The Importance of Life-time Changes in Fluorescence Depolarization

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The rotational relaxation time, ρ , calculated from measurements of fluorescence depolarization, is clearly dependent on the assumed mean life-time, τ , of the excited state. However, variations in τ with experimental conditions (temperature and solvent composition) occur and the effect of such alterations in τ is demonstrated. In particular it should be noted that, unless life-time changes can be excluded, the occurrence of linear plots of reciprocal degree of polarization against the temperature/viscosity ratio does not necessarily indicate the absence of intramolecular freedoms. An attempt to correct for such life-time changes by measurement of the fluorescence intensity is made for the bovine serum albumin-1-dimethylaminonaphthalene-5-sulphonyl chloride system. The value of $\rho/3\tau$ thus obtained for this system at 20° is approx. 4.7, as against approx. 3.4 obtained by several workers in the absence of life-time corrections.

THEORETICAL

Introduction. If the fluorescence from a solution of a fluorescent substance is observed at right angles to the exciting unpolarized light, it is generally found to be partially polarized. The degree of polarization, P , is defined as:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (1)$$

where $I_{\parallel}$ is the intensity of the emitted light with electric vectors in the plane of the electric vectors of the exciting light and $I_{\perp}$ is the intensity of the emitted light with electric vectors perpendicular to this plane. Following Weber (1953), who reviewed both the theory and the application of this phenomenon, it is usually assumed that:

$$\frac{1}{P} + \frac{1}{3} = \left(\frac{1}{P_0} + \frac{1}{3} \right) \left[1 + \beta \left(\frac{T}{\eta} \right) \right] \quad (2)$$

where $\beta = (3\tau/\rho)(\eta/T)$, T is the absolute temperature, η is the solvent viscosity, τ is the mean life-time of the excited state, ρ is the harmonic mean of the principal rotational relaxation times of the molecule and P_0 is a constant, being the value of P when ρ is infinite, i.e. when there is no movement of the fluorescent chromophore.

The effect of intramolecular rotations, particularly about the dye-macromolecule bond, has been considered. Generally it is believed that apart from the usual η/T -dependence such rotations are

temperature-activated but not a function of solvent viscosity. Gill & Omenn (1965) demonstrated that this is so for a synthetic polypeptide in a solvent of viscosity up to 9 centipoise. For this situation a plot of $1/P$ versus T/η , which should be linear in the simple case, becomes dependent on the way in which T/η is altered. At constant temperature, the asymptote at high values of T/η is given by:

$$\frac{1}{P} + \frac{1}{3} = \left(\frac{1}{P_A} + \frac{1}{3} \right) \left[1 + \beta \left(\frac{T}{\eta} \right) \right] \quad (3)$$

which is the equation of Wahl & Weber (1967) modified for excitation by natural light, and where $1/P_A$ is the point of intersection of the asymptote with the ordinate.

It is apparent that several assumptions implicit in the present theories of fluorescence depolarization are invalid, at least for the BSA†-dansyl (1-dimethylaminonaphthalene-5-sulphonyl) chloride system, which exhibits heterogeneous emission (Chen, 1968). However, it is not clear what practical effect a more sophisticated theory will have. Meanwhile a further complication, caused by the variation of τ with experimental conditions, is considered here on the basis of eqns. (2) and (3).

Fluorescent life-time. Fluorescence is usually assumed to be the result of an emission from the lowest vibrational level of the singlet excited state to the different vibrational levels of the ground state of the molecule. The natural life-time of the fluorescent state may be calculated from a knowledge of the molecule's spectral properties and of the medium's

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refractive index, to the square of which the life-time is inversely proportional (Strickler & Berg, 1962). Life-times so obtained are those that would be observed if all the excited molecules were to emit. However, the quantum yield, to which the observed life-time is proportional (Perrin, 1926), is frequently decreased by the presence of competitive processes. In this case, provided that no spectral shifts of the fluorescence occur, the observed life-time, τ , is given by:

$$\tau = \left(\frac{I}{I_s} \right) \tau_s \quad (4)$$

where I is the intensity of the fluorescent light and the subscript s refers to standard defined conditions. If the molecular states involved in the radiative transition do alter, then eqn. (4) on its own is not sufficient, and some doubts have been expressed about the general reliability of the equations by which the life-time may be calculated (Ware & Baldwin, 1964). However, in many cases an approximate correction for fluorescence-intensity changes can be made.

