

Fluorescence yield.

Much of the usefulness of the fluorescence observation for the chemist and biologist stems from the fact that most of the molecules absorbing in the ultraviolet and visible regions of the spectrum do not emit conspicuous fluorescence. However, the difference between fluorescent and 'non fluorescent' molecules is quantitative rather than qualitative, and may be more precisely characterized by the fluorescence yield. This can be defined in terms of either energy, or quanta:

$$\text{Energy yield} = \text{Energy emitted} / \text{Energy Absorbed} = Q \quad (1)$$

$$\text{Quantum yield} = \text{Quanta emitted} / \text{Quanta Absorbed} = q \quad (2)$$

The former is of little interest because the value of the energy yield is systematically dependent upon the wavelength of excitation. On the other hand the quantum yield is, in most cases, independent of the exciting wavelength, and one can then speak without ambiguity of the quantum yield of a pure fluorophore after specifying the solvent composition and the temperature.

As already indicated there is a direct relation between the quantum yield and the relative merit of the radiative and radiationless rate of deactivation of the excited state, namely

$$k_d/r = q^{-1} - 1 \quad (3).$$

or in another form

$$\tau = \tau_0 q \quad (4)$$

The last equation is valid only for a homogeneous population. In many cases even in pure compounds in solution, and in virtually all cases of biological samples we have to deal with heterogeneous molecular populations.

Fluorescence yields of heterogeneous populations.

For each homogeneous group within the population we can set a separate fluorescence yield q_i and lifetime τ_i related by the equation

$$\tau_i = \tau_{ei} q_i \quad (5)$$

The observed average lifetime will be weighted according to the intensity of fluorescence from each component so that

$$\langle \tau \rangle = \sum \tau_i n_i q_i / \sum n_i q_i = \sum \tau_{ei} n_i q_i^2 / \sum n_i q_i \quad (6).$$

where n_i is the number of elements of the i th. component excited under the experimental conditions used.

The observed average yield is given by

$$\langle q \rangle = \sum n_i q_i / \sum n_i \quad (7)$$

and by the definition of average.

$$q_i = \langle q \rangle + \delta q_i ; \quad \sum n_i \delta q_i = 0. \quad (8).$$

Introducing eqs. (8) and (7) in (5), so that

$$q_i^2 = \langle q \rangle^2 + 2 \langle q \rangle \delta q_i + \delta q_i^2. \quad (9).$$

we find.

$$\langle \tau \rangle = \langle \tau_{ei} \rangle \langle q \rangle + 2 \sum n_i \tau_{ei} \delta q_i / \sum n_i + \sum n_i \tau_{ei} \delta q_i^2 / \sum n_i \quad (10).$$

If - and only if - all the groups have the same emissive lifetime, that is if $\tau_{ei} = \tau_e$ the second term in the right side of (10) disappears and.

$$\langle \tau \rangle = \langle \tau_{ei} \rangle \langle q \rangle + \sum n_i \tau_{ei} \delta q_i^2 / \langle q \rangle. \quad (11)$$

The second term ^{of (11)} is always positive or zero and for these cases.

$$\langle \tau \rangle \geq \langle \tau_e \rangle \langle q \rangle \quad (12).$$

the equality applying to a homogeneous and the inequality to a heterogeneous population. If a pure fluorophore exists in solution as an equilibrium mixture of forms with appreciable differences in quantum yield, so that $\langle \tau_{ei} \rangle = \tau_e$, the equality (12) will not be obeyed. At present sufficiently accurate methods of experimental determination of $\langle \tau \rangle$, but not of $\langle q \rangle$ or τ_e exist, but with accurate independent measurements of the three quantities, eq. (12) can be used to detect the presence of conformers of different yield in solutions of a seemingly pure fluorophore. Under present experimental conditions this equation can be used to establish comparisons among compounds which chemical structure and absorption

(spectrum show not to differ appreciably in emissive lifetimes, It is also useful in distinguishing between static and dynamic chemical quenching (q.v.).

Determination of the absolute fluorescence yield.

- The energy yield can be directly determined by the
- * Calorimetric method first introduced by Alekseev. In this method the exciting radiation is absorbed completely by the fluorescent solution; the fluorescence leaves the system and does not contribute to the thermal equilibrium, but this is modified by the heat released to the solvent as a result of non-radiative competing processes. This heating is compared with that produced when an absorber devoid of fluorescence is similarly illuminated. To derive the quantum yield a calculation of the energy losses by thermalization of the excited state is required, and this is given by the difference in energy between the exciting wavelength and the mean fluorescence wavelength. In the original Alekseev method actual differences in temperature between absorbing and fluorescent solutions are measured. In the ingenious
 - * more recent calorimetric method of Saybold et al. the thermal expansion of the illuminated fluorescent solution is measured and compared with the larger expansion owing to the non-fluorescent absorber. Other proposed methods rely on the comparison of the intensity of the fluorescence with that of the exciting radiation. The first method of this kind, due to S.I. Wavilov has also historical interest as it led to the demonstration that fluorescent

Compounds in solution - fluorescein in this case - could re-radiate most of the absorbed energy - rather than a small fraction of it, as many at that time believed. Wavilov, and more recently Melhuish, used an integrating sphere. This is a closed chamber - not necessarily spherical - coated with a light-diffusing material of negligible absorption for the fluorescence and exciting radiation. In practice a coating of MgO provides good diffusivity and low absorption both in the ultraviolet and visible regions of the spectrum. A detector placed somewhere in the spherical wall samples the radiation density, and it is assumed that conditions are such that even radiation distribution inside the sphere is reached regardless of the directional emission of the source. (diffusing plate or fluorescent sample). A second method of the same type proposed by Weber and Teale does away with the integrating sphere and compares the fluorescence signal with the signal from a non-fluorescent scattering solution, both of them adjusted to give the same apparent absorbance at the exciting wavelength. . . . All these methods of comparison of excitation and fluorescence signals require correction for the difference in spectral response of the photomultipliers over the wavelengths of excitation and emission, which in turn call for

an exact determination of the molecular fluorescence spectrum. To obviate these sources of error, often underestimated, Weber and Teale interposed between source and photomultiplier a solution of Rhodamine B ^{in ethylene glycol}, intended to act as a proportional quantum counter for the scattering and fluorescent emissions. As Rhodamine B solutions do not absorb strongly beyond 600 nm. This arrangement is limited to fluorophores with marginal emission at wavelength longer than this.

The emitted radiation has the same symmetry properties for both fluorescence and scattering (π , eqs. 8-12), and in both the signal, measured at right angle to the excitation by unpolarized light, is related to the total emission I_s or I_f respectively, by the relations

$$I_s = \frac{2S'}{(3+p_s)} \quad ; \quad I_f = \frac{2F}{(3+p_f)}$$

where p_s and p_f denote the linear polarizations of the right-angle emitted scattering or fluorescence respectively

It follows that

$$q = \frac{I_f}{I_s} = \frac{F}{S} \frac{(3+p_s)}{(3+p_f)}$$

Recently Demass has proposed a new geometrical arrangement in which the proportional quantum counter solution is enclosed in a cylindrical container

the hollow axis of which is filled with the fluorescent solution. The exceptional parallelism of laser beam excitation ensures the absence of direct excitation of the fluorescent quantum counter and defines a rigorous geometry for the scattering of fluorescence.

Reliability and accuracy of quantum yield determinations

The use of these various methods has resulted in qualitative though not ^{complete} quantitative agreement with respect to all fluorophores investigated. It is acknowledged that the quantum yields of solutions of ^{some} compounds with strong absorption in the visible and near ultraviolet regions (e.g. fluorescein, Janus acridine) have yields which must be close to unity. Thus various measurements with alkaline fluorescein have given values of 0.9-0.95. The agreement is much less satisfactory with compounds of weaker absorption and smaller yields. Quinine sulfate is reported by Melhuish to have a yield of 0.55, from measurements with an integrated sphere. Scott et al. calculated a value of 0.70 for this compound, and point out that it is easy to underestimate the fluorescence yield, because of the difficulty of giving its true weight to the emission in the orange or red regions on account of the notoriously poor response of most photomultiplier tubes in this

spectral region. For compounds with lower quantum yields the discrepancy may become even worse. Thus tryptophan is credited with yields between 0.13 (Comparison with quinine sulfate) and 0.20 (scattering method with proportional photon counter). While reexamination of the experimental assumptions used in each case is required, it may be well to recall that many 'absolute fluorescent yields' quoted in the literature are based upon photometric comparison between the unknown fluorescence and some standard compound for which an absolute yield has been independently measured. Such photometric comparisons rely heavily on corrections for the transmission of the monochromator and ^{the} photomultiplier response. Although the usual assumption is that these corrections do not introduce appreciable cumulative errors, the discrepancies between ^{the} values of relative fluorescence yields reported makes it obvious that ⁱⁿ the comparison of the yield of compounds emitting in different regions of the spectrum, a standard error of the order of $\pm 10\%$ is to be expected.

Cause of the variable fluorescence yield.

The upper limit of the fluorescence yield is unity, often approached in practice. The lower limit is never strictly zero as we do not expect radiationless transition processes faster than 10^{13} s^{-1} , and these would not reduce the yield to a value inferior to 10^{-5} . Such very low yields are not measurable in practice. Yields of order of 10^{-2} are estimated without any difficulty and even those of order 10^{-3} can be gauged as a well defined difference between the signals from a specially purified solvent with or without the compound under study. The measurement of ^{yet} smaller yields is increasingly difficult and unrewarding in practice.

As shown by equation (3) the fluorescence yield is determined by the rate of non radiative competing processes operating during the fluorescence lifetime. According to this equation, if $q = 0.5$, an increase of k_d by a factor of 10 would reduce q to ~ 0.1 . Our knowledge of the properties of excited states is far from having reached the stage at which it can predict from either first principles or empirical rules to change in the probability of radiationless transition to this magnitude. Following the initial work of Kasha it is accepted that crossing to the triplet state - the so-called intersystem crossing - is one of the most common processes that reduce the fluorescence yield. The most telling demonstration

of its existence is the reduction of the fluorescence yield upon introduction of heavy atoms in the fluorescent molecules, which should be expected to increase the rate of intersystem crossing through the enhanced spin-orbit coupling, well known from atomic spectroscopy (Herzberg). Thus, introduction of Br or I in the aromatic nucleus leads uniformly to smaller fluorescence yields and chelation of heavy metals by fluorescent compounds produces virtually complete disappearance of the fluorescence. e.g. the Fe or Cu porphyrins, or flavins, in contradistinction to the Mg or Zn adducts.

Population of the triplet state is usually revealed by two different phenomena: Firstly the appearance of transient absorption, lasting microseconds to milliseconds, resulting from transitions from T_1 , the triplet state populated by crossing from S_1 , to the upper triplet states $T_2, T_3 \dots$. These $T \rightarrow T$ transitions like the familiar $S \rightarrow S$ transitions involve no change in multiplicity and are therefore allowed. Secondly, particularly at low temperature the exciting flash is followed by the emission of (phosphorescence) luminescence with spectral distribution at longer wavelengths than the fluorescence and with a lifetime of microseconds to seconds. This emission is phosphorescence that can be traced to the forbidden transition $T_1 \rightarrow S_0$, and for which a lifetime of 10^6 to 10^9 times longer than the $S_0 \rightarrow S_1$ is predicted.

