.

The foregoing conclusions apply to absorption by solute or solvent and to fluorescence by solute and solute impurities. As pointed out previously fluorescence by solvent ordinarily presents no problem, as this effect is eliminated by the usual subtraction of measurements on solution and solvent.

The usefulness and accuracy of the light scattering method is appreciably extended by these findings.

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