It is known that the absorption and emission spectra of some dyes change markedly with the environment (Laurence, 1952). The fluorescence of dansyl chloride and fluorescein isothiocyanate derivatives have been studied as a function of temperature and solvent by Omenn & Gill (1966). The fluorescence intensity of a synthetic polypeptide-dansyl chloride conjugate decreased by about 20% as the temperature was raised (0–50°). In the presence of 50% (w/w) glycerol the intensity was higher and decreased by about 40% over the same temperature range. The fluorescein derivative showed much smaller intensity changes.

Correction to the apparent relaxation time. If the life-time changes with varying environmental conditions, the ordinary plot of $1/P$ versus T/η is of little value. A method is required of adjusting the observed values of $1/P$ to those values, $1/P_c$, that would be observed if the life-time, τ_s were constant at the value it possesses in some standard state, s .

If it is assumed that ρ and P_0 are unaffected by those conditions that cause τ_s to become τ , then from eqn. (2) we obtain:

$$\rho = 3\tau_s \left(\frac{\frac{1}{P_0} + \frac{1}{3}}{\frac{1}{P_c} - \frac{1}{P_0}} \right) = 3\tau \left(\frac{\frac{1}{P_0} + \frac{1}{3}}{\frac{1}{P} - \frac{1}{P_0}} \right) \quad (5)$$

and therefore:

$$\frac{1}{P_c} - \frac{1}{P_0} = \frac{\tau_s}{\tau} \left(\frac{1}{P} - \frac{1}{P_0} \right) \quad (6)$$

Hence the degree of polarization should be corrected by parallel measurements of τ_s/τ , and such corrected plots of $1/P_c$ versus T/η may now be interpreted in terms either of the simple theory or of the various possibilities of intramolecular and intermolecular interactions. Wahl (1966a) recognized that a correction for life-time changes could be applied, but his method destroyed the simple relation of the abscissa to the frictional forces of the solvent, a mode of presentation that possesses certain advantages. In a subsequent paper (Wahl & Weber, 1967) no mention was made of the possible effects of life-time changes.

Two particular experimental situations are now considered in which T/η is varied (1) by changing T at constant solvent composition and (2) by changing the solvent composition at constant temperature. Consider case (1) in which, further, the plot of $1/P$ versus T/η gives a straight line of gradient S , extrapolating to $1/P_0$ at $T/\eta = 0$. Let:

$$\frac{\tau_s}{\tau} = a + b \left(\frac{T}{\eta} \right) + c \left(\frac{T}{\eta} \right)^2 + \dots \quad (7)$$

where a , b and c are constants. Substituting this expression into eqn. (6) and differentiating with respect to T/η gives the slope of the plot of $1/P_c$ versus T/η , i.e.:

$$\frac{\partial \left(\frac{1}{P_c} \right)}{\partial \left(\frac{T}{\eta} \right)} = aS + 2bS \left(\frac{T}{\eta} \right) + \dots \quad (8)$$

Thus for a straight-line $1/P$ versus T/η plot, it follows from eqn. (8) that the plot of $1/P_c$ versus T/η will be curved unless b and higher coefficients are zero. If polydispersity and pronounced molecular asymmetry can be excluded, then such a curve in which b is positive indicates the development of new freedoms (internal flexibility or dissociation) with increasing temperature. However, a particular curved plot of $1/P$ versus T/η may yield a straight line for $1/P_c$ versus T/η , extrapolating to $1/P_0$ and this would be attributed to a molecule free from intramolecular rotation. More generally, however, a curved plot of $1/P$ versus T/η would be expected to yield a plot of $1/P_c$ versus T/η of modified curvature.

In general, it may be assumed that intramolecular rotations are greatly restricted as $T \rightarrow 0$ so that the tangent at $T = 0$ on the plot of $1/P_c$ versus T/η gives the relaxation time of the macromolecule alone. For the case described by eqn. (8), the gradient at $T/\eta = 0$ is aS , to be compared with the value S obtained for a plot of $1/P$ versus T/η .