While it is easy to demonstrate qualitatively by these tests that the lowest triplet state of many fluorophores can be populated by excitation to the singlet manifold it is quite another matter to give a good estimate of the rate of this process, and therefore a clear demonstration that the $S_1 \rightarrow T_1$ intersystem crossing is the radiationless process that actually decreases the fluorescence yield. On the other hand there are many well authenticated cases of small or negligible fluorescence, triplet-triplet absorption and phosphorescence, which require for their explanation the supposition of direct $S_1 \rightarrow S_0$ non-radiative transitions. A wealth of indirect evidence, foremost isotopic substitutions and theoretical analysis (Siebrand, Tortner) shows that this process of direct conversion of electronic into vibrational energy is made possible by anharmonic nuclear vibrations, Those involving C-H (or C-D) bonds.

Particularly obscure appear at present the very large variations in yield that many substances particularly some N-phenyl substituted amines undergo in various solvents, which may involve factors of 100 to 1000. We have here specific, quasi chemical interactions which must involve in many cases reversible electron transfer of the excited molecule to a suitable acceptor. Both processes of excited state ^{electron} transfer are well authenticated but the determination of their absolute rates is not, in general, possible.

Decay of the fluorescence polarization after a brief light pulse

If the solution is briefly illuminated with a light pulse lasting only a fraction of the fluorescence lifetime, at any time after the illumination has ceased we can set $f_{||}(t) = f_{\perp}(t) = 0$.

≠ The system (34) can be then solved to give

$$(*) \quad I_{||}(t) = \frac{I_0}{3} \exp(-rt) + \left(I_{||}(0) - \frac{I_0}{3} \right) \exp(-(r+6R)t). \quad (39).$$

$$I_{\perp}(t) = \frac{I_0}{3} \exp(-rt) + \left(I_{\perp}(0) - \frac{I_0}{3} \right) \exp(-(r+6R)t)$$

where $I_0 = I_{||}(0) + 2I_{\perp}(0)$.

Forming the anisotropy $A(t)$, as in (37)

$$A(t) = A(0) \exp(-6Rt). \quad (40).$$

According to (39) and (40) the decay of each of the polarized components is the sum of two exponential terms but the decay of the anisotropy is given by a single exponential. As first realized by Jablonski the decay of the polarized components in real time can be used to determine the rate of rotation of the molecules. The ^{polarized} decays ~~curves~~ (39) permit computation of the apparent lifetimes of the polarized fluorescence $t_{||}^*$ and $t_{\perp}^*$ by means of eq (17, τ), giving

$$t_{||}^* = \tau_0 \frac{1 + 2A(0) (r/(r+6R))^2}{1 + 2A(0) r/(r+6R)} \quad (41)$$

$$t_{\perp}^* = \tau_0 \frac{1 - A(0) (r/(r+6R))^2}{1 - A(0) r/(r+6R)}$$

Equations (41) show that if $A_0 > 0$, the apparent lifetime of the parallel component is decreased and that of the perpendicular component is increased with respect to τ_0 , and that the opposite happens if $A_0 < 0$. These differences in the decay times may be explained as follows: The fluorescence intensity observed through a polarizer oriented to favor one axis, decays as a result of two processes, emission and rotation, because a rotation of 90° makes the subsequent emission unobservable through the fixed polarizer. At the instant of the excitation if $P_0 > 0$, $I_{||} > I_{\perp}$, therefore $I_{||}$ loses to $I_{\perp}$ by rotational diffusion more than it receives from it, the net result being $t_{||}^* < \tau_0$. The converse is true for $I_{\perp}$, so that $t_{\perp}^* > \tau_0$. Evidently if $P_0 < 0$, $I_{||} < I_{\perp}$ and the opposite relations follow: $t_{||}^* > \tau_0$, $t_{\perp}^* < \tau_0$. If the observing polarizer is set at an angle θ to $I_{||}$ such that $\cos^2 \theta = \frac{1}{3}$, ($54^\circ 16'$) we have.

$$I_{54}(t) = \frac{1}{3} I_{||}(t) + \frac{2}{3} I_{\perp}(t).$$

$$I_{54}(0) = \frac{I_0}{3} \quad (42)$$

and the second term in (34) disappears. We have by this means weighted the components so that there is neither loss nor gain of intensity by the rotations. The observed decay is then τ_0 . This gives a practical method to measure the fluorescence lifetime free from the errors that can be introduced by the rotational motion of the molecules. In fact, in the absence of an observation polarizer, the decay of the fluorescence emitted at right angle to the polarized excitation equals $I_{||}(t) + I_{\perp}(t)$, or according to (38),

* For excitation with natural light ($\tau_0 \leq 0.15$) the overestimation would be at most 6%.

$$I(t) = \frac{I_0}{3} \left(\exp - \Gamma t + A_0 \exp - (\Gamma + 6R)t \right) \quad (43).$$

The error incurred by ignoring the second term is maximal when $\Gamma \sim 6R$. For excitation with a wavelength yielding a high limiting polarization ($A_0 > 0.3$) it would then lead to an overestimation of the fluorescence lifetime of 10%.

Equations (41) show that whatever the value of $A(0)$ the polarized decays will approach to if either $6R \gg \Gamma$ or $6R \ll \Gamma$. In the first case the rotational diffusion erases the difference between $I_{||}$ and $I_{\perp}$ before any emission can take place, In the second the rotations are so slow that the fluorescence decays without appreciable exchange of intensity between the components. The greatest effect of the rotations are observed when $\Gamma = 6R$.

These effects would be maximal for $A(0) = 2/5$ and in that case equations (41) give $t_{||}^* = 6/7 * \tau_0$; $t_{\perp}^* = 9/8 * \tau_0$. or $t_{\perp}^* / t_{||}^* = 1.31$. From these figures it can be gauged that quite precise observations of the rate of polarized decay are necessary to obtain a reliable value of the rotational rate.

Although the rate of emission is absent in eq (40) the range of values of R that is determinable by anisotropy measurements is critically related to the fluorescence lifetime. The upper limit of measurable R is determined by the width of the exciting pulse and the response time of the equipment. With to-day's means a typical value would be $1-2 \cdot 10^9 \text{ s}^{-1}$. The lower limit of measurable rate of rotation is determined by the precision with which an anisotropy value reliably different from A_0 may be measured long time after excitation. The error of an intensity measurement increases with the increase of the square root of the intensity, and as the fluorescence intensity decays exponentially with time, if the error of measurement of A_0 at $t=0$ equals dA_0 , its measurement at time t after excitation entails a standard error $dA_0 \exp(\tau t/2)$. A measurement $A(t)$ will significantly differ from A_0 if

$$A_0(1 - \exp(-6Rt)) \geq 3dA_0 \exp(\tau t/2)$$

In the limit,

$$1 \geq \frac{3dA_0}{A_0} \exp(\tau t/2)$$

$$t \leq \frac{2}{\tau} \ln\left(\frac{A_0}{3dA_0}\right)$$

If $dA_0/A_0 = 0.03$, a very good performance,

$$t \leq 4.6 \tau.$$

Beyond four and a half lifetimes the error is too large to permit determination of a significant decrease in the anisotropy

At this time we require

$$\frac{A_0}{A(t)} \sim 1.1 = \exp(6R\tau \times 4.6).$$

which gives

$$6R\tau \sim \frac{1}{45} \quad \text{or} \quad R \sim \frac{1}{250}$$

The slowest rotational rate detectable would be of the order of 10^6 s^{-1} .

Polarized fluorescence under sinusoidal excitation.

In this case $f_{||}(t)$ and $f_{\perp}(t)$ are periodic functions of the time. If excitation is by a single beam of light we can write

$$f_{||}(t) = \gamma_{||} f(t).$$

$$f_{\perp}(t) = \gamma_{\perp} f(t).$$

If $f(t)$ depends upon a single frequency $f = \omega/2\pi$,

$$f(t) = a + b \sin \omega t. \quad (44)$$

where $1 > b/a > 0$, represents the modulation of the excitation.

The solution of the system (34) under these excitation conditions constitutes the 'forced response' of the polarized components to a periodic impulse. It can be expected to have the form shown in (27c), namely

$$I_{||}(t) = A_{||} + B_{||} \sin(\omega t + \delta_{||}).$$

$$I_{\perp}(t) = \dots \quad (45)$$

This method of solution would lead to a set of equations like (28c). It is simpler to derive the relevant quantities, phase and modulation, employing the relation between the impulse and harmonic responses (36), (37). For the phase delays there give:

$$\tan \delta_{||} \equiv \omega \tau_{||}^P = \frac{\omega}{\Gamma} \left(\frac{1 + 2A_0\beta}{1 + 2A_0\beta(1 + 6R/\Gamma)} \right) \quad (46)$$

$$\tan \delta_{\perp} \equiv \omega \tau_{\perp}^P = \frac{\omega}{\Gamma} \left(\frac{1 - A_0\beta}{1 - A_0\beta(1 + 6R/\Gamma)} \right)$$

$$\text{where } \beta = \frac{\Gamma^2 + \omega^2}{\omega^2 + (\Gamma + 6R)^2} \quad (47)$$

The above relations show that $\tan \delta_{||}$ and $\tan \delta_{\perp}$ are not simply proportional to the frequency, so that the equations

$$\tan \delta_{||} = \omega t_{||}^* ; \tan \delta_{\perp} = \omega t_{\perp}^* \quad (48)$$

with $t_{||}^*, t_{\perp}^*$ given by eqs (41) are not generally valid. However, if the frequency of the excitation is sufficiently small so that ω^2 may be neglected in comparison with Γ^2 eqs (46) reduce to eqs (41). For this reason the polarized lifetimes derived from the $\sin$ harmonic response (Eqs (46)) should be carefully distinguished from the quantities obtained from the impulse response (Eqs (41)). The former are designated $\tau_{||}^P, \tau_{\perp}^P, \tau_{||}^M, \tau_{\perp}^M$ to denote their origin from phase or modulation data. The strong dependence of these quantities upon the frequency of the excitation, when this frequency is comparable to, or higher than Γ , is easy to understand: as $\omega\tau$ rises above 1 the fluorescence becomes progressively demodulated and the modulated part is the result of emissions at times progressively shorter than τ . It is at these times that the polarized components differ the most and can thus generate the larger difference in apparent lifetime by the rotational exchange. These effects are very appreciable. For $\omega/\Gamma = 2$, and $\Gamma = 6R$, $A_0 = 2/5$, conditions that can be approached in practice, $\tau_{||}^P = 3/4$, $\tau_{\perp}^P = 3/2$ and $\tau_{\perp}^P/\tau_{||}^P = 2$, a value that greatly exceeds the

(maximum ratio 1.31 of $t_{\perp}^*/t_{\parallel}^*$ - Fig shows a plot of $\tau_{\parallel}^P/\tau_0$ and $\tau_{\perp}^P/\tau_0$ as a function of the ratio $6R/(\Gamma+6R)$. For reasons already discussed these ratios approach unity when $6R \ll \Gamma$ or $6R \gg \Gamma$. The maximum splitting of the polarized decays $(\tau_{\parallel}^P - \tau_{\perp}^P)/\tau_0$ is found at $\Gamma \sim 6R$ when $\omega\tau \sim 1$ but shifts to higher rotational rates as ω increases above Γ .

