Polarization of the Fluorescence of Macromolecules

1. THEORY AND EXPERIMENTAL METHOD

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According to the theory developed by Perrin (1926), the partial polarization of the fluorescent light emitted by molecules in solution depends upon the relation of their rotational relaxation time to the lifetime of the excited state of the fluorescence.

The measurements of Gaviola (1927), Szymanowski (1935) and Dushinsky (1936) show that the lifetime of the excited state of the fluorescence is a quantity of the order of 10^{-8} sec. for many dyes in water solution. As the relaxation time of the rotation of these molecules in water is much shorter than 10^{-8} sec., almost completely depolarized radiation is to be expected and is in fact observed. On the other hand, fluorescent macromolecules, e.g. proteins, should already emit partially polarized fluorescence in water solution as their relaxation times are of the required order of magnitude. Measurements of the degree of polarization of the fluorescence should in consequence afford a convenient means of determining the relaxation times of macromolecules in dilute solution under a variety of circumstances.

In this paper are described the general principles which attend the application of this method to the study of macromolecules. The following paper of this series (Weber, 1952) describes experiments done with proteins rendered fluorescent by the conjugation with a small fluorescent molecule.

THEORY

Perrin (1926, 1936) has developed the theory of the polarization of the fluorescence of solutions on the assumption that the emitting molecules carry rigidly bound linear oscillators of absorption and emission and that the molecular rotations are described by Einstein's (1906) equation. The first assumption can be considered experimentally proved (Feofilov, 1943). Although the classical experiments of J. Perrin (1909) have shown that the rotation of large, visible particles follows the Einstein equation, some doubt may be entertained as to its validity in the case of dissolved molecules which do not greatly differ in size from the molecules of the solvent. The protein molecules fall midway between these two extremes, and the measurements

of their relaxation times by the dielectric dispersion method (Oncley, 1942) indicate that the equation of Einstein cannot seriously be at fault. However, the fact that the real shape of the particles is not known and that the measurements have been carried out over a limited range of temperatures and viscosities does not allow a decision as to how closely the theory is followed.

Let us consider a system of co-ordinates $O(xyz)$. The fluorescent solution is placed at O in a square cell with faces oriented normally to the co-ordinate axes. The exciting light travels in the xO direction, and unless otherwise stated we shall assume it to be completely polarized with its electric vector in the Oz direction. The fluorescent light emitted in the Oy direction, i.e. at right angles both to the direction of propagation of the exciting light and to the direction of the electric vector is observed. Its partial polarization p is defined by the equation

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

where $I_{\parallel}$ is the component of the intensity emitted in the direction Oy with its direction of vibration parallel to that of the exciting light, while $I_{\perp}$ is the component normal to the former, i.e. in the Ox direction.

According to Perrin (1926) the value of p for a spherical molecule is given by

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \left(1 + \frac{RT}{\eta V} \tau_0 \right), \quad (2)$$

where R is the gas constant, T the absolute temperature, η the viscosity of the solvent, V the molecular volume of the fluorescent molecule and τ_0 the lifetime of the excited state of the fluorescence; p_0 is clearly the value of p when $T/\eta \rightarrow 0$, i.e. when no depolarization by molecular rotation takes place. In practice it differs little from the polarization observed in a medium of high viscosity like glycerol. According to the above equation, if $1/p$ is plotted against T/η a straight line is obtained cutting the $1/p$ axis at $1/p_0$. Experimentally, for fluorescent dyes with $V \simeq 500$ in glycerol-water mixtures it is found that the relation just mentioned obtains down to viscosities of 10–15 centipoises, but for

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viscosities below these values the polarization is lower than predicted (Perrin, 1926; Wawilow, 1936). Several explanations have been advanced, notably that Einstein's law is not valid in these conditions (Perrin, 1936). Whatever the explanation, if a change in molecular volume has the same effect as a corresponding change in viscosity, Perrin's law should hold accurately for molecules of $V > 10^4$ in media of viscosity of 1 centipoise.

Simultaneous excitation of several oscillators

If several oscillators corresponding to one or more molecular species in solution are simultaneously excited, the observed polarization $\bar{p}$ is related to the individual polarizations p_i that would obtain if each type of oscillator were the only one excited, in the following manner:

Setting $I_{ii} + I_{i\perp} = F_i$,

$$p_i = \frac{I_{ii} - I_{i\perp}}{I_{ii} + I_{i\perp}} = \frac{I_{ii} - I_{i\perp}}{F_i}.$$

Moreover $I_{\parallel} = \sum_i I_{ii}$ and $I_{\perp} = \sum_i I_{i\perp}$.

$$\text{Therefore } \bar{p} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} = \frac{\sum_i F_i p_i}{\sum_i F_i}. \quad (3)$$

Addition law of the polarizations of several oscillators

The total intensity of the radiation emitted by a point of the fluorescent source can be represented by three orthogonal components. Choosing for the directions of the components the co-ordinate axes, we have in the case of non-polarized radiation,

$$I_{\parallel} = I_{\perp} = i,$$

the total intensity being proportional to $3i$. If the fluorescence excited by polarized light as described, becomes now partially polarized to the extent p_i , while the total emitted intensity remains constant, Curie's law of symmetry (Perrin, 1929) shows that the total radiation is then proportional to

$$I_{\parallel} + 2I_{\perp} = 3i.$$

This requires

$$I_{\parallel} = i + \Delta, \quad (4)$$

$$I_{\perp} = i - \frac{1}{2}\Delta. \quad (5)$$

From the last equations, 4 and 5, together with 1, we have for the i th oscillator species in solution:

$$\frac{\Delta_i}{2} = \frac{2i_i}{3 \left(\frac{1}{p_i} - \frac{1}{3} \right)},$$

$$F_i p_i = \frac{2i_i}{\left(\frac{1}{p_i} - \frac{1}{3} \right)}.$$

Defining $f_i = \frac{i_i}{\sum_i i_i}$ as the contribution of the i th oscillator species to the total fluorescent intensity, the last equation becomes

$$F_i p_i = 2 \left(\sum_i i_i \right) \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}}.$$

On the other hand, from Eqn. 3,

$$\frac{1}{\bar{p}} - \frac{1}{3} = \frac{\sum_i F_i}{\sum_i F_i p_i} - \frac{1}{3} = \frac{\sum_i F_i p_i \left(\frac{1}{p_i} - \frac{1}{3} \right)}{\sum_i F_i p_i}.$$

Introducing in the latter the value of $F_i p_i$ just derived,

$$\frac{1}{\bar{p}} - \frac{1}{3} = \frac{1}{\sum_i \frac{f_i}{p_i - \frac{1}{3}}}. \quad (6)$$

Thus $1/\bar{p} - \frac{1}{3}$ is the harmonic mean of the quantities $1/p_i - \frac{1}{3}$ weighed according to the contribution to the total fluorescent intensity of the solution.