Hence the relaxation time of the macromolecule is given by [cf. eqn. (5)]:

$$\frac{\rho}{3\tau_s} = \frac{\frac{1}{P_0} + \frac{1}{3}}{aS\left(\frac{T}{\eta}\right)} = \left(\frac{1}{a}\right)\left(\frac{\rho}{3\tau}\right) \quad (9)$$

Thus the value of $\rho/3\tau$ obtained from a straight-line plot of $1/P$ versus T/η should be divided by the value of τ_s/τ at $T/\eta = 0$ in order to obtain the true relaxation time of the macromolecule, corrected for changes in the life-time.

If the experimental results are obtained at constant temperature by varying the solvent composition (case 2) through the addition of, for example, sucrose, the appropriate analysis, assuming invariant life-time, is given by Wahl & Weber (1967) [see eqn. (3)]. Let the observed asymptote to the plot of $1/P$ versus T/η have a gradient M , and pass through $1/P_A$ at $T/\eta = 0$. Correspondingly, let the asymptote to the plot of $1/P_c$ versus T/η of gradient, M_c , pass through $1/P_{Ac}$ at $T/\eta = 0$. Choosing the standard conditions, denoted by subscript s , to be the solution in the absence of sucrose at the particular temperature, let the fluorescent life-time be given by:

$$\frac{\tau_s}{\tau} = 1 + m \left[1 - \frac{T(\eta)}{\eta(T)} \right] + n \left[1 - \frac{T(\eta)}{\eta(T)} \right]^2 + \dots \quad (10)$$

where m and n are constants. The relaxation time of the macromolecule in the standard conditions is given by:

$$\frac{\rho}{3\tau_s} = \frac{\frac{1}{P_{Ac}} + \frac{1}{3}}{\frac{1}{P_c} - \frac{1}{P_{Ac}}} = \frac{\frac{1}{P_c} + \frac{1}{3}}{M_c\left(\frac{T}{\eta}\right)_s} - 1 \quad (11)$$

Differentiating eqn. (6) gives:

$$\frac{\partial\left(\frac{1}{P_0}\right)}{\partial\left(\frac{T}{\eta}\right)} = \left(\frac{1}{P} - \frac{1}{P_0}\right) \frac{\partial\left(\frac{\tau_s}{\tau}\right)}{\partial\left(\frac{T}{\eta}\right)} + \frac{\tau_s}{\tau} \cdot \frac{\partial\left(\frac{1}{P}\right)}{\partial\left(\frac{T}{\eta}\right)}$$

which at $(T/\eta)_s$ (which occurs in the region of the asymptote) is the value of M_c :

$$M_c = \left(\frac{\partial\left(\frac{1}{P_c}\right)}{\partial\left(\frac{T}{\eta}\right)} \right)_s = -\left(\frac{1}{P} - \frac{1}{P_0}\right)_m \left(\frac{\eta}{T}\right)_s + M \quad (12)$$

In differentiating eqn. (10), terms on the right-hand side higher than the second have been neg-

lected. Finally, introducing eqn. (12) into eqn. (11), remembering that at $(T/\eta)_s$, $P = P_c$, we obtain:

$$\frac{\rho}{3\tau_s} = \frac{\frac{1}{P_A} + \frac{1}{3} + \left(\frac{1}{P} - \frac{1}{P_0}\right)_m}{\frac{1}{P} - \frac{1}{P_A} - \left(\frac{1}{P} - \frac{1}{P_0}\right)_m} \quad (13)$$

Thus to obtain the true relaxation time from experiments performed at constant temperature, a knowledge of the effect of solvent changes on the fluorescent life-time (i.e. of coefficient m) is needed.

As noted above, when direct determination of the life-time is impracticable, a useful indication of the ratio τ_s/τ may frequently be obtained from measurements of the fluorescence intensity (cf. eqn. 4) and of the refractive index when changes are involved.

EXPERIMENTAL

Materials

Bovine serum albumin was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, and dansyl chloride from British Drug Houses Ltd., Poole, Dorset. Sephadex G-25 (fine grade) was supplied by Pharmacia (G.B.) Ltd., London W.13. Other reagents were of analytical quality, and triple-distilled water was employed for the preparation of all aqueous solutions.