Differential polarized phase fluorescence

Instead of the separate measurements of the phase and modulation of each polarized components with respect to the exciting light one can make a direct measurement of the phase difference or the modulation ratio of one with respect to the other. Such a procedure offers in principle the advantages of all differential methods, of reducing the errors arising from the different magnitudes of the quantities subtracted and their difference, and virtually eliminating those errors that arise from successive rather than simultaneous measurement of the subtracted quantities. In this particular case a further source of error is completely eliminated; the one arising from a residual phase difference between the exciting light and fluorescence owing to differences in the time response of the photomultiplier to quanta of different energy.

The differential phase $\delta_{\parallel} - \delta_{\perp}$ can be calculated by means of the equation

$$\tan \Delta \equiv \tan(\delta_{\parallel} - \delta_{\perp}) = \frac{\tan \delta_{\parallel} - \tan \delta_{\perp}}{1 + \tan \delta_{\parallel} \tan \delta_{\perp}} \quad (49)$$

together with (46), or more directly from the Fourier transforms (36 τ) and (37 τ) which give for it

$$\tan \Delta = (P_{\parallel} Q_{\perp} - P_{\perp} Q_{\parallel}) / (P_{\parallel} P_{\perp} + Q_{\parallel} Q_{\perp}) \quad (50)$$

In this case, after some elementary simplifications

$$\tan \Delta = \frac{-2R\omega A_0}{\frac{1}{9}(\Gamma^2 + \omega^2)(1-A_0)(1+2A_0) + \frac{2}{3}(2+A_0)R\Gamma + 4R^2} \quad (51)$$

Replacing Γ by $1/\tau$ and writing (51) as a quadratic equation in $x \equiv 2R\tau$,

$$x^2 + x \left\{ \left(\frac{2+A_0}{3} \right) - \omega\tau \left| \frac{A_0}{\tan \Delta} \right| \right\} + m_0^2 = 0. \quad (52)$$

$$\text{where } m_0 = \frac{1}{3} \sqrt{(1+\omega^2\tau^2)(1-A_0)(1+2A_0)}$$

The sign of $\tan \Delta$ is determined by those of $\tan \delta_{||}$ and $\tan \delta_{\perp}$. Both are negative as they represent delay with respect to the emission. If $A_0 > 0$, $|\tan \delta_{||}| > |\tan \delta_{\perp}|$ and $\tan \Delta > 0$. If $A_0 < 0$, $\tan \Delta < 0$. Therefore $A_0/\tan \Delta > 0$ whether $A_0 \leq 0$. Because of this we employ the absolute value $|A_0/\tan \Delta|$ in eq. (52).

Fig. shows plots of $\tan \Delta$ against $\log 2R\tau$ for values of $\omega\tau = 1, 2, 3$ and $P_0 = 0.40$ ($A_0 \sim 0.30$). The quadratic dependence of $\tan \Delta$ upon $2R\tau$ implies that one value of $\tan \Delta$ is compatible with two values, sometimes very different of $2R\tau$. To decide between them one only needs to remember that R is a monotonically increasing function of the temperature. Therefore a second measurement of $\tan \Delta$ at a sufficiently higher temperature permits to select the unique values of $2R\tau$ corresponding to the two values of $\tan \Delta$. The maxima seen in the plots of $\tan \Delta$ against $\log 2R\tau$ correspond to

$$\tan \Delta_{\max} = \frac{3\omega\tau A_0}{A_0 + 2(1 + 3m_0)} \quad (53)$$

and occurs at a value of $2R\tau$ given by.

$$(2R\tau)_{\max} = m_0. \quad (54).$$

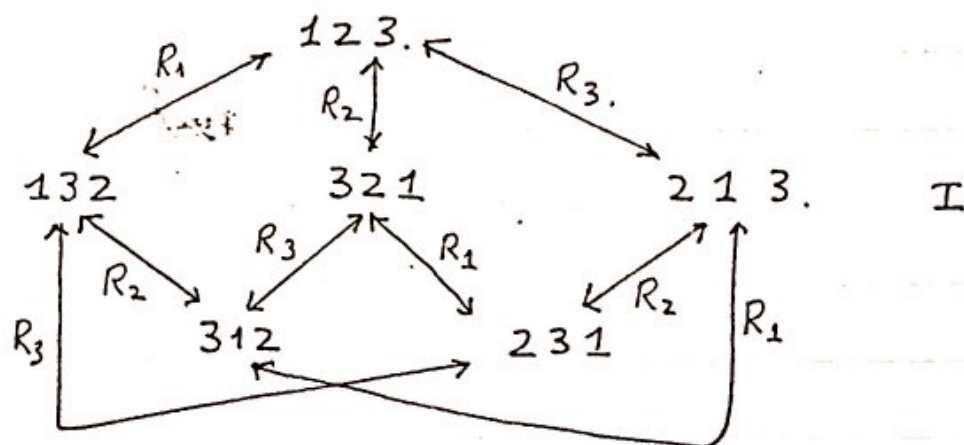
At values of $\omega\tau$ conspicuously higher than 1 $\tan \Delta_{\max}$ approaches a limit of practically $\sim 25^\circ$. Eq (53) shows that $\tan \Delta_{\max}$ is independent of R and therefore the plots of all spherical rotors differ only in the value of the temperature and viscosity of the solvent at which $\tan \Delta_{\max}$ is found.

This maximum (Eq (53)) is not obtainable if two spherical fluorophores of different size are responsible for the emission. The effect of the heterogeneity of rotational rates is shown in fig which give the plot of $\tan \Delta$ for a mixture of two spherical fluorophores contributing equally to the emitted intensity and with volume ratios that vary from 1:1 to 100:1. Volume ratios of 3:1 are required for appreciable effects, but at higher ratios these effects are very pronounced and at volume ratios higher than 20:1 a splitting into independent curves is noticeable.

Changes in orientation during anisotropic diffusion. -

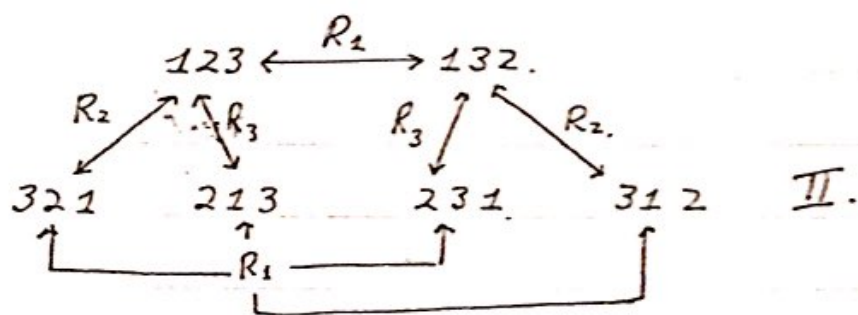
To facilitate the intuitive understanding of the anisotropic molecular motions we shall adopt a description based on the assumption that only a small number of molecular orientations are allowed and consider the possible ways in which these permissible orientations can be reached. For this purpose we attach to the molecule a rigid coordinate system with axes numbered

1, 2 and 3 respectively. The permissible orientations of our molecule are those that bring into coincidence in any manner these molecular coordinates and the laboratory coordinates X, Y and Z . If axis 1 is brought into coincidence with X , axis 2 must coincide with either Y or Z . The two orientations so generated will be called 123 or 132 respectively as we agree that the ordering of the triad discloses the molecular axes coincident with X, Y and Z respectively. Evidently coincidence of X and 2 generates the orientations 213 and 231 and coincidence of X and 3 generates the two final orientations 312 and 321. These six orientations constitute the permitted set. The process of diffusion starting with one fixed orientation e.g. 123 takes place as indicated in the scheme below. (I).



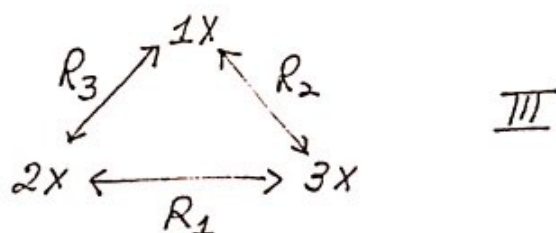
123 generates the orientations 132, 321, 213 by rotation about the axes 1, 2, and 3 respectively. Evidently these first generated orientations can appear at very different rates if R_1 , R_2 and R_3 , differ greatly. On the other hand the two remaining orientations 312 and 231 must appear and grow at the same rate since they both arise from the three pairs of possible successive rotations R_1, R_2 ; R_2, R_3 ; R_3, R_1 .

Any experiment that reveals anisotropic diffusion must generate by some means a population of oriented molecules and follow its decay into a final distribution of orientation, which in the case of free diffusion is one of random alignment. There exists no experimental method which permit selection of one alone among our six orientational states. Optical selection or application of an external electric field can only result in a population in which one direction is preferentially aligned. and the diffusion process as schematized below (II)



In photoselection, direction 1 may be considered as that of the absorption oscillator of the molecules, and the pair 123, 132 corresponds to the set of molecules preferentially excited. Rotation about the direction of the absorption oscillator, at rate R_1 , results in exchange between these two. The two members of the pair 321, 312 on one hand, and the members of the pair 213, 231 ^{on the other}, must each appear at the same rate, as each pair "is generated" by rotation about the same axis (R_2 or R_3) from 2 species, 123 and 132 which exist at all times in identical amounts. Detection of the anisotropic process will be possible only if the two pairs appear at different rates, which in turn requires R_2 and R_3 to be perceptibly different. At the beginning of the diffusion, rotation about R_1 does not contribute to the disorientation, but at further times: - it "works" to equalize the concentration of the species in the second row of the Scheme II. -- If R_1 is comparable to the faster of the other two rates, differences in the amounts of the species of the second row will be difficult or impossible to detect. In other words not only we need $R_2 \gg R_3$ (or $R_3 \gg R_2$) but also $R_3 \gg R_1$ (or $R_2 \gg R_1$). Evident anisotropic diffusion will be observed if our rate is appreciably faster

than the other two, but not if two rates are much faster than the third. The scheme II. can be simplified further: It will be noticed that $123 + 132 = 1X$, the set of molecules with coincident 1 and X, regardless of other features. Similarly $213 + 231 = 2X$ and $312 + 321 = 3X$, and the diffusion process may be reduced to scheme III.



Specification of the proportions of components $1X$, $2X$, $3X$ at any given time after excitation is sufficient to construct the Λ matrix (29 π) which contains the complete information about the orientations. Notice however, that according to scheme III one cannot expect to observe more than two successive decay processes in any experiment. The exact solution of the rotational diffusion equations for molecules devoid of symmetry shows that this is not the case, but also permits the conclusion that observation of more than two decay processes is experimentally very difficult if not impossible. Thus consideration of a discontinuous diffusion process is ~~all~~ virtually all that is required in practice.

The inertial ellipsoid.

Molecules have an almost infinite variety of shapes but it is not necessary to refer to them explicitly in considering their motions as revealed by the different experimental methods.