Eqns. 4 and 5 refer to excitation with polarized light. In this case the electric vector of the exciting light is an axis of symmetry and $3i = I_{\parallel} + 2I_{\perp}$. If the fluorescence is excited with natural light the direction of propagation is now an axis of symmetry (Perrin, 1929) and the total radiation of the source is proportional to $3i = 2I_{\parallel} + I_{\perp}$. Eqns. 4 and 5 become now respectively,

$$I_{\parallel} = i + \frac{1}{2}\Delta; \quad I_{\perp} = i - \Delta, \quad (7)$$

and consequently

$$\frac{1}{\bar{p}_n} + \frac{1}{3} = \frac{1}{\sum_i \frac{f_i}{\frac{1}{p_{in}} + \frac{1}{3}}}, \quad (8)$$

where the subscript n refers to the same quantities as before but on excitation with natural light. Perrin's law for the excitation with natural light reads

$$\frac{1}{p_n} + \frac{1}{3} = \left(\frac{1}{p_{0n}} + \frac{1}{3} \right) \left(1 + \frac{RT}{\eta V} \tau_0 \right). \quad (9)$$

Therefore in the equations to follow it will only be necessary to write $1/p_n + \frac{1}{3}$ and $1/p_{0n} + \frac{1}{3}$ instead of $1/p - \frac{1}{3}$ and $1/p_0 - \frac{1}{3}$ respectively, to pass from the case of excitation with polarized light to that of excitation with natural light.

If $1/\bar{p} - \frac{1}{3}$ is plotted against T/η in the case of a system of several components the slope, obtained by differentiating Eqn. 6, is

$$\left. \begin{aligned} \frac{d}{d(T/\eta)} \left(\frac{1}{\bar{p}} - \frac{1}{3} \right) &= \sum_i \frac{d \left(\frac{1}{\bar{p}} - \frac{1}{3} \right)}{d \left(\frac{1}{p_i} - \frac{1}{3} \right)} \frac{d \left(\frac{1}{p_i} - \frac{1}{3} \right)}{d(T/\eta)}, \\ \frac{d \left(\frac{1}{\bar{p}} - \frac{1}{3} \right)}{d \left(\frac{1}{p_i} - \frac{1}{3} \right)} &= \frac{\frac{f_i}{\left(\frac{1}{p_i} - \frac{1}{3} \right)^2}}{\left(\sum_i \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}} \right)^2}. \end{aligned} \right\} \quad (10)$$

If the polarization of the fluorescence given by the i th oscillator species when alone in solution is described by Perrin's equation,

$$\left. \begin{aligned} \frac{1}{p_i} - \frac{1}{3} &= \left(\frac{1}{p_{0i}} - \frac{1}{3} \right) \left(1 + \frac{3\tau_i}{\rho_i} \right), \quad \rho_i = \frac{3\eta V_i}{RT}, \\ \frac{d \left(\frac{1}{p_i} - \frac{1}{3} \right)}{d(T/\eta)} &= \left(\frac{1}{p_{0i}} - \frac{1}{3} \right) \frac{3\tau_i}{\rho_i} \left(\frac{1}{T/\eta} \right), \end{aligned} \right\} \quad (11)$$

introducing the values of the partial differentials into Eqn. 10 and performing the additions,

$$\frac{d}{d(T/\eta)} \left(\frac{1}{\bar{p}} - \frac{1}{3} \right) = \frac{\sum_i \frac{f_i}{\left(\frac{1}{p_i} - \frac{1}{3} \right)^2} \left(\frac{1}{p_{0i}} - \frac{1}{3} \right) \frac{3\tau_i}{\rho_i}}{\frac{T}{\eta} \left[\sum_i \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}} \right]^2}. \quad (12)$$

Similarly, we may calculate

$$\frac{d^2}{d(T/\eta)^2} \left(\frac{1}{\bar{p}} - \frac{1}{3} \right) = \frac{-2 \left[\sum_i \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}} \left(\frac{d \left(\frac{1}{p_i} - \frac{1}{3} \right)}{d(T/\eta)} \right)^2 \right] \left[\sum_i \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}} \right] + 2 \left[\sum_i \frac{f_i}{\left(\frac{1}{p_i} - \frac{1}{3} \right)^2} \frac{d \left(\frac{1}{p_i} - \frac{1}{3} \right)}{d(T/\eta)} \right]^2}{\left[\sum_i \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}} \right]^3}.$$

Replacing $\frac{d \left(\frac{1}{p_i} - \frac{1}{3} \right)}{d(T/\eta)}$ by its value given in Eqn. 11 and performing the multiplication of the series,

$$\frac{d^2 \left(\frac{1}{\bar{p}} - \frac{1}{3} \right)}{d(T/\eta)^2} = \frac{-\sum_i \frac{f_i f_j}{\left(\frac{1}{p_i} - \frac{1}{3} \right) \left(\frac{1}{p_j} - \frac{1}{3} \right)} \left(\frac{3\tau_i/\rho_i}{1 + 3\tau_i/\rho_i} - \frac{3\tau_j/\rho_j}{1 + 3\tau_j/\rho_j} \right)^2}{\left(\frac{T}{\eta} \right)^2 \left(\sum_i \frac{f_i}{\frac{1}{p_i} - \frac{1}{3}} \right)^3} \quad (i \neq j). \quad (12a)$$

Therefore the first derivative is always positive and the second either zero or negative and the plotting of $1/\bar{p}$ against T/η is either a straight line or a curve concave towards the T/η axis. Eqn. 11 shows also that if the oscillators differ only in the value of the limiting polarization, p_{0i} , no curvature is to be observed. It is in fact easy to deduce that in such case the observed polarization is given by the equation

$$\frac{1}{\bar{p}} - \frac{1}{3} = \frac{1}{\left(\sum_i \frac{f_i}{\frac{1}{p_{0i}} - \frac{1}{3}} \right)} \left(1 + \frac{\tau_0}{\rho} \right). \quad (12b)$$

It is found experimentally that p_0 varies with the wavelength of the exciting light. Eqn. 12b shows, however, that the calculated value of the relaxation time of the rotation will be the same whether the light used for the excitation is monochromatic or not. This has been experimentally demonstrated by Perrin (1929). Eqn. 12b also shows that if the excitation is due to more than one wavelength a

change in the observed polarization may in certain cases be due to a change in the absorption spectrum of the fluorescent solution leading to a different value of $\bar{p}_0$, and not to a change in the lifetime of the excited state or the rotational relaxation time.