Methods

A conjugate of bovine serum albumin with dansyl chloride was prepared as described by Weber (1952). Free dye was removed from the reaction mixture by passing it down a column of Sephadex G-25 previously equilibrated with 0.05M-sodium phosphate buffer, pH 7.3. In the experiments described below the degree of labelling was about 12 moles of dye/mole of protein on the basis of $E_{1\%}^{1\text{cm}}$, 171 at 330 nm. given for dansyl chloride by Weber (1952).

The degree of polarization of the fluorescence from the conjugate was determined with substantially the apparatus of Johnson & Richards (1962) with natural exciting light. However, the mirrors (M_1 and M_2 in their Fig. 1) enclosing the cell were withdrawn since it was found that they introduced small but significant effects on the observed depolarization. Generally, five determinations of $1/P$ were made at each temperature, giving a standard deviation of 0.04 at a value of $1/P$ about 5. The temperature of the cell contents was measured to $\pm 0.1^\circ$ with a thermistor placed directly in the cell, whose resistance was measured in a Wheatstone Bridge circuit (Johnson, 1957). Calibration was performed against a National Physical Laboratory standard thermometer.

The fluorescence intensity relative to that of a dilute sealed-up solution of fluorescein was obtained from the measurements used to determine the degree of polarization by the equation:

$$I_{90} = \sqrt{I_1^2 + I_2^2} \quad (14)$$

I_{90} was corrected to the intensity, I , which would be

observed if the emitted light were completely depolarised (Singleterry & Weinberger, 1951):

$$I = I_{90} \left(1 + \frac{P}{3} \right) \quad (15)$$

Account was also taken of the change of volume of the solution, and hence of the concentration of the fluorescent species, as sucrose or glycerol was added.

The concentration of glycerol was determined by measurement of the specific gravity of the solution with a small glass weight, and that of sucrose by measurement of the refractive index of the solution with an Abbé refractometer, by using in each case the tabulated data in the Handbook of Chemistry and Physics (Hodgman, 1952). In both cases a simple correction was applied to allow for the presence of dissolved protein and buffer salts.

RESULTS

The results of two separate experiments are plotted as $1/P$ versus T/η in Fig. 1 for the preparation BSA-dansyl chloride in the absence of sucrose or glycerol. The line gives the value $\rho_{20}^0/3\tau = 3.37 \pm 0.03$ when the extrapolated value for $1/P_0$ is used, where ρ_t^0 indicates the relaxation time in water at t° . If the degree of polarization observed from a solution containing 60% (w/w) sucrose at 0° is taken as the P_0 value, the calculated value of $\rho_{20}^0/3\tau$ becomes 4.04. Fig. 2 shows that there was a considerable change in the fluorescence intensity with temperature. Visually, there was little if any change in the colour of the fluorescence; hence it is probable that there was a notable change in the quantum yield with temperature. If the value of I_{20}/I at $T/\eta = 0$ is taken as 0.66, the corrected values of $\rho_{20}^0/3\tau$ become 5.1 and 6.1 respectively according to eqn. (9). A plot of $1/P_c$ versus T/η , given in Fig. 3, may be obtained by combining the information given in Figs. 1 and 2 in accordance with eqns. (4) and (6). The plot is no longer a

straight line extrapolating through the $1/P_0$ value. Provided that the Perrin relation as expressed by eqn. (4) holds, the curved plot of $1/P_c$ versus T/η must indicate the development of an intramolecular rotation as the temperature was raised. The dotted line drawn in Fig. 3, a possible tangent to the curve at $T/\eta = 0$, corresponds to a value of $\rho_{20}^0/3\tau = 4.70$ (see below). To obtain this value by the application of eqn. (9) to the value $\rho_{20}^0/3\tau = 3.37$, I_{20}/I must equal 0.72 at $T/\eta = 0$. A lower value of I_{20}/I at $T/\eta = 0$ implies that the tangent in Fig. 3 should be of smaller slope.

If either sucrose or glycerol was added to the system and the temperature treated as the variable as previously, there tended to be a slight increase in the $1/P_0$ value and a more marked increase in the slope of the plot of $1/P$ versus T/η (Fig. 4). Typical results, obtained by least-squares analysis on the assumption that the experimental points fell

Fig. 2. Plot of fluorescence intensity at 20° divided by the observed intensity, (I_{20}/I) versus T/η for BSA-dansyl chloride in 0.05M-phosphate buffer, pH 7.3. Differently marked points were obtained on different occasions by changing the temperature (constant solvent composition).