Reciprocally these motions cannot directly give information about the molecular shape but only about the inertial characteristics of the molecule that are required to produce the experimental effects. At best one can only reconstruct the so-called inertial ellipsoid associated to the molecules. This is an ideal "rigid body of ellipsoidal shape which, subjected to a given impulse will perform a motion of amplitude and rate equal to those of the molecule that it represents. The rotational rates about the principal axes of the ellipsoid are R_1, R_2, R_3 respectively. Their arithmetic mean $R = (R_1 + R_2 + R_3)/3$ is the average rotational rate of the body and the relative residuals are defined by the equations

$$\delta_i = (R_i - R)/R \quad (55)$$

$$i = 1, 2, 3.$$

The variance of the rates is

$$V = \sum_i \delta_i^2 \quad (56)$$

It will be seen that for our purpose it is convenient to define a similar quantity, σ^2 as one sixth of the variance,

$$\sigma^2 = \frac{1}{6} \sum_i \delta_i^2. \quad (57)$$

By the definition of average,

$$\sum_i \delta_i = 0 \quad (58)$$

** These are the ellipsoids of revolution, oblate and prolate, and the totally asymmetric ellipsoid.

so that one of the three residuals may be eliminated in the last but one equation, giving

$$3\sigma^2 = \delta_i^2 + \delta_j^2 + \delta_i\delta_j \quad (59)$$

The last equation is that of an ellipse. Each point on the ellipse defines an inertial ellipsoid of characteristic variances but different ratios of the axis. The points numbered in fig. correspond to models of particular interest.

* * Here! are the oblate ellipsoid, the prolate ellipsoid and the totally asymmetric ellipsoid. In the ellipsoids of revolution there are only two principal rates. R_{eq} for rotation about an equatorial axis and R_{ax} for rotation about the axis of revolution, and $R = (2R_{eq} + R_{ax})/3$. It follows from eqs (55) (57) that an oblate ellipsoid of revolution has $0 < \sigma \leq 1/2$, and a prolate ellipsoid of revolution $0 < \sigma \leq 1$. Intermediate between the figures of revolution is the ellipsoid of maximum asymmetry in which one rate coincides with the average rate and the other two diverge equally giving, $\delta_1 = 0$, $-\delta_2 = \delta_3 = \pm\delta$. For this model $\sigma = |\delta|/\sqrt{3}$ and since $|\delta| < 1$ $0 < \sigma < 1/\sqrt{3}$ (0.577...). Even in this extreme case the totally asymmetric ellipsoid is close to the oblate spheroid of revolution. Values of σ greater than $1/\sqrt{3}$ are possible only if one rate becomes progressively larger than the other two, thus approaching the prolate ellipsoid of revolution. For molecules with $\sigma > 0.7$ we can speak without ambiguity of prolate molecules, or molecules in which the rate of

(**

The ellipse is represented in fig. 2. Its axes are at 45° of the coordinate axis, and their lengths are in the ratio $1/\sqrt{3}$. Rotational models with differences significant enough to define distinct rotational types are those at points in the ellipse numbered 1 to 6. At points 2 and 6 one of the residuals, δ_i or δ_j , become zero and the remaining two must then be equal and of opposite sign. This also obtain at 4, where $\delta_i = -\delta_j$ and thus $\delta_k = 0$. These points 2, 4 and 6 represent the 'most asymmetric ellipsoid' in which one rate of rotation coincides with the mean and the other two diverge by equal and opposite amounts. Points 1, 3 and 5 (and their conjugate 1', 3', 5') define degenerate ellipsoids of inertia in which two of the principal rotational rates become equal. At points 3 and 5' δ_j and δ_i attain their maximum value, It can be seen from equation (59) that the condition of maximum for δ_i implies $\delta_j = -\delta_i/2$. Thus 1', 3 and 5' represent prolate ellipsoids of revolution in which the non-degenerate principal rate of rotation is the first rate, while 1, 3' and 5 represent oblate ellipsoids of revolution with a non-degenerate slow rate. It is seen that the twelve points numbered in the figure correspond to three significant rotational types only.

rotation is eight or more times faster than that of the other two. It is in these molecules that we expect to observe evident anisotropic rotations.

Impulse response of the fluorescence anisotropy in irregular molecules.

Given an inertial ellipsoid with rotational rates R_1 , R_2 and R_3 about the principal axes, and the orientation of the absorption and emission oscillators with respect to these axes, diffusion theory permits the calculation of the impulse response of the polarized components of the emission to an instantaneous excitation. (Belford et al. Chuang and Eisenthal, Ehrenberg and Rigler). These may be written as

$$I_{||}(t) = e^{-t/\tau} \left(\frac{1}{3} + S(t) \right) \quad (60)$$

$$I_{\perp}(t) = e^{-t/\tau} \left(\frac{1}{3} - \frac{S(t)}{2} \right) \quad (61)$$

The anisotropy of the emission decays with time according to the equation

$$A(t) = \frac{3}{2} S(t) \quad (62)$$

with

$$S(t) = \sum_{i=1}^5 C_i \exp(-m_i t) \quad (63)$$

Let α_j, ϵ_j ($j=1,2,3$) designate the direction cosines of the transition moments in absorption and emission respectively, with the principal axes of the inertial ellipsoid, then.

$$C_j = \frac{4}{5} \alpha_K \alpha_L \epsilon_K \epsilon_L \quad ; \quad m_j = 6R \left(1 + \frac{\delta_j}{2} \right) \quad j=1,2,3 \quad j \neq K \neq L \neq j$$

$$C_4 = \frac{1}{5} (F+G) \quad ; \quad m_4 = 6R(1-\sigma) \quad (64)$$

$$C_5 = \frac{1}{5} (F-G) \quad ; \quad m_5 = 6R(1+\sigma) \quad (65)$$

In the last equations

$$F = \sum_{j=1}^3 \alpha_j^2 \epsilon_j^2 - \frac{1}{3}; \quad G = \frac{1}{3\sigma} \sum_{j=1}^3 \delta_j (\alpha_j^2 \epsilon_j^2 + \alpha_k^2 \epsilon_l^2 + \alpha_l^2 \epsilon_k^2) \quad (6)$$

and δ_i and σ defined by equations

of the decay constants m_1 to m_5 .

Since $\delta_i + \delta_j + \delta_k = 0$,

$$(\delta_j + \delta_k)^2 = \delta_j^2 + \delta_k^2 + 2\delta_j\delta_k = \delta_i^2 \quad (67)$$

Also

$$\delta_j^2 + \delta_k^2 \geq 2\delta_j\delta_k$$

and

$$\delta_j^2 + \delta_k^2 \geq \delta_i^2/2.$$

or

$$\sigma^2 = \frac{1}{6}(\delta_i^2 + \delta_j^2 + \delta_k^2) \geq \frac{3}{12} \delta_i^2$$

and

$$\sigma^2 \geq \delta_i^2/4 \quad \text{or} \quad \sigma \geq \frac{|\delta_i|}{2} \quad (68)$$

Therefore of the five decays ^{the} slowest and fastest are m_4 and m_5 respectively, and the other three are bracketed by these two.

~~An ellipsoid of revolution is characterized by $\sigma = (1 + \delta_{ax}) / (1 + \delta_{eq})$ where δ_{ax} and δ_{eq} are the relative residuals of the rotational rate about the axis of revolution and an equatorial axis respectively. δ_{ax} is greater than zero for a prolate ellipsoid and δ_{eq} is less than zero for an oblate ellipsoid and in either case $\delta_{eq} = -\delta_{ax}/2$. The upper limits for σ are in consequence 1 and $1/2$ for the prolate and oblate ellipsoids of revolution respectively. With these values eq. show that~~

The ratio m_4/m_5 can become arbitrarily small as σ approaches unity. In practice this means that if m_4 is comparable to Γ

the rate of emission, $\dots m_5$ will be negligible relative
 to m_4 and the polarization will decrease to a value that remains constant for the
 remaining part of the emission. In contradistinction the
 extreme oblate ellipsoid with $\sigma = 1/2$ is characterized by a ratio
 m_4/m_5 of only $1/3$ which in the present state of the
 experimental art is barely discernable from the case of isotropic
 rotation. An inertial ellipsoid of maximum asymmetry has $\sigma \leq 1/\sqrt{3}$
 and the ratio m_4/m_5 is only marginally smaller than for
 an oblate ellipsoid of revolution. Thus, as we foretold
 from an examination of the discontinuous process, the molecules
 that show evident anisotropic rotation are those in which one
 principal rate of rotation is ^{much} faster than the other two, and these
 are represented by prolate ellipsoids of inertia of which the
 limiting case is the prolate ellipsoid of revolution.

In an ellipsoid of revolution the two orthogonal equatorial
 diameters can serve as principal axes and these can be
 oriented so that one of them is normal to the absorption
 or emission oscillator, giving $\alpha_3 = 0$ or $E_3 = 0$, and
 reducing the non-zero amplitudes to those of the lines with
 decay ^{constant equal to} $6R(1+\sigma)$, $6R(1-\frac{\sigma}{2})$ and $6R(1-\frac{\sigma}{2})$. Even
 when $\sigma \rightarrow 1$ the first two will be in a ratio smaller than
 $4/1$ and can be considered unresolvable under ordinary,
 and even extraordinary experimental conditions.

(Compute lines here and plot decays to show).

Figure shows the decay of the fluorescence anisotropy with time, measured in $1/6R$ units, or 'correlation times', for a prolate ellipsoid of revolution with $\sigma = 0.9$ and parallel oscillators of absorption and emission at a variable angle α to the axis of revolution. As might be expected there is little curvature in the plot of $\ln(A(t)/A(0))$ for $\alpha = 45^\circ$ or less as we approach in these conditions the case of a spherically symmetric rotor. The largest curvature is observed for the ellipsoid containing the oscillators in the equatorial plane. ($\alpha = 90^\circ$) and this is only moderately different from the case with $\alpha = 75^\circ$ although the time dependent anisotropy results from the sum of two exponential terms in the former case and three in the latter. A decision between these two cases requires an analysis of the portion of the decay at times $t > 2/6R$ since for shorter times the curves are well-nigh indistinguishable. Optimal conditions for the observation of anisotropic rotational effects are met for $\tau \sim 1/6R$, so that relevant differences between the two curves occur at $t > 2\tau$.

The precision that can be achieved in the measurement of the time-dependent anisotropy following a light pulse is determined by both its absolute value and the intensity of the emission, or equivalently, ^{by} the number of photons counted to determine it.