*Molecules of different size carrying
the same fluorescent oscillator*

There is in this case only one lifetime of the excited state and one limiting polarization, i.e. $p_{0i} = p_0$ and $\tau_i = \tau_0$. Eqn. 6 can be written in the form

$$\frac{1}{\bar{p}} - \frac{1}{3} = \frac{1}{\sum_i \frac{f_i}{\left(\frac{1}{p_0} - \frac{1}{3} \right) \left(1 + \frac{3\tau_0}{\rho_i} \right)}} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \frac{1}{\sum_i \frac{f_i}{1 + \frac{3\tau_0}{\rho_i}}}.$$

Adding to both members of the last equation the quantity

$$\left(\frac{1}{p_0} - \frac{1}{3} \right) \left\{ 1 + 3\tau_0 \sum_i \frac{f_i}{\rho_i} - \sum_i f_i \left(1 + \frac{3\tau_0}{\rho_i} \right) \right\} = 0,$$

and rearranging the right-hand side

$$\frac{1}{\bar{p}} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \left\{ 1 + 3\tau_0 \sum_i \frac{f_i}{\rho_i} - \left[\sum_i f_i \left(1 + \frac{3\tau_0}{\rho_i} \right) - \frac{1}{\sum_i \frac{f_i}{1 + \frac{3\tau_0}{\rho_i}}} \right] \right\}. \quad (13)$$

At values of T/η , such that all values of $3\tau_0/\rho_i$ are small compared to unity, the term between the square brackets is negligible. Therefore the initial slope in the plotting of $1/\bar{p}$ against T/η is proportional to the harmonic mean of the relaxation times of the particles weighted according to their contribution to the total emitted intensity. At values of T/η such that the term between the square brackets becomes significant, the value of $1/\bar{p} - \frac{1}{3}$ is always smaller than that corresponding to the initial slope, and the relative decrease is, according to Eqn. 13,

$$\delta \left(\frac{1}{\bar{p}} - \frac{1}{3} \right) = \frac{\sum_i f_i \left(1 + \frac{3\tau_0}{\rho_i} \right) - \frac{1}{\sum_i \frac{f_i}{1 + \frac{3\tau_0}{\rho_i}}}}{\frac{1}{\sum_i \frac{f_i}{1 + \frac{3\tau_0}{\rho_i}}}} = \left(\sum_i f_i \left(1 + \frac{3\tau_0}{\rho_i} \right) \right) \left(\sum_i \frac{f_i}{1 + \frac{3\tau_0}{\rho_i}} \right) - 1.$$

Writing

$$1 = \left(\sum_i f_i \right) \left(\sum_i f_i \right) = \sum_i f_i^2 + 2 \sum_{i \neq j} f_i f_j$$

and performing the multiplication of the series,

$$\delta \left(\frac{1}{\bar{p}} - \frac{1}{3} \right) = \sum_{i \neq j} \frac{f_i f_j 9\tau_0^2 \left(\frac{1}{\rho_i} - \frac{1}{\rho_j} \right)^2}{\left(1 + \frac{3\tau_0}{\rho_i} \right) \left(1 + \frac{3\tau_0}{\rho_j} \right)} = \sum_{i \neq j} \frac{f_i f_j}{\left(\frac{1 + 3\tau_0/\rho_j}{3\tau_0/\rho_j} \right)^2 \left(\frac{1}{n-1} \right)^2 + \left(\frac{1 + 3\tau_0/\rho_j}{3\tau_0/\rho_j} \right) \left(\frac{1}{n-1} \right)}, \quad (14)$$

in which

$$n = \frac{\rho_j}{\rho_i}, \quad n \geq 1.$$

In the case of only two molecular species in solution the last equation reads

$$\delta \left(\frac{1}{\bar{p}} - \frac{1}{3} \right) = \frac{f_1 f_2}{\left(\frac{1 + 3\tau_0/\rho_1}{3\tau_0/\rho_1} \right)^2 \left(\frac{1}{n-1} \right)^2 + \left(\frac{1 + 3\tau_0/\rho_1}{3\tau_0/\rho_1} \right) \left(\frac{1}{n-1} \right)}. \quad (15)$$

From Eqn. 2

$$\frac{1 + 3\tau_0/\rho_1}{3\tau_0/\rho_1} = \frac{\frac{1}{p_1} - \frac{1}{3}}{\frac{1}{p_1} - \frac{1}{p_0}}$$

Substituting this in Eqn. 15 we have

$$\frac{\delta\left(\frac{1}{\bar{p}} - \frac{1}{3}\right)}{\left(\frac{1}{\bar{p}} - \frac{1}{3}\right)} = \frac{f_1 f_2}{\left(\frac{1}{p_1} - \frac{1}{3}\right)^2 \left(\frac{1}{n-1}\right)^2 + \left(\frac{1}{p_1} - \frac{1}{3}\right) \left(\frac{1}{p_1} - \frac{1}{p_0}\right) \left(\frac{1}{n-1}\right)}$$

The presence of experimentally significant curvature requires that the lowest polarizations attained depart from the values corresponding to the initial tangent by an amount

$$\frac{\delta\left(\frac{1}{\bar{p}} - \frac{1}{3}\right)}{\frac{1}{\bar{p}} - \frac{1}{3}} \geq 2.5\epsilon,$$

where ϵ is the standard error of the observations. If we assume $\epsilon = 0.02$ and $1/p_1 < 10$, as in any case $1/p_0 \geq 2$, the condition for observable curvature becomes

$$\frac{f_1 f_2}{\frac{25}{16} \left(\frac{1}{n-1}\right)^2 + \frac{5}{4} \left(\frac{1}{n-1}\right)} \geq 0.05,$$

n is a minimum if $f_1 = f_2 = \frac{1}{2}$, and therefore $n > \frac{3}{2}$. Thus in a solution of two spherical molecules a deviation from the linear law will only be present under the experimental conditions described if the ratio of the relaxation times of the rotation is greater than $\frac{3}{2}$, and conversely if one molecular species carry two different oscillators in equal amounts a deviation from the linear law requires a similar ratio for the lifetimes of the excited state.