Fig. 1. Plot of $1/P$ versus T/η for BSA-dansyl chloride in 0.05M-phosphate buffer, pH 7.3. Differently marked points were obtained on different occasions by changing the temperature (constant solvent composition).

Fig. 3. Plot of $1/P_c$ versus T/η for BSA-dansyl chloride in 0.05M-phosphate buffer, pH 7.3. Differently marked points were obtained on different occasions by changing the temperature (constant solvent composition). ----, Theoretical line for $\rho_{20}^0/3\tau = 4.70$.

on a straight line, are summarized in Table 1. The standard deviation of the slope was approx. 2% of its value and the standard deviation of $1/P_0$ was about 0.02 at the lower concentrations of sucrose or glycerol. With this information it was possible to derive a plot of $1/P$ versus T/η at constant temperature (isotherms), as shown in Fig. 5. At low concentrations of sucrose or glycerol it seems that this plot is represented by a straight line. There was no marked deviation from the line even at high sucrose concentrations (up to 60%, w/w) and the parameters of three such lines are given in Table 2. However, above 40% (w/w) glycerol, i.e. $T/\eta < 10^4$ at 30°, there was a pronounced deviation from the straight line. This may be accounted for by changes in the quantum yield of the fluorescence and in the refractive index of the medium. On applying eqn. (6), with $\tau_s/\tau = (\mu/\mu_s)^2 I_s/DI$ essentially straight lines (Fig. 6) were obtained from the plot of $1/P_c$ versus T/η (I is calculated from the same measurements of $I_{\parallel}$ and $I_{\perp}$ as P is for the solution of refractive index, μ ; D is the volume dilution factor caused by the addition of sucrose or glycerol; and the subscript s refers to the standard state of phosphate buffer solution at 20°). By using the extrapolated value of $1/P_c$ at $T/\eta = 0$ for each line, the equivalent $\rho_{20}^0/3\tau$ values for the molecule were found to be 4.71, 4.59 and 4.78 from the 20°,

30° and 40° lines respectively. These values are to be compared with a value near 5.0 (Table 2), which was obtained without making any correction for fluorescence-intensity changes, and which was also obtained in the absence of sucrose or glycerol when account was taken of life-time changes. The magnitude of these changes is given by a value of m (eqn. 10) of the order of -0.055 at 20°, decreasing to -0.026 at 40°, for both additives at low concentrations. At higher viscosities (i.e. higher concentrations of additive) the fluorescence intensity was much more enhanced by glycerol than by sucrose at the same temperature. Assuming that the rotational relaxation time of the macromolecule at 20° was 150nsec. (Wahl, 1966b; Moser, Squire & O'Konski, 1966) and taking the soundest value of $\rho_{20}^0/3\tau$ as 4.7, the effective fluorescent life-time of the BSA-dansyl chloride conjugate at 20° in 0.05M-phosphate buffer solution becomes 10.6nsec.

DISCUSSION

Numerical interpretation of fluorescence-depolarization observations depends on a knowledge of the mean life-time of the excited state of the fluorescent chromophore over the whole range of experimental conditions. Several direct determinations (Steiner

Fig. 4. Plot of $1/P$ versus T/η for BSA-dansyl chloride in 0.05M-phosphate buffer, pH 7.3. Each line, at the constant glycerol concentration (w/w) indicated by percentages on the figure, was obtained by varying the temperature.

Fig. 5. Plot of $1/P$ versus T/η for BSA-dansyl chloride in 0.05M-phosphate buffer, pH 7.3, with added sucrose ($\blacktriangle$ and $\blacktriangledown$) or glycerol ($\triangle$, $\diamond$ and ∇); each line shows results obtained at constant temperature.