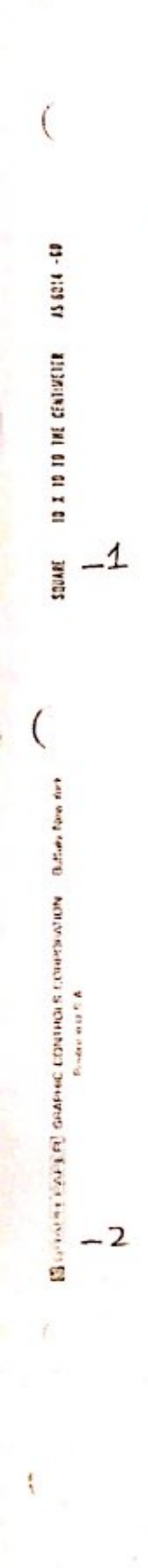
Setting $\rho \equiv I_{\parallel}/I_{\perp}$, (69)

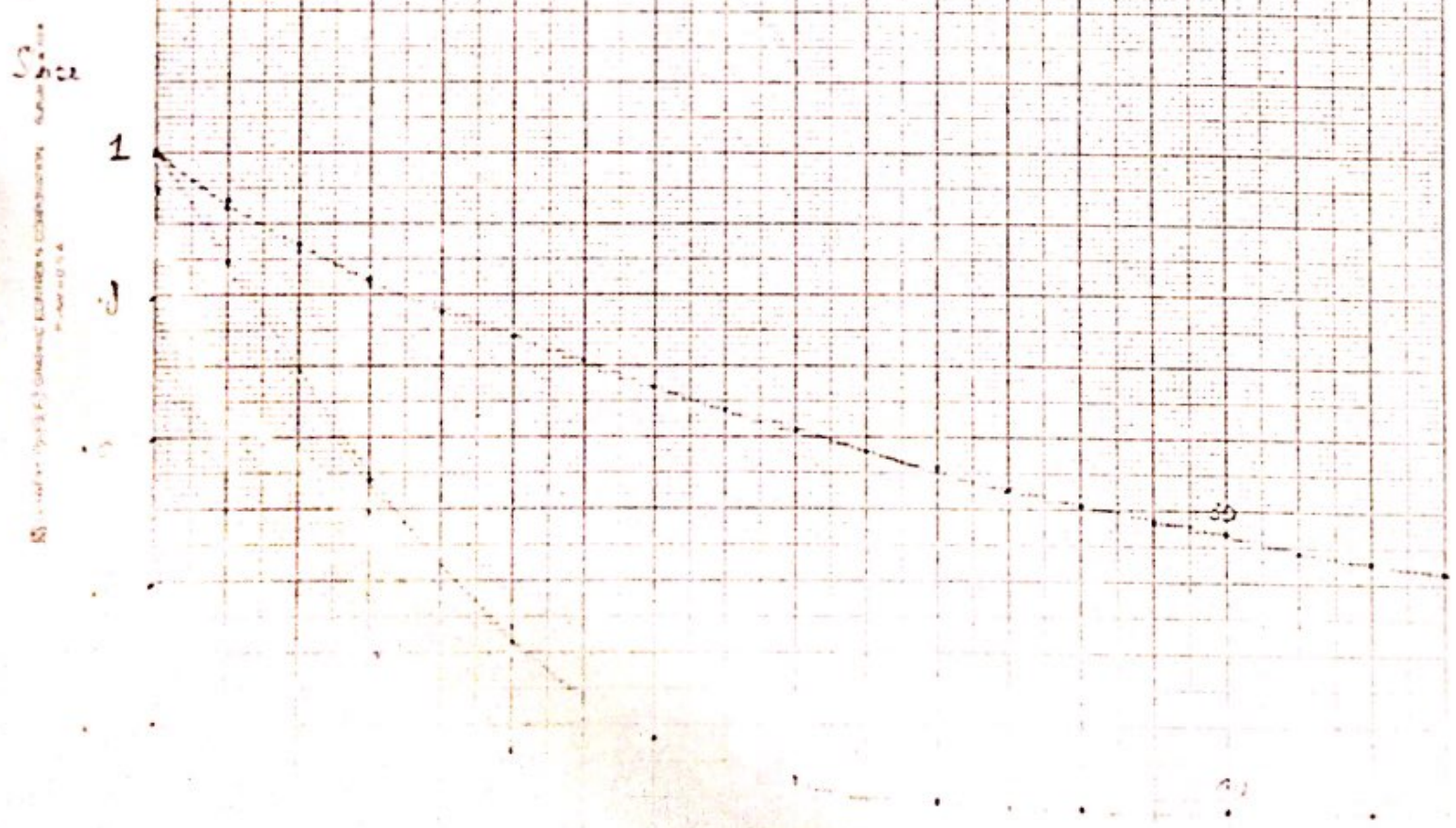
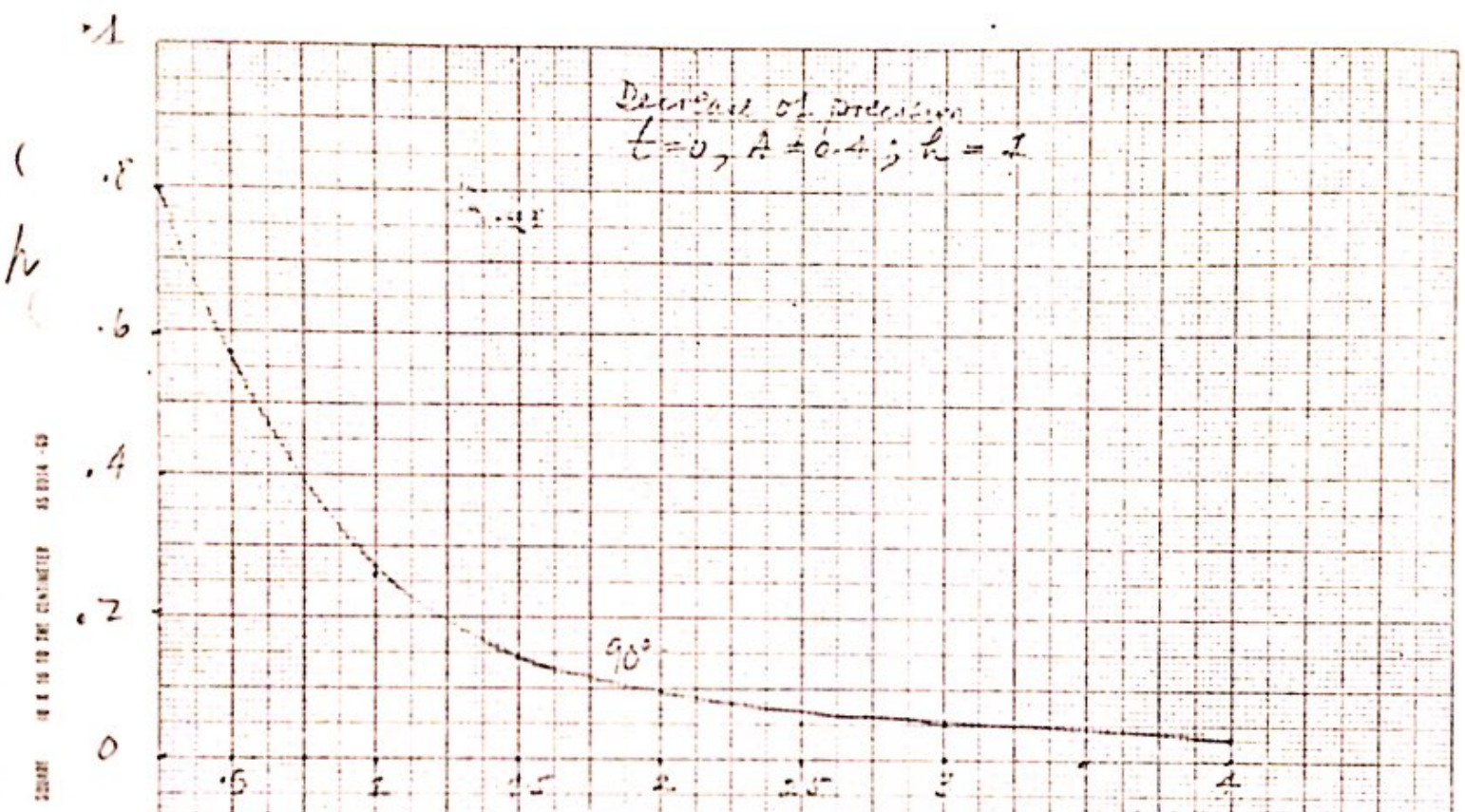
$$\rho = (1 + 2A)/(1 - A)$$

and $d\rho/\rho = [3A/(1-A)(1+2A)] dA/A \approx 3A d\ln A$. (70)

0.125

()