Effect of the presence of a fluorescent impurity of low molecular weight

In the case of two spherical molecules of relaxation times ρ_1 and ρ_2 Eqn. 13 becomes

$$\frac{\frac{1}{\bar{p}} - \frac{1}{3}}{\frac{1}{p_0} - \frac{1}{3}} = 1 + \left[\frac{3\tau_0}{\rho_2} (f_1 q + f_2) \frac{\left(1 + \frac{3\tau_0}{\rho_2} \left(\frac{q}{f_1 q + f_2}\right)\right)}{\left(1 + \frac{3\tau_0}{\rho_2} (f_1 + f_2 q)\right)} \right], \quad (16)$$

with $q = \rho_2/\rho_1$, $q \leq 1$. The slope S on the plotting of $1/\bar{p} - \frac{1}{3}$ against T/η can be obtained by differentiating Eqn. 16:

$$S = \frac{(f_1 q + f_2) \frac{3\tau_0}{\rho_2} + 2q \left(\frac{3\tau_0}{\rho_2}\right)^2 + q(f_1 + f_2 q) \left(\frac{3\tau_0}{\rho_2}\right)^3}{\left(1 + \frac{3\tau_0}{\rho_2} (f_1 + f_2 q)\right)^2 \left(\frac{T}{\eta}\right)} \quad (17)$$

The tangent to the curve at a given value of T/η cuts the ordinate axis at the value $1/p'_0$ defined by the equation

$$\frac{\frac{1}{p'_0} - \frac{1}{p_0}}{\frac{1}{p_0} - \frac{1}{3}} = \frac{\frac{1}{\bar{p}} - \frac{1}{3}}{\frac{1}{p_0} - \frac{1}{3}} - S \frac{T}{\eta} = \frac{\left(\frac{3\tau_0}{\rho_2}\right)^2 (1-q)^2 f_1 f_2}{\left(1 + \frac{3\tau_0}{\rho_2} (f_1 + f_2 q)\right)^2} \quad (18)$$

If $q \ll 1$ and $3\tau_0/\rho_2 \gg 1$ we have the case in which a macromolecule and a low molecular weight impurity coexist in solution. Eqn. 18 shows that then a very simple relation obtains approximately between the relative increase in the intercept and the contribution of the impurity to the total fluorescent emission, namely:

$$f_2 \approx \frac{\frac{1}{p'_0} - \frac{1}{p_0}}{\frac{1}{p_0} - \frac{1}{3}} \quad (19)$$

Moreover, if

$$\frac{3\tau_0}{\rho_2} f_1 \gg 1, \quad \frac{\frac{3\tau_0}{\rho_2} (f_1 q + f_2)}{1 + \frac{3\tau_0}{\rho_2} (f_1 + f_2 q)} \approx \frac{f_2}{f_1} \quad (20)$$

and the right-hand side of Eqn. 16 reduces to

$$\frac{1}{f_1} \left(1 + \frac{3\tau_0}{\rho_1}\right).$$

From the last, and Eqn. 19, we have

$$\left(\frac{1}{\bar{p}} - \frac{1}{3}\right) \approx \left(\frac{1}{p'_0} - \frac{1}{3}\right) \left(1 + \frac{3\tau_0}{\rho_1}\right). \quad (21)$$

Eqn. 21 has the same form as 2, p'_0 taking the place of p_0 . Therefore the linear law is followed over the range of values of T/η such that $(3\tau_0/\rho_2) f_1 \gg 1$ and the relaxation time calculated from the slope and intercept is the true relaxation time of the rotation of the macromolecule.

Polarization of the fluorescence emitted by ellipsoidal molecules carrying randomly oriented oscillators

Perrin (1936) has developed the theory of the polarization of the fluorescence emitted by molecules of any shape. There appears to be no simple theory for ellipsoids of revolution. The value of p is in that case an explicit function not only of the principal relaxation times of the rotation, but also

of the three angles determined by the oscillators of absorption and emission of light and the axis of revolution of the ellipsoid.

The general equation (Perrin, 1929),

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \frac{2}{3 \overline{\cos^2 \omega} - 1}, \quad (22)$$

where $\overline{\cos^2 \omega}$ is the average cosine square of the angle swept by the emission oscillator between the times of absorption and emission of light, may, however, be applied in certain cases. If OA (Fig. 1) denotes the

Fig. 1. Explanation in the text.

direction of the oscillator of absorption, OE the direction of the emission oscillator and OR the major axis of the ellipsoid, for the last equation to be valid it is necessary that $\overline{\cos^2 \omega}$ be independent of azimuth about OE . This requires all positions of the ellipsoid with OE in a particular direction to have equal weight. The weight of each position is given by $\cos^2 \theta$, where θ is the angle between the absorption oscillator and the electric vector of the exciting light Oz . The condition that all positions of the molecule obtained by rotation about OE have equal weight can only be fulfilled in two particular instances:

(a) OA and OE are coincident;
 (b) OA and OE make a fixed angle λ between them, but for a given direction of OE and OR all azimuths of OA about OE are equally probable. Clearly this is impossible if the axis of revolution of the molecule makes fixed angles with the oscillators, but it will represent the actual case when a small fluorescent molecule is attached to a much larger non-fluorescent one by adsorption or covalent bonding, provided there is no particular orientation of the molecules with respect to each other. If the larger molecule is an ellipsoid of revolution, $\overline{\cos^2 \omega}$

will be a function of the angle α between OR and OE and the two principal relaxation times of the rotation of the ellipsoid. Moreover, it will be necessary to sum over all values of α since random orientation of the second molecule with respect to the first has been assumed. By application of Eqn. 6 we then have