Table 1. Effect of additives on the plot of $1/P$ versus T/η obtained by varying the temperature for BSA-dansyl chloride

Glycerol (% , w/w)	...	0	7	22	40	50.8	66.5	
$1/P_0$		3.79	3.70	3.87	3.88	3.95	4.04	
$10^4 \times \text{Slope (poise/}^\circ\text{K)}$		0.421	0.472	0.460	0.613	0.763	1.672	
Sucrose (% , w/w)	...	0	14.5	29.4	40.6	50.2	59.6	63.2
$1/P_0$		3.79	3.90	3.87	3.96	3.95	3.98	3.97
$10^4 \times \text{Slope (poise/}^\circ\text{K)}$		0.421	0.492	0.635	0.894	1.470	3.33	3.96

Table 2. Parameters for $1/P$ versus T/η isotherms for BSA-dansyl chloride system in the presence of sucrose

Temp. (t) ($^{\circ}\text{C}$)	...	...	20	30	40
$1/P_A$			4.12	4.19	4.32
$10^4 \times \text{Slope (poise}/^{\circ}\text{K})$			0.298	0.318	0.320
$\rho_{20}^0/3\tau$			5.14	3.77	3.05
$\rho_{20}^0/3\tau$			5.14	4.88	4.98

Fig. 6. Plot of $1/P_c$ versus T/η for BSA-dansyl chloride in 0.05M-phosphate buffer, pH 7.3, with added sucrose (▲ and ▼) or glycerol (Δ, ○ and ∇); each line shows results obtained at constant temperature.

& McAlister, 1957; Frey & Weill, 1967) indicate that the fluorescent life-time of the dye dansyl chloride is about 12nsec. However, although Frey & Wahl (1966) found that the effective life-time for BSA-dansyl chloride was a function of the number of dye molecules, N , per protein molecule, varying from 22.2nsec. when $N=1.2$ to 15nsec. when $N=8.4$; Steiner & McAlister (1957) reported $\tau=11.5$ nsec. with $N=2.7$. As yet the source of the discrepancy is not known, although the work of Chen (1968) supports the higher values obtained by Frey & Wahl (1966). Wahl & Frey (1966) demonstrated that the variation in τ with N was matched by a variation in the plot of $1/P$ versus T/η obtained by altering the temperature for the differently labelled BSA samples, and that an almost constant value of ρ_{20}^0 of 150nsec. was obtained when the τ value appropriate for the particular preparation was used. Since, as indicated above, τ seems to change appreciably also with solvent and temperature, it is surprising that this ρ_{20}^0 value is in such close agreement with that obtained by another method (Moser *et al.* 1966).

Weber (1952) reported that the value of $\rho_{20}^0/3\tau$ for the BSA-dansyl chloride system ($N=2.3$) was virtually unaltered by the presence of 20% (w/v) sucrose. However, Wahl & Frey (1966), studying the same system over a range of N values down to 1.24, observed that sucrose affected the depolariza-

tion properties as though it were promoting intramolecular freedom. They found that τ decreased on adding sucrose. Similarly Wahl (1966a), studying lysozyme labelled with dansyl chloride, found a 14% decrease in τ as the sucrose concentration was raised from 0 to 29%. Some decrease is also to be expected owing to the increase in the refractive index of the medium (Birks & Dyson, 1963), but such a change in τ would be expected to lower the $1/P$ value. However, at the same T/η value, the value of $1/P$ observed in the presence of sucrose or glycerol is generally greater than the value obtained in the buffer solution only (see, e.g., Fig. 4). This is consistent with the observation reported above and by Gill, McLaughlin & Omenn (1967) that sucrose and glycerol increase the fluorescence intensity and hence, presumably, the mean fluorescence life-time.

If life-time changes are ignored, it is apparent that constant-temperature (varying composition) plots of $1/P$ versus T/η are linear and yield a value for $\rho_{20}^0/3\tau$ (≈ 5.0) that does not vary significantly between 20° and 40°. On the other hand, constant-composition plots of $1/P$ versus T/η (varying temperature), though linear, yield a substantially lower value of $\rho_{20}^0/3\tau$ (≈ 3.4). Some modification of the protein by sucrose cannot be completely ruled out, but unless life-time changes in the presence of sucrose or glycerol are unexpectedly large, it seems that the presence of a temperature-activated rotation is required to explain the lowered value obtained in the latter case. Wahl & Weber (1967) and Weltman & Edelman (1967) drew a similar conclusion for γ -globulin-dansyl chloride conjugates.