72 $\frac{F_{10}}{A}$

Decay of Intensity in Ellipsoid of Revolution

$$\kappa = \epsilon = 90^\circ \quad \beta = 0$$

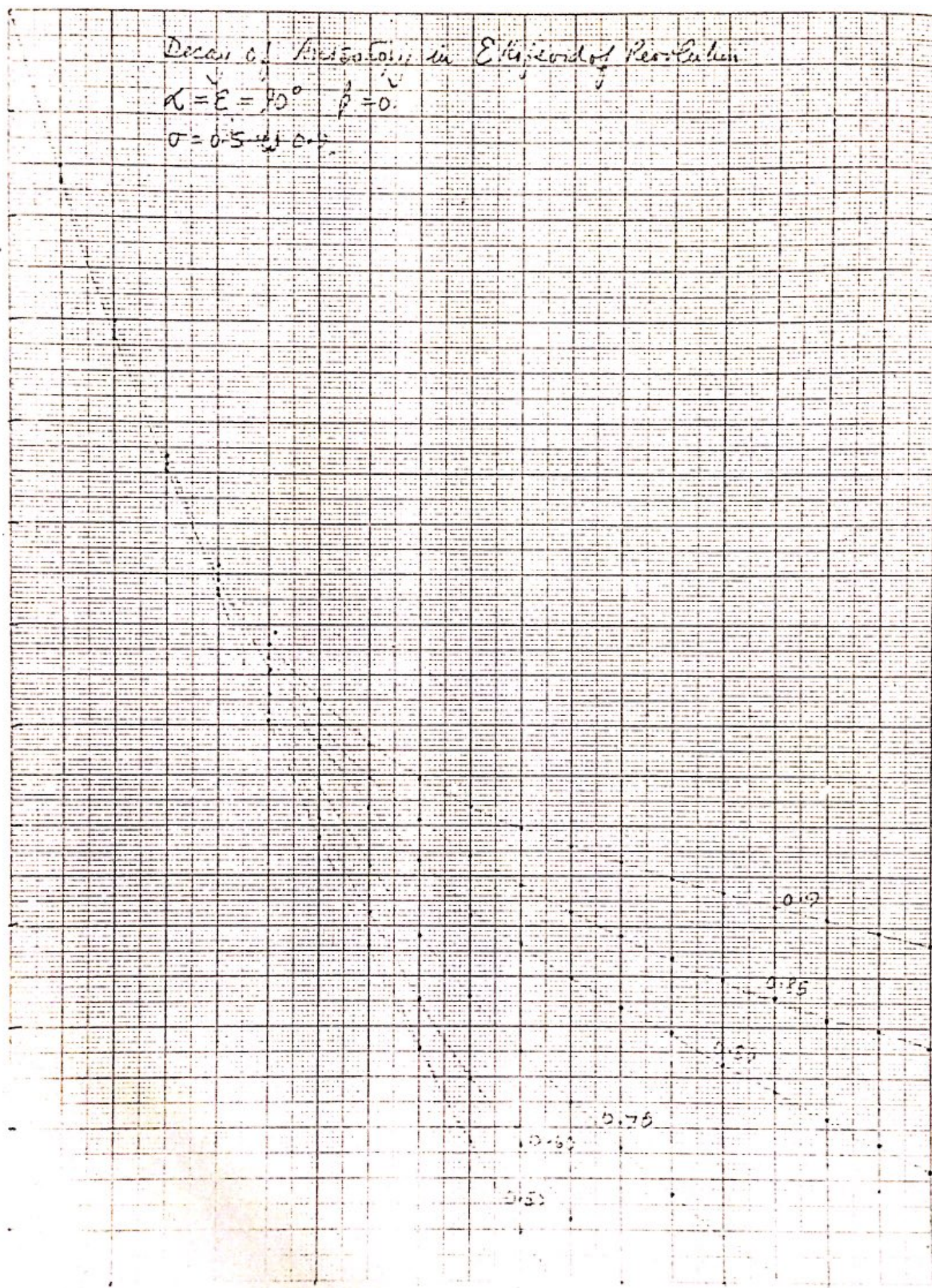
$$\sigma = 0.5 - 0.4 \text{ cm}^{-1}$$

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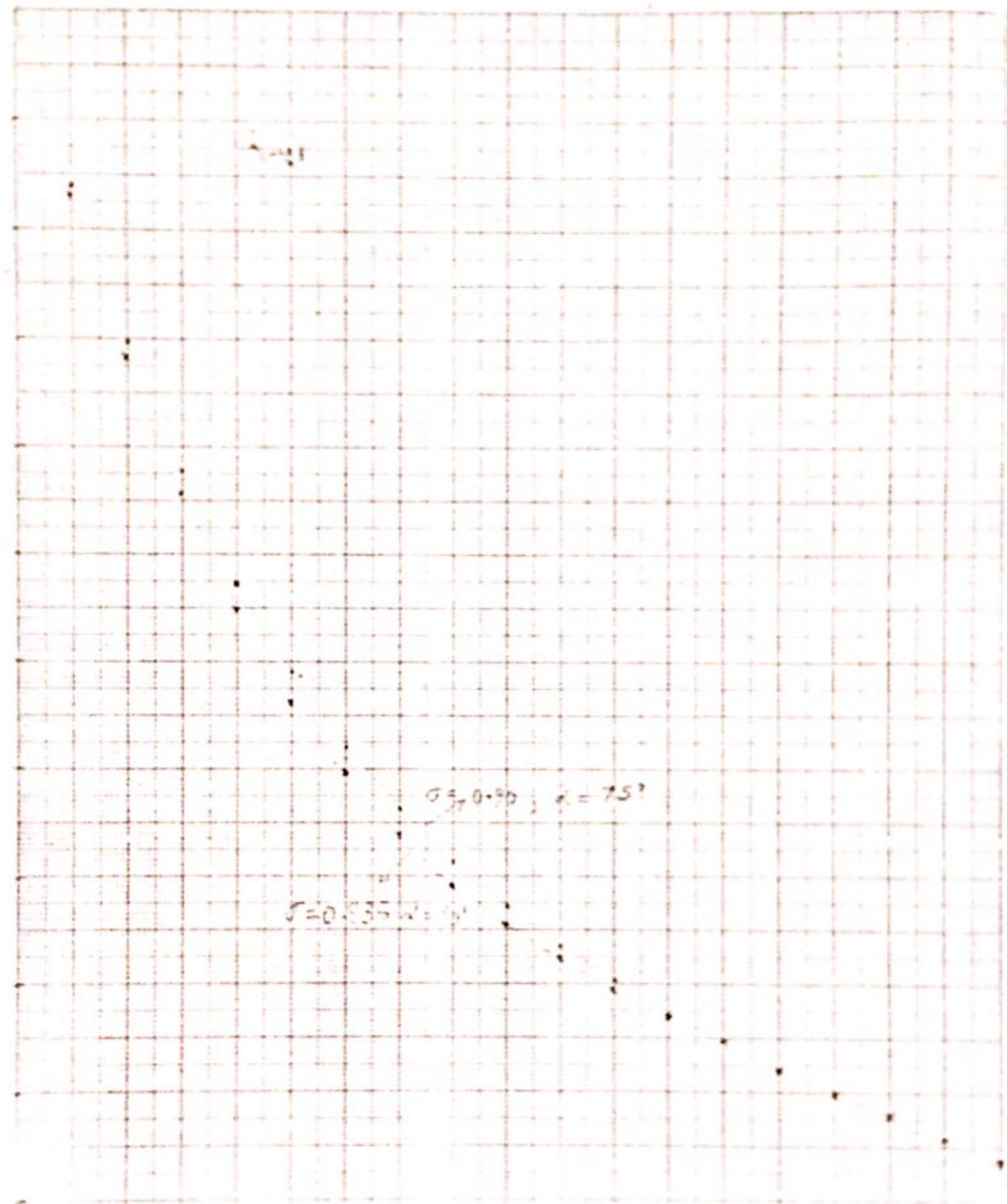
$$d\rho/\rho \approx \sqrt{\frac{2}{N_I(t)}} = \sqrt{\frac{2}{N_I(0)}} * \exp(t/2\tau). \quad (71)$$

where $N_I(t)$ and $N_I(0)$ are the numbers of photons counted as perpendicular polarized intensity at times t and 0 respectively. From the last two equations

$$d \ln A(t) \approx \frac{1}{3A(t)} * \exp(t/2\tau) * \sqrt{\frac{2}{N_I(0)}}. \quad (72)$$

As time increases, the precision of the measurements of the α polarized components decrease exponentially with $t/2$ and compounds the imprecision caused by decrease in the absolute value of the anisotropy. In the curves shown in fig. the precision in the measurement of $\ln A(t)$ has dropped to $1/10^{\text{th}}$ of the precision of $A(0)$ at $t=2\tau$ and $1/30^{\text{th}}$ at $t=4\tau$. Thus we do not expect in practice to be able to distinguish the additional decay term present for $\alpha=75^\circ$. It should be noticed that we have chosen one of the most favorable cases to detect this additional decay term as the three decay constant for the ellipsoid of revolution with $\sigma=0.9$ are in the ratio $1:5.5:19$ and the three amplitudes are significant for $\alpha=75^\circ$. Moreover the decay curve for $\alpha=75^\circ$ $\sigma=0.9$ can be approximated further by the two term curve by choosing $\alpha=90^\circ$, $\sigma=0.835$, as shown in figure. Discrimination between these two curves would require $d \ln A = \pm 0.033$ at $t=1.5\tau$, where $A(t)=0.1$. and according to the last equation, $N_I(0) \approx 6. \times 10^5$.

ln $\frac{1}{\rho}$



$$G = 0.90; k = 7.5?$$

$$r = 0.335 W = 10$$

The insufficiency of the decay data to single out a unique inertial ellipsoid should not surprise us. According to eq. 62-65 complete determination of the system requires no less than seven independent parameters but the experimental data can furnish at best two decay constants and two amplitudes, plus the angle between oscillators known from the limiting polarization. Employing for the analysis of the data the model of an ellipsoid carrying an oscillator along a principal axis, we can obtain from the two decay rates observed a characteristic value of σ . This will always be a minimum value for the real inertial ellipsoid, because the two decay constants of our assumed model are the largest and smallest of those appearing in the equation for the general ellipsoid ((63)-(65)) and the effect of the intermediate terms would be to reduce the apparent difference between the extreme ones. The minimum value of σ thus calculated may be sufficient for practical purposes. Particularly if $\sigma > 0.7$ we can conclude with certainty that the ellipsoid is a prolate one, in which one rate of rotation greatly exceeds the other two. Referring to the example of figure we find that the calculated values of σ would vary in these cases from 0.33, for $\alpha = 45^\circ$ to 0.85 for $\alpha = 90^\circ$. Such figures are reasonable if one remembers that they characterize not the inertial ellipsoid but a 'depolarization ellipsoid' in which the apparent decay rates are determined by the ^{of the oscillations} orientation with

respect to the principal axes of rotation.

	Slope at 5π	Slope at 0.25π .	σ
30	0.15921	0.40268	0.433
45	0.35131	0.70515	0.332.
60	0.45697	1.0002	0.372.
75	0.150	1.20616.	0.778
90	0.10066	1.2784	0.854.

The effects of the variable amplitudes C_1 to C_5 .

In the above discussion we have not referred to the amplitude of the several terms, assuming them ^{to be} to

be sufficient to permit detection and comparison of the decay rates. The amplitudes are primarily dependent upon the orientation of the oscillators. If either of these coincide with a principal axis of inertia the first three terms are eliminated as $C_1 = C_2 = C_3 = 0$. The decay has only two terms, if the coincidence axis is not the axis of revolution of the ellipsoid, and these two terms are those of the largest divergence of the decay constants. If the coincidence axis is an axis of revolution, there is only one term and the anisotropy disappears altogether. Table gives the values of C_1 and C_4 for the ellipsoids of revolution with an equatorial oscillator, and for the ellipsoids of maximum asymmetry. This latter case is characterized by $\sigma = 1/\sqrt{3}$ and the ratio of the

constants of the decay of the anisotropy is twice as large than for the oblate ellipsoid of revolution. If one adopts the model of a prolate ellipsoid of revolution for the analysis of the case of obvious anisotropic behaviour, equations show that, by choosing one equatorial axis, say axis 3, to be in the plane of the axis of revolution and one of the transition moments, α_3 or β_3 becomes zero, reducing to three terms the decay of the anisotropy. These have the rate constants $6R(1+\sigma)$, $6R(1-\sigma)$ and $6R(1+\frac{\sigma}{2})$. For the extreme case in which $\sigma \rightarrow 1$ the first and

third are in the ratio 2:1.5 and can be considered unresolvable under ordinary, and even extraordinary conditions. experimental conditions.

Computation of a series of curves giving decay of the log of the impulse response of the anisotropy against time for ellipsoids of revolution with oscillator of emission at a variable angle to the axis. Suppose number of counts for separation of extreme rates and rates of constants $1+\sigma$ and $1+\sigma/2$.

Another limiting case dependent upon the orientation of the oscillator occurs when one of the transition moment makes an indeterminate angle with the three principal axes of inertia giving $\alpha_1^2 = \alpha_2^2 = \alpha_3^2 = 1/3$ (or $\epsilon_1^2 = \dots = 1/3$). In these cases $F=G=0$ $\rho_{JK} = \frac{4}{15} \epsilon_K \epsilon_L$ and one two or three terms may be active in the impulse response. For a completely asymmetric ellipsoid with $\sigma = \frac{1}{\sqrt{3}}$, if the three decays are active they will be in the ratio 0.71:1:1.29, and the anisotropy would be 'perhaps' detectable, as a difference between the fastest and slowest decays. For an ellipsoid of revolution the extreme rates would approach the ratio $(1+\sigma)/(1-\sigma/2)$ which is only 4 for an infinitely elongated molecule. Experimental decay rates larger than these demand a prolate ellipsoid with orientation of the oscillator at an angle larger than 54° to the axis of revolution.

We see that for the evident case of anisotropic rotation the model of a prolate ellipsoid that carries the oscillation in an equatorial plane is almost obligatory, and we can understand why no experimental study has been reported in which the decay of the anisotropy consists of more than two terms.

Table of Ratio of.
Extreme rates and.
"middle" to extremes

Oblate

T.A.E.

Prolate

Stationary fluorescence polarization.

By direct integration of eq (63) we obtain the average value of the anisotropy on steady illumination:

$$A = \frac{3}{10} \left(\sum_{i=1}^3 \frac{\beta_i}{1+m_i\tau} + \frac{F+G}{1+m_4\tau} + \frac{F-G}{1+m_5\tau} \right) \quad (73).$$

In the general case the ^{stationary} anisotropy is the sum of five terms generated by the corresponding ones of the time-dependent anisotropy. In a plot of A^{-1} , or $1/p$ against T/η or $6RT$ the presence of more than one appreciable term in (73) results in curvature concave to the T/η axis. Such a curvature can be detected in observations of the fluorescence polarization of the ^{unsaturated} aromatic hydrocarbons, (fig. 1) and is the more pronounced the greater the differences among the m constants in (73). It then follows that, like in the time dependent case, the curvature in the plots of A^{-1} is largely due to the contributions from the last two terms and analysis in terms of the pseudomechanical polarization ellipsoid can yield a value of σ , the limitation of which have already been discussed. Figure shows plots of A_0/A against $6RT$ for an ellipsoid of revolution carrying parallel oscillators at an angle α to the axis of revolution. The presence of an intermediate value of m between m_4 and m_5 will be here even more difficult to detect than in the time dependent anisotropy.