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \frac{2}{\frac{6}{\pi} \int_0^\pi \overline{\cos^2 \omega}(\alpha) d\alpha - 1}, \quad (23)$$

$$\text{where } \overline{\cos^2 \omega}(\alpha) = \frac{1}{\tau_0} \int_0^\infty \overline{\cos^2 \omega}(\alpha, t) e^{-t/\tau_0} dt. \quad (24)$$

Calculation of $\overline{\cos^2 \omega}$. Consider two positions of the ellipsoid; in the first the principal axes of the ellipsoid are coincident with the co-ordinate axes and the components of the oscillators of emission along the co-ordinates are $\cos \alpha$, $\cos \beta$ and $\cos \gamma$ respectively. After a rotation about the centre the components are $\cos \alpha'$, $\cos \beta'$ and $\cos \gamma'$. Therefore ω , the angle determined by the two directions of the oscillator of emission, is defined by,

$$\cos \omega = \cos \alpha \cos \alpha' + \cos \beta \cos \beta' + \cos \gamma \cos \gamma'.$$

If $\cos_{ij}$ ($i = 1, 2, 3$; $j = 1, 2, 3$) denotes the angle between the direction of a principal axis i of the ellipsoid in its second position and an axis j in the first,

$$\begin{aligned} \cos^2 \omega = & [\cos^2 \alpha \cos_{11} + \cos \alpha \cos \beta \cos_{12} \\ & + \cos \alpha \cos \gamma \cos_{13} + \cos \alpha \cos \beta \cos_{12} \\ & + \cos^2 \beta \cos_{22} + \cos \beta \cos \gamma \cos_{23} \\ & + \cos \alpha \cos \gamma \cos_{13} + \cos \beta \cos \gamma \cos_{23} \\ & + \cos^2 \gamma \cos_{33}]^2. \end{aligned}$$

Taking into account that the mean value of the double products of the form $\overline{\cos_{ij} \cos_{jk}}$, where one or more indices appear only once are null by symmetry (Perrin, 1936), and setting

$$\cos^2 \alpha = z, \quad \cos^2 \beta = \cos^2 \gamma = \frac{1}{2}(1 - z),$$

we have

$$\begin{aligned} \overline{\cos^2 \omega} = & z^2 \overline{\cos_{11}^2} + \frac{(1 - z)^2}{4} (\overline{\cos_{22}^2} + \overline{\cos_{33}^2}) \\ & + z(1 - z) (\overline{\cos_{21}^2} + \overline{\cos_{31}^2} + \overline{\cos_{11} \cos_{22}} \\ & + \overline{\cos_{12} \cos_{21}} + \overline{\cos_{11} \cos_{33}} + \overline{\cos_{13} \cos_{31}} \\ & + \frac{(1 - z)^2}{2} (\overline{\cos_{22}^2} + \overline{\cos_{23} \cos_{33}} + \overline{\cos_{33} \cos_{23}}), \end{aligned} \quad (25)$$

where the bar denotes average value.

To obtain $\overline{\cos^2 \omega}(\alpha, t)$ a function of the time t , we introduce the values calculated by Perrin (1936) for the average cosine square and double products of the angles determined by the axes of the ellipsoid of

revolution submitted to brownian movement. These are

$$\left. \begin{aligned} \overline{\cos^2_{11}} &= \frac{1}{3} + \frac{2}{3} e^{-3t/\rho_1}, \\ \overline{\cos^2_{22}} = \overline{\cos^2_{33}} &= \frac{1}{3} + \frac{1}{6} e^{-3t/\rho_1} - \frac{1}{2} \exp \left[- \left(\frac{4}{\rho_2} - \frac{1}{\rho_1} \right) t \right], \\ \overline{\cos^2_{12}} = \overline{\cos^2_{21}} = \overline{\cos^2_{13}} = \overline{\cos^2_{31}} &= \frac{1}{3} - \frac{2}{3} e^{-3t/\rho_1}, \\ \overline{\cos^2_{23}} = \overline{\cos^2_{32}} &= \frac{1}{3} + \frac{1}{6} e^{-3t/\rho_1} - \frac{1}{2} \exp \left[- \left(\frac{4}{\rho_2} - \frac{1}{\rho_1} \right) t \right], \\ \overline{\cos_{22} \cos_{11}} + \overline{\cos_{12} \cos_{21}} &= \overline{\cos_{33} \cos_{11}} + \overline{\cos_{13} \cos_{31}} \\ &= \exp \left[- \left(\frac{2}{\rho_1} + \frac{1}{\rho_2} \right) t \right], \\ \overline{\cos_{22} \cos_{33}} + \overline{\cos_{23} \cos_{32}} &= \exp \left[- \left(\frac{4}{\rho_2} - \frac{1}{\rho_1} \right) t \right], \end{aligned} \right\} \quad (26)$$

ρ_1 and ρ_2 are the relaxation times of the rotation of the ellipsoid about the long and short axis respectively. By introduction of these values in Eqn. 25 and integration of Eqn. 24:

$$\overline{\cos^2 \omega}(\alpha) = \frac{1}{3} + \frac{(1-3z)^2}{6} \frac{1}{\left(1 + \frac{3\tau_0}{\rho_1}\right)} + \frac{(1-z)^2}{2} \frac{1}{\left(1 + \left(\frac{4}{\rho_2} - \frac{1}{\rho_1}\right)\tau_0\right)} + 2z(1-z) \frac{1}{\left(1 + \left(\frac{2}{\rho_1} + \frac{1}{\rho_2}\right)\tau_0\right)}, \quad (27)$$

and finally integrating Eqn. 23 after introducing $\overline{\cos^2 \omega}(\alpha)$

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \frac{AB}{C} \left(\frac{8}{3 + 6 \frac{B}{C} - \frac{B^2}{C^2}} \right), \quad (28)$$

where

$$A = 1 + \frac{3\tau_0}{n_1\rho_0}, \quad B = 1 + \left(\frac{4}{n_2} - \frac{1}{n_1} \right) \frac{\tau_0}{\rho_0}, \quad C = 1 + \left(\frac{2}{n_1} + \frac{1}{n_2} \right) \frac{\tau_0}{\rho_0},$$

$$\frac{\rho_1}{\rho_0} = n_1, \quad \frac{\rho_2}{\rho_0} = n_2, \quad \rho_0 = \frac{3\eta V_e}{RT}, \quad V_e = \text{volume of the ellipsoid.}$$