Weltman & Edelman (1967) gave detailed experimental results of an investigation by fluorescence depolarization with plane-polarized exciting light of human γ G-immunoglobulin conjugated with dansyl chloride. On changing the temperature, plots of $1/P$ versus T/η gave apparently straight lines whose slope increased as the sucrose concentration was raised. They also analysed the results obtained at a series of constant temperatures by the method of Wahl & Weber (1967), finding that as the temperature was raised the apparent rotating volume (as indicated by ρ) increased. Recognizing that the intensity and hence life-time of the fluores-

Fig. 7. Plot of I_s/I versus T/η for human γ G-immunoglobulin-dansyl chloride at constant solvent composition. Data from Weltman & Edelman (1967).

Fig. 8. Plot of $1/P_c$ versus T/η for human γ G-immunoglobulin-dansyl chloride at constant solvent composition. ---, Theoretical line for $\rho = 220$ nsec. at 25° . Data from Weltman & Edelman (1967).

cence was changing, Weltman & Edelman (1967) multiplied the values of ρ calculated from sucrose isotherms by the intensity ratio, I/I_{25} , determined in the absence of sucrose, and thus they apparently eliminated the trend in the observed volume with increasing temperature. However, it is possible, as suggested here, to correct individual values of $1/P$ to $1/P_c$. For this purpose the experimental values of I_s/I , where the standard condition is water at 25° , have been obtained from Table 3 of Weltman & Edelman (1967), and are plotted here in Fig. 7. Again it can be seen that a considerable variation of intensity with temperature occurs. Assuming with Weltman & Edelman (1967) that their observed values of $1/P$ are linear with T/η , $1/P$ at any value of T/η may be calculated from eqn. (2) by inserting the appropriate values (i.e. $1/P_0 = 2.41$, $\tau = 13$ nsec., $\rho_{25}^0 = 131$ nsec.) and hence values of $1/P_0$ are obtained by means of eqn. (6). (An experimental value of $1/P_0$ was not given, but since the plots of $1/P$ versus T/η at different sucrose concentrations all extrapolated to $1/P$ about 2.40, it is likely that little error will be introduced by assuming this value for $1/P_0$.) Fig. 8 gives a plot of $1/P_c$ versus T/η thus obtained. Clearly the line is convex to the T/η axis, which indicates that intramolecular rotation developed as the temperature was raised. Also drawn in Fig. 8 is the line that would be given by a rigid particle of relaxation time 220 nsec. at 25° and $\tau = 13$ nsec. (the value of ρ determined experimentally by several workers; see, e.g., Weltman & Edelman, 1967), which is compatible with the tangent of the plot of $1/P_c$ versus T/η at $T/\eta = 0$. Though the latter cannot be determined accurately, the size of the rotating unit at low T/η values is clearly considerably larger than that determined from the traditional analysis. The value of a (eqn. 7) cannot be accurately determined from Fig. 7, but it probably lies between 0.60 and

0.71, and with the uncorrected ρ_{25}^0 of 131 nsec., the relaxation time for the macromolecule given by eqn. (9) is between 218 and 185 nsec. Since intensity data were not given as a function of sucrose concentration, it is not possible to apply eqn. (13) to the constant-temperature plots of $1/P$ versus T/η , as has been done here for BSA.

Hence it should be stressed that apparent linearity of the varying-temperature plot of $1/P$ versus T/η is not necessarily, in general, an indication of the absence of intramolecular rotation, but that life-time changes may effectively cancel out the curvature normally expected to arise from this cause. Nor is it possible to correct such data accurately in view of the difficulty of obtaining life-time values at or near $T/\eta = 0$, and an uncertain extrapolation is involved.

The feasibility of temperature-activated intramolecular rotations is generally accepted, and independent confirmation from paramagnetic-resonance techniques has recently been provided. Thus Stone, Buckman, Nordio & McConnell (1965) demonstrated that a pyrrolidine nitroxide derivative combined with BSA at two distinct types of sites. They deduced that the added group was free to rotate rapidly at one site, but that it had much less freedom of motion at the other type of site.

In principle, the technique of fluorescence depolarization presents a powerful method for the determination of rotational relaxation times of molecules in solution. However, before its potentialities can be realized, further knowledge of the life-time of the excited state, of its variation over a wide range of experimental conditions and of its connexion with fluorescence-intensity changes is required.

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