Oscillations in Equatorial plane.

A. Depolarization by phenomenological ellipsoid.

$$A = \frac{3}{10} \left[\frac{F-G}{1+x(1+\sigma)} + \frac{F+G}{1+x(1-\sigma)} \right] = \left[\frac{2F + 2x(F+G\sigma)}{1+2x+x^2(1-\sigma^2)} \right] \left[\frac{3}{10} \right]$$

$$A_0 (x \rightarrow 0) = 2F \left(\frac{3}{10} \right) \quad x = 6R\tau.$$

$$\frac{A_0}{A} = \frac{1 + 2x + x^2(1-\sigma^2)}{1 + x \left(1 + \frac{G\sigma}{F} \right)}$$

$$\text{Initial slope } (x \ll 1) \text{ is } 2 - \left(1 + \frac{G\sigma}{F} \right) = 1 - \frac{G\sigma}{F}$$

$$G\sigma = \frac{1}{3} (\delta_1 \cos^2 E + \delta_3 \sin^2 E) = \frac{1}{3} \left(\frac{R_1}{R} \cos^2 E + \frac{R_3}{R} \sin^2 E - 1 \right)$$

$$F = \frac{1}{3} (3 \cos^2 E - 1)$$

$$1 - \frac{G\sigma}{F} = 1 - \frac{R_1 \cos^2 E + R_3 \sin^2 E - R}{3R \cos^2 E - R} = \frac{(R_1 + R_2 + R_3) \cos^2 E - R_1 \cos^2 E - R_3 \sin^2 E}{3R \cos^2 E - R}.$$

$$6R\tau \left(1 - \frac{G\sigma}{F} \right) = \left(\frac{R_2 \cos^2 E + R_3 (\cos^2 E - \sin^2 E)}{3 \cos^2 E - 1} \right) \cdot 6\tau.$$

$$\boxed{\frac{A_0}{A} = 1 + \frac{6R\tau}{3 \cos^2 E - 1} \left(\frac{R_2}{R} \cos^2 E + \frac{R_3}{R} (2 \cos^2 E - 1) \right)}$$

B. Depolarization by ellipsoid of Revolution.

Retaining only terms in x , the three term equation gives:

$$A = \left\{ \frac{4C_3 + 2F + 2x \left[4C_3 + 2F + \frac{\sigma}{2} (2G - F) \right]}{1 + 3x \left(1 - \frac{\sigma}{6} \right)} \right\}$$

$$\text{Since } A_0 = 4C_3 + 2F$$

$$\frac{A_0}{A} = \frac{1 + 3x \left(1 - \frac{\sigma}{6} \right)}{1 + 2x \left[1 + \frac{\sigma}{2} \left(\frac{2G - F}{4C_3 + 2F} \right) \right]}$$

The slope for $x \ll 1$ is,

$$1 - \frac{1}{3}\sigma \left\{ \frac{1}{2} + \frac{2G-F}{2F+C_3} \right\}.$$

which tends to

$$1 - \frac{G\sigma}{F} \quad \text{when } C_3 = 0.$$

For a molecule carrying the oscillators in the equatorial plane of the ellipsoid:

$$\alpha_3 = \epsilon_3 = 0. \quad \alpha_2 = \frac{\pi}{2} - \alpha_1 \quad \epsilon_2 = \frac{\pi}{2} - \epsilon_1.$$

$$F = \cos^2 \alpha \cos^2 \epsilon + \sin^2 \alpha \sin^2 \epsilon - \frac{1}{3}$$

$$F = \frac{1}{2} \left[(2\cos^2 \alpha - 1)(2\cos^2 \epsilon - 1) + \frac{1}{3} \right]$$

$$G = \frac{1}{3\sigma} \left\{ \delta_1 \cos^2 \alpha \cos^2 \epsilon + \delta_2 \sin^2 \alpha \sin^2 \epsilon + \delta_3 (\sin^2 \alpha \cos^2 \epsilon + \cos^2 \alpha \sin^2 \epsilon) \right\}.$$

$$G = \frac{1}{3\sigma} \left\{ \delta_1 (3\cos^2 \alpha \cos^2 \epsilon - \cos^2 \alpha - \cos^2 \epsilon) + \delta_2 (3\cos^2 \alpha \cos^2 \epsilon - 2\cos^2 \alpha - 2\cos^2 \epsilon + 1) \right\}$$

$$G = \frac{1}{3\sigma} \left[\left(\frac{\delta_1 + \delta_2}{2} \right) \left(\frac{3}{2} (2\cos^2 \alpha - 1)(2\cos^2 \epsilon - 1) - \frac{1}{2} \right) + \left(\frac{\delta_1 - \delta_2}{2} \right) [(2\cos^2 \alpha - 1) + (2\cos^2 \epsilon - 1)] \right]$$

if $\delta_1 = \delta_2$ (ellipsoid of revolution).

$$G = \frac{1}{3\sigma} \left\{ \pm \sigma \left(\frac{3}{2} (2\cos^2 \alpha - 1)(2\cos^2 \epsilon - 1) - \frac{1}{2} \right) \right\}$$

$$G = \pm \left[\frac{1}{2} (2\cos^2 \alpha - 1)(2\cos^2 \epsilon - 1) - \frac{1}{6} \right]$$

+ : oblate, - prolate.

$$F \pm G = \Lambda \epsilon (F+G) \quad 1/6 (F-G) \quad (\text{oblate})$$

$$\Lambda \epsilon (F-G) \quad 1/6 (F+G) \quad (\text{prolate})$$

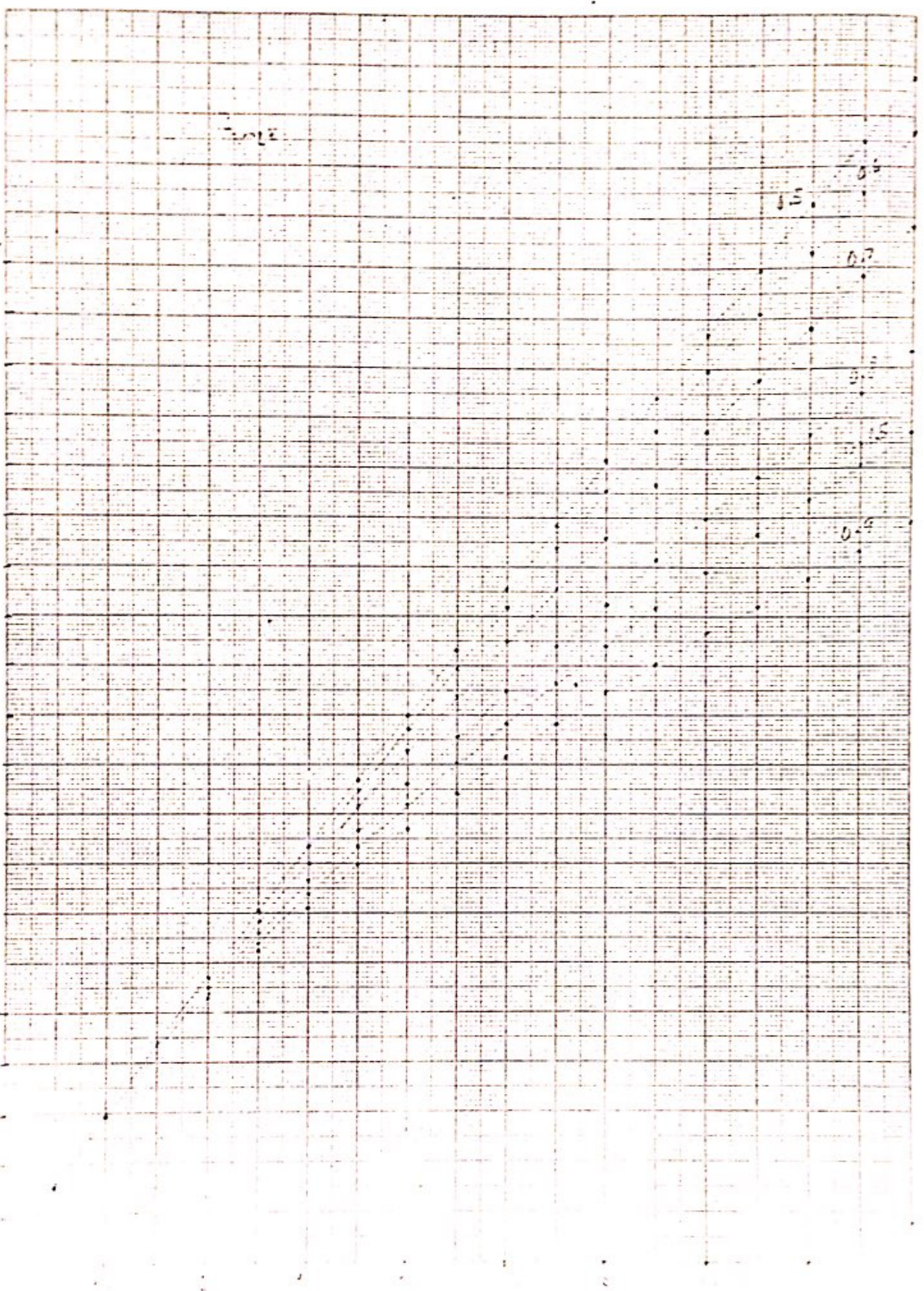
$$\Lambda = 2\cos^2 \alpha - 1, \quad \epsilon = 2\cos^2 \epsilon - 1.$$

5

1

3

2



Differential polarized phase fluorometry

The sine and cosine Laplace transforms of eq (61), Q and P of eqs. (36) may be written,

$$\begin{aligned} P_{\parallel} &= \tau(X+Y) & Q_{\parallel} &= -\omega\tau^2(X+Z) \\ P_{\perp} &= \tau(X-\frac{Y}{2}) & Q_{\perp} &= -\omega\tau^2(X-\frac{Z}{2}) \end{aligned} \quad (74)$$

In the above equations

$$X = \frac{1}{3} (1 + \omega^2\tau^2)^{-1}$$

$$Z = \sum_{i=1}^5 \frac{\beta_i}{(1+m_i\tau)^2 + \omega^2\tau^2} ; \quad Y = \sum_{i=1}^5 \frac{\beta_i(1+m_i\tau)}{(1+m_i\tau)^2 + \omega^2\tau^2} \quad (75)$$

Employing equation (50) we obtain

$$\tan \Delta = \frac{3\omega\tau(Y-Z)}{\frac{2}{3} + Y + \omega^2\tau^2 Z - 3(Y^2 + \omega^2\tau^2 Z^2)(1 + \omega^2\tau^2)} \quad (76)$$

Here like in the case of the time-dependent or the stationary anisotropy the presence of the firelocks in Y and Z results in effects quite similar to those introduced by heterogeneity of a population of spherical rotors. The larger the differences in the m constants the larger the spread of the values of $\tan \Delta$ along the $2R\tau$ axis, and the more evident the 'tangent defect' or deficit in the maximum value of $\tan \Delta$ with respect to the spherical value of eq. (53). This tangent defect will be largely dependent upon the contribution of

m_4 and m_5 to the values of Y and Z . Just as in the case of the time-dependent or the stationary anisotropy. Figure shows plots of $\tan \Delta$ against $2k\tau$ for an ellipsoid of revolution with $\sigma = 0.9$ carrying oscillators at various angles α to the axis of revolution. Once more we cannot expect to distinguish the effect of the intermediate terms when we analyze the results and in practice we can only interpret them by reference to a phenomenological polarization ellipsoid.