The last equation can also be written

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \frac{\left(1 + \frac{3\tau_0}{n_1\rho_0} \right) \left(1 + \left[\frac{4}{n_2} - \frac{1}{n_1} \right] \frac{\tau_0}{\rho_0} \right)}{1 + \frac{1}{2} \left(\frac{5}{n_2} + \frac{1}{n_1} \right) \frac{\tau_0}{\rho_0} + \frac{9}{8} \frac{\frac{\tau_0^2}{\rho_0^2} \left(\frac{1}{n_2} - \frac{1}{n_1} \right)^2}{\left(1 + \left[\frac{2}{n_1} + \frac{1}{n_2} \right] \frac{\tau_0}{\rho_0} \right)}}, \quad (29)$$

which clearly shows that on plotting $1/p - \frac{1}{3}$ against τ_0/ρ_0 or, which is equivalent, against T/η , the ratio of the initial slope to that of the sphere of the same volume is

$$\frac{S}{S_0} = \frac{1}{2} \left(\frac{1}{n_1} + \frac{1}{n_2} \right), \quad (30)$$

the harmonic mean of the ratios of the relaxation times of the ellipsoid to the relaxation time of the sphere of the same volume. If the last term of the denominator of Eqn. 29 is neglected the equation describes within 1% the depolarization due to

molecules of any elongation up to $\tau_0/\rho_0 = 0.13$ and within 5% up to $\tau_0/\rho_0 = 0.5$. A better and more interesting approximation is given by the equation

$$\left. \begin{aligned} \frac{1}{p} - \frac{1}{3} &= \left(\frac{1}{p_0} - \frac{1}{3} \right) \left(1 + \frac{3\tau_0}{\rho_0 n_p} \left[\frac{1 + \frac{3\tau_0}{\rho_0 n_a}}{1 + \frac{3\tau_0}{\rho_0 n_p}} \right] \right), \\ \frac{1}{n_p} &= \frac{1}{2} \left(\frac{1}{n_1} + \frac{1}{n_2} \right), \quad n_a = \frac{n_1 + n_2}{2}. \end{aligned} \right\} \quad (31)$$

The last is obtained immediately from Eqn. 6 in the case of two spherical molecules present in equal amounts ($f_1 = f_2 = \frac{1}{2}$) and having relaxation times ρ_1 and ρ_2 . The random distribution of the directions of the oscillators in an ellipsoidal molecule result in almost complete uncoupling of the effects of both relaxation times, so that qualitatively and almost quantitatively the situation is much the same as if both relaxation times belonged to different molecules.

Depolarization by flat and elongated molecules

If the molecule is a flat ellipsoid of revolution its two principal relaxation times never differ between themselves by more than 10% (Perrin, 1934). Consequently, on plotting $1/p$ against T/η the departure from linearity is negligible and a flat ellipsoidal molecule cannot be distinguished from a spherical molecule. On the other hand, the two relaxation times of the rotation of an elongated ellipsoid may differ greatly and therefore a curvature concave towards the T/η axis may be obtained.

Fig. 2 shows the plot of $(1/p - \frac{1}{3})/(1/p_0 - \frac{1}{3})$ against τ_0/ρ_0 calculated from Eqn. 28 for ellipsoids of revolution of increasing axial ratio; n_1 and n_2 have been calculated from the equations of Perrin (1934). There is a mistake in the values of n_2 in equations 96 and 96 bis in Perrin's (1934) paper. $2\rho^2 - 1$ must be changed into $1 - 2\rho^2$, the mistake coming from equation 95 where the coefficient of S in the denominator of n_2 should be $b^2a(a^2 - 2b^2)$ instead of $b^2a(2b^2 - a^2)$. From Fig. 2 it appears that the curvature is pronounced only if the accessible range of values of τ_0/ρ_0 is sufficiently large. Thus if $\tau_0/\rho_0 < 0.5$ no elongation smaller than 6 is likely to be detected, and if $\tau_0/\rho_0 < 0.2$ any elongation will fail to produce curvature.

times may be approximated by the use of Eqn. 16. In order to see the significance of f_1 and f_2 we may consider the likely case where the attached molecule executes a very rapid rotation restricted to a few degrees, independent of the slow rotation of the macromolecule. Since $3\tau_0/\rho_2 \gg 1$, the plot of $1/p$ against T/η will yield a straight line extrapolating to $1/p'_0 > 1/p_0$. After Eqn. 22 if θ is the average amplitude of the rapid rotation of the small molecule,

$$\frac{1}{p'_0} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \frac{2}{3 \cos^2 \theta - 1}. \quad (32)$$

So that Eqn. 19 yields

$$f_2 = \frac{2}{3} \sin^2 \theta,$$

Fig. 2. Theoretical curves (Eqn. 28) for prolate ellipsoids of revolution. The number of each curve gives the elongation = $\frac{\text{long axis}}{\text{short axis}}$.

It may be concluded that in the case of molecules not very asymmetrical, i.e. the globular proteins, the simple Eqn. 1 should apply if ρ_0 is substituted by ρ_p , the harmonic mean of the principal relaxation times of the rotation.

Depolarization of the fluorescence by intramolecular rotation

The considerations that lead us to the use of Eqn. 22 allow a qualitative treatment of the case in which the small molecule attached to the macromolecule has a certain freedom of rotation with respect to the latter. If the macromolecule follows the linear law, i.e. if its depolarization can be described as resulting from one relaxation time $\rho_p = \rho_1$ and the small molecule has relaxation time ρ_2 about its conjugating bond or one nearby to it, the composition of the effects of these two relaxation

relating f_2 to the amplitude of the rapid rotation. If $(3\tau_0/\rho_2)f_1 \gg 1$ the linear law is followed, just as in the case of an impurity of low molecular weight, and the relaxation time calculated from the slope and intercept is the relaxation time of the rotation of the macromolecule as a whole.

It is evident from the preceding discussion that the study of the polarization of the fluorescence alone cannot distinguish between the effects of intramolecular rotations and the presence of a small amount of an impurity of low molecular weight. In both cases the net effect is a decrease in the value of p_0 , so that the relaxation time of the rotation of the macromolecule can be calculated without difficulty.

In the preceding discussion we have assumed that the amplitude of the internal rotations does not change with temperature. It is conceivable, however, that the amplitude allowed to such rotations

may increase due to thermal breaking of intramolecular bonds, i.e. f_2 increases with the temperature. The last two equations then give

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) \left(\frac{1}{1 - f_2(T)} \right) \left(1 + \frac{3\tau_0}{\rho_p} \right).$$

On plotting $1/p$ against T/η a curvature convex towards the latter axis will appear, just as in the case of thermal dissociation of the molecule into rigid units. If a maximum value of f_2 is reached a straight line (s_2) or a curve concave towards the T/η axis may again obtain at higher temperature. If a definite slope is finally reached $1/p'_0$ can be determined by extrapolation. If $1/p_0 = 1/p'_0$ the curvature cannot be due to internal rotations and must result from molecular fission; s_2/s_1 , the ratio of the initial to the final slope, is then the ratio of the average relaxation times of the original and resulting particles. If $1/p_0 \neq 1/p'_0$, and if intramolecular rotations alone are the cause of the curvature, from Eqn. 21

$$\frac{s_2}{s_1} = \frac{\frac{1}{p_0} - \frac{1}{3}}{\frac{1}{p'_0} - \frac{1}{3}} \quad (33)$$

must be satisfied within the limits of the experimental error.

EXPERIMENTAL METHOD

The arrangement used for the determination of the polarization of the fluorescence is shown in Fig. 3. Light from a mercury arc is made parallel and then polarized by the

Fig. 3. Experimental arrangement for the determination of the polarization of the fluorescence. H , Hg discharge lamp; L_1 and L_2 , lenses ($f_1 = 8$ cm.; $f_2 = 12.5$ cm.); N_1 and N_2 , Nicol prisms; K , cell containing the fluorescent solution; B , water bath with glass windows; F_c and F_f , complementary filters; A , Arago compensator; S , Savart polariscope; M_1 and M_2 , mirrors; W_c and W_f , windows.

Nicol prism N_1 . A suitable filter F_c is placed between the latter and the cell K containing the fluorescent solution. This is an ordinary glass cell with parallel faces of 1 cm. depth; it

stands inside a small square water bath of some 50 ml. capacity provided with glass windows for the exciting and fluorescent light, W_c and W_f , respectively. The temperature of the bath is kept constant by means of a small heater and rheostat, or by cooling with ice as the case requires. The filter for the fluorescent light F_f , the Arago compensator A of four glass plates, the Savart polariscope S and its Nicol N_2 are mounted on a small optical bench at right angles to that carrying the lenses, the Nicol N_1 and the bath. The Arago compensator stands on a graduated circle and a lateral vernier allows readings of 0.1 degree. The Savart plate is mounted so that it can be tilted slightly. In this way the interference fringes can be seen moving over the field with a good improvement in sensitivity. Two small mirrors, M_1 and M_2 , can be placed against the back and side of the glass cell respectively, as described by Perrin (1929). The first increases the number of excited molecules and makes the field more homogeneous while the second increases the light reaching the eye by a factor of nearly two.

Calibration of the compensator of Arago

A source of known polarization is required. This is provided by the light reflected from the face of a glass prism of known refractive index, at different incidence angles. The polarization is given by the well-known Fresnel equations (Gaviola & Pringsheim, 1924). A check on the consistency of the calibration curve can easily be done by the following method:

Let the electric vector of the linearly polarized exciting light make an angle θ with the Oz direction instead of being coincident with it as we have assumed till now. The fluorescent light emitted in the Oy direction has components $I_{\theta 1}$ and $I_{\theta \perp}$ related to I_1 and $I_{\perp}$, the components that obtain at $\theta = 0$, in the following manner:

$$I_{\theta 1} = I_1 \cos^2 \theta + I_{\perp} \sin^2 \theta,$$

$$I_{\theta \perp} = I_{\perp},$$

$$\frac{1}{p_{\theta}} = \frac{I_1 \cos^2 \theta + I_{\perp} (1 + \sin^2 \theta)}{(I_1 - I_{\perp}) \cos^2 \theta} = \frac{1}{p} + \tan^2 \theta \frac{2I_{\perp}}{I_1 - I_{\perp}},$$

and remembering that $I_1/I_{\perp} = \frac{1+p}{1-p}$, we have

$$\frac{1}{p_{\theta}} = \frac{1}{p} + \left(\frac{1}{p} - 1 \right) \tan^2 \theta. \quad (34)$$

If a solution showing considerable polarization of the fluorescence, e.g. fluorescein in glycerol, is excited with light vibrating at a variable angle θ to the Oz axis, and the observed values of $1/p_{\theta}$ are plotted against $\tan^2 \theta$ a straight line is obtained, the slope and intercept of which should be in the relation indicated by Eqn. 34 if no systematic error is present in the calibration of the compensator.

Some sources of error in the determination of the polarization

From Eqn. 34 it is found that if $\theta = 2^\circ$ the polarization differs by less than 1/1000 from that of $\theta = 0$. Therefore the inaccuracy in setting the plane of polarization of the exciting light should not result in any appreciable error in the determined polarization.

Convergence of the exciting beam. If a solution is illuminated with a plane polarized, parallel beam BO (Fig. 4), the direction of which has latitude θ and longitude ϕ , and the direction of the electric vector of which makes an angle θ with Oz , an observer receiving light from the source along Oy would record a polarization $p(\theta, \phi)$ defined by

$$p(\theta, \phi) = \frac{i_{\parallel} - i_{\perp}}{i_{\parallel} + i_{\perp}},$$

where $i_{\parallel}$ and $i_{\perp}$ are the emitted intensity components polarized along Oz and Ox respectively. The polarization p observed at right angles to the directions of propagation and vibration of the beam being as usual

$$p \equiv \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}.$$

From Fig. 4,

$$\left. \begin{aligned} i_{\parallel} &= I_1 \cos^2 \theta + I_{\perp} \sin^2 \theta, \\ i_{\perp} &= I_1 \cos^2 \phi \sin^2 \theta + I_{\perp} (\cos^2 \theta \cos^2 \phi + \sin^2 \phi). \end{aligned} \right\} \quad (35)$$

The polarization $\bar{p}(\theta, \phi)$ observed on excitation by a convergent beam of homogeneous intensity, of maximum latitude θ and maximum longitude ϕ is, by Eqn. 3,

$$\bar{p}(\theta, \phi) = \frac{\int_0^{\phi} \int_0^{\theta} (i_{\parallel} - i_{\perp}) d\theta d\phi}{\int_0^{\phi} \int_0^{\theta} (i_{\parallel} + i_{\perp}) d\theta d\phi}.$$

Introducing the values of $i_{\parallel}$ and $i_{\perp}$ given by Eqn. 35 into the last one and performing the integrations,

$$\bar{p}(\theta, \phi) = \frac{(I_{\parallel} - I_{\perp}) \frac{1}{4} \left(1 + 3 \frac{\sin 2\theta}{2\theta} - \frac{\sin 2\phi}{2\phi} + \frac{\sin 2\theta \sin 2\phi}{2\theta} \right)}{(I_{\parallel} + I_{\perp}) - (I_{\parallel} - I_{\perp}) \frac{1}{4} \left(1 - \frac{\sin 2\theta}{2\theta} - \frac{\sin 2\phi}{2\phi} + \frac{\sin 2\theta \sin 2\phi}{2\theta} \right)}. \quad (36)$$

For conical convergence $2\theta = 2\phi = x$,

$$\frac{1}{\bar{p}(x)} = \frac{1}{p} \frac{1}{\frac{1}{4} \left(1 + \frac{\sin x}{x} \right)^2} - \frac{\left(1 - \frac{\sin x}{x} \right)^2}{\left(1 + \frac{\sin x}{x} \right)^2}.$$

The second term can be neglected since it is smaller than 1/1000 even for $x = \frac{1}{2}\pi$, and consequently,

$$\bar{p}(x) = \frac{1}{4} p \left(1 + \frac{\sin x}{x} \right)^2 \geq p \frac{\sin x}{x}.$$

Proceeding in a similar way, it is possible to show that in the case of an excitation with natural light the polarization $\bar{p}_n(x)$ is related to that observed on excitation by a perfectly parallel beam p_n , by the equation,

$$p_n(x) = p_n \left\{ \frac{\sin x}{x} - \frac{1}{4} \left(\frac{1 - \sin^2 x}{x^2} \right) \right\} \geq p_n \left\{ \frac{3 \sin x}{2x} - \frac{1}{2} \right\}. \quad (37)$$

Therefore convergences below 10° give rise to negligible errors in the observed polarization.

Fig. 4. Convergence of the exciting beam (for explanation see text).

Size of the source. A point in the centre of the source emits a cone of light of solid angle θ determined by the aperture of the Nicol N_s and its

distance to the source. If the Arago compensator is rotated through an angle ϕ it is easy to see that the normal to the latter makes with the divergent light angles varying from $\phi - \frac{1}{2}\theta$ to $\phi + \frac{1}{2}\theta$. It is then possible to see interference bands due to overcompensation at one end of the source before they have been fully compensated at the opposite end. It is possible to use this fact to obtain a sharp end point in the compensation by the interposition of a lens between the source and the compensator so that the necessary divergence is obtained.

Scattering of the exciting and fluorescent light. If the filters F_e and F_f for the exciting and fluorescent light are truly complementary any scattered or reflected exciting light will not reach the eye. The scattering of the fluorescent light merits further attention. Two effects have here to be considered: the lateral scattering which would result in an increase in the observed polarization and the forward scattering which should have the opposite result. As might be

expected, the first effect is negligible compared to the second, and it is found experimentally that the polarization of the fluorescence from a turbid solution is always lower than one of the same characteristics from a transparent medium. Fortunately, the Tyndall effect shown by dilute protein solutions seems to have no detectable effect on the polarization. It is a simple matter to detect in any given case whether depolarization by forward scattering takes place. With the mirror M_2 in position, and the exciting beam traversing the middle of the cell, the average path of the fluorescent light inside the solution is about twice the path in the absence of the mirror. If the polarization of the fluorescence is the same with and without the mirror any depolarization by turbidity can be excluded.

SUMMARY

1. A simple addition law for the polarizations of several fluorescent components in solution has been derived.

2. It is shown that a system of components which differ in molecular size or in their lifetime of the excited state, but which follow independently Perrin's law of depolarization give in the plot of $1/p$ against T/η a curve concave with respect to the latter axis. (p = polarization; T = absolute temperature; η = viscosity of the solvent.)

3. An extension of Perrin's theory of depolarization to the case of ellipsoidal molecules carrying randomly oriented oscillators of absorption and emission is described. In such case the polarization of the fluorescence is an explicit function of the principal relaxation times of the rotation of the ellipsoid, the lifetime of the excited state and the limiting polarization.

4. A qualitative treatment of the depolarization by intramolecular rotations is given.

5. The experimental determination of the polarization is described and some causes of error discussed in detail.

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Polarization of the Fluorescence of Macromolecules

2. FLUORESCENT CONJUGATES OF OVALBUMIN AND BOVINE SERUM ALBUMIN

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In the preceding paper (Weber, 1952) the theory of the polarization of the fluorescence given by macromolecules in solution carrying randomly oriented linear oscillators has been examined. If the relaxation time of the rotation of proteins is to be obtained by this method, the fluorescent oscillators must be rigidly bound to the molecule. Proteins as such are wholly non-fluorescent, but the radiation emitted by a stable complex of the protein with a small fluorescent molecule should have the same optical characteristics as a fluorescence of the protein molecules themselves.

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Such a stable fluorescent complex can in theory be obtained in several ways: (i) by coupling of a small molecule through a covalent bond and subsequent elimination of the non-coupled fluorescent molecules by the ordinary methods of protein purification; (ii) by adsorption equilibrium of fluorescent molecules on the protein. It will be necessary in the latter case to determine the fraction bound to the protein by an independent method or to consider only the polarization values obtained under conditions such that the whole of the dye is bound to the protein. This last condition requires an affinity of the protein for the fluorescent molecule which is