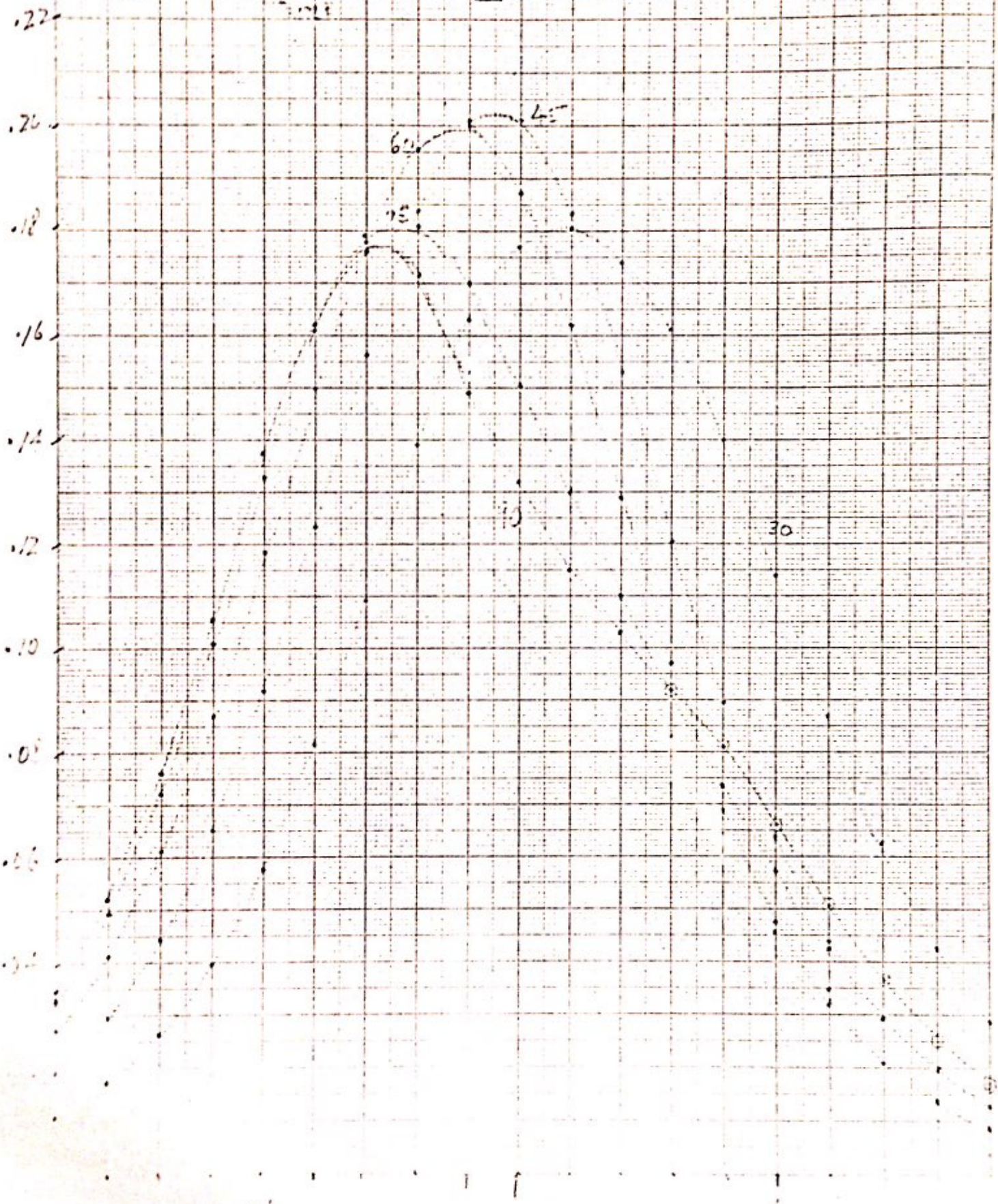
DPD =

Properties of Resolution

$d = \frac{\lambda}{2 \sin \theta}$

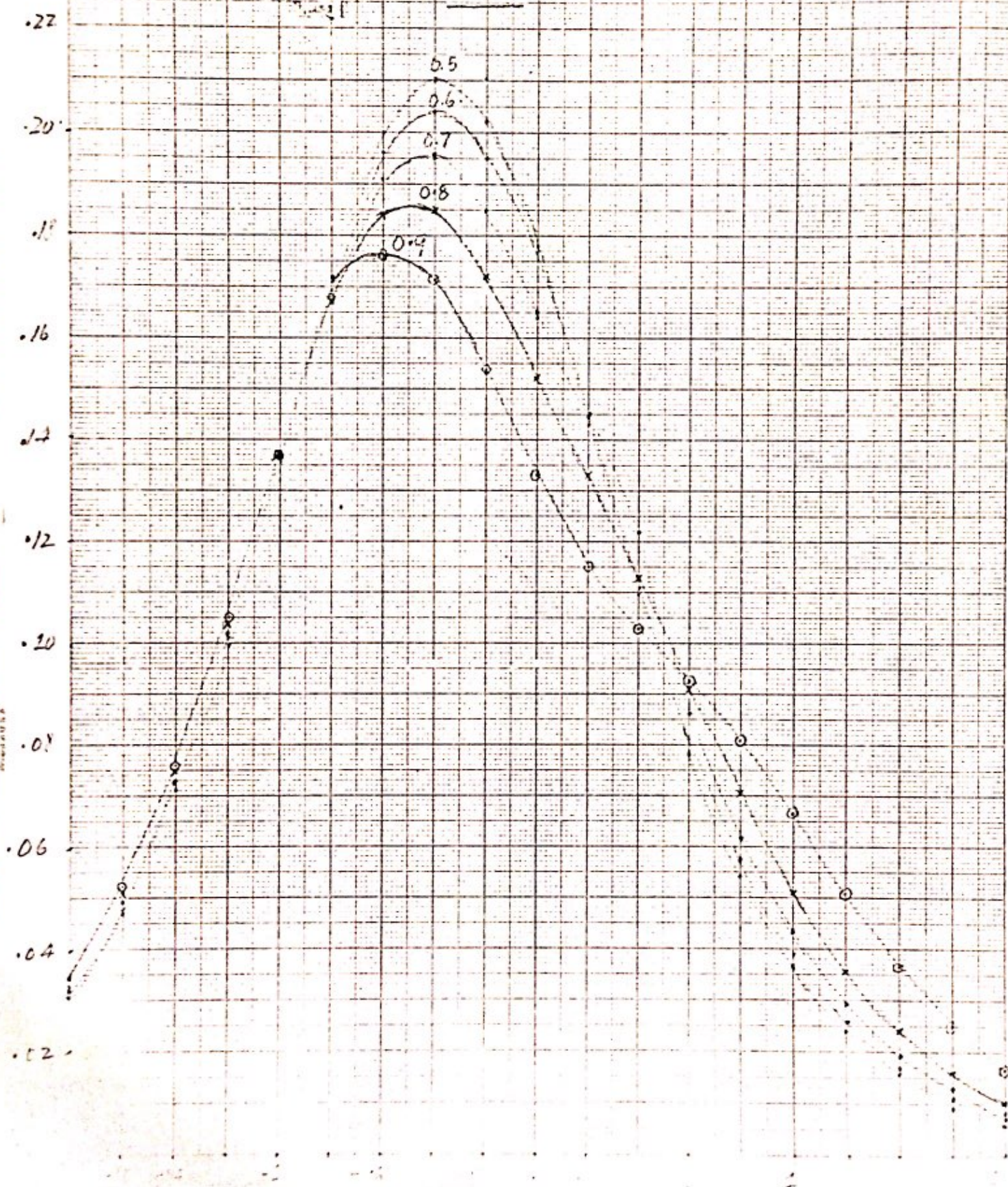
$\beta = 0$

$\omega Z = 1 \quad \sigma = 0.9$



DPF

$\alpha = 8 = 90^\circ$
 $\sigma = 0.5 - 0.9$
 $(\sigma = 0.5)$



Reduction of general ellipsoid equation for differential polarized phase fluorometry to the spherical case.

$$\tan \Delta = \frac{3\omega\tau(Y-Z)}{\frac{2}{3} + Y + \omega^2\tau^2 Z - 3(Y^2 + \omega^2\tau^2 Z^2)(1 + \omega^2\tau^2)}$$

The following substitutions are used:

$$x = 2R\tau, \quad \omega\tau = b.$$

$$Z = \sum \beta_i / (1+3x)^2 + b^2 = \frac{2}{3} \frac{A_0}{(1+3x)^2 + b^2}.$$

$$Y = (1+3x)Z$$

$$\tan \Delta = \frac{3b \cdot 3xZ}{\frac{2}{3} + Z(1+3x+b^2) - 3Z^2(1+b^2)((1+3x)^2 + b^2)}$$

$$Z^2[(1+3x)^2 + b^2] = Z \cdot \frac{2}{3} A_0.$$

$$\frac{2}{3Z} = \frac{(1+3x)^2 + b^2}{A_0}.$$

$$\tan \Delta = \frac{9bx}{\frac{(1+3x)^2 + b^2}{A_0} + 3x + (1+b^2)(1-2A_0)}$$

$$\tan \Delta = \frac{9bx A_0}{1+b^2 + 6x + 9x^2 + 3xA_0 + (1+b^2)(1+A_0-2A_0^2)}$$

$$\tan \Delta = \frac{bx A_0}{\frac{1}{9}(1+b^2)(1-A_0)(1+2A_0) + \frac{x}{3}(2+A_0) + x^2}$$

which gives the sphere equation on substitution
of b by $w\tau$ and x by $2R\tau$.

From our examination of the depolarization of the fluorescence in the stationary state and in real time it can be concluded that such measurements do not allow the direct determination of the ellipsoid of inertia associated to the fluorophore. The experimental observations permit only the estimation of a phenomenological depolarization ellipsoid. This may be defined as a general ellipsoid carrying one of the oscillators along a principal axis of rotation and producing depolarization effects indistinguishable from those of the real fluorophore. Such an ellipsoid is not explicitly defined beyond the value of σ , the standard deviation of the principal rotational rates computed from the experimental data on the assumption that these are expressed by only the terms containing m_4 and m_5 in the equations. The set of rotational types consistent with a minimum value of σ is defined by using the ellipse of residuals. The lower bound for any residual is $-R$ and therefore the possible models are those that lie within the triangle limited by the lines $\delta_1 = -1$, $\delta_2 = -1$, $\delta_3 = -1$ in fig. If $\sigma < 0.5$ all points of the ellipse are possible models. As σ increases from 0.5 to $1/\sqrt{3}$ the ellipsoids of inertia comprised between the oblate ellipsoid and the most asymmetric ellipsoid are

progressively excluded. As σ increases beyond $1/\sqrt{3}$ the allowed models get increasingly close to the prolate ellipsoid of revolution which is the only model with $\sigma = 1$. One limit to the range of models allowed by values of σ greater than $1/\sqrt{3}$ is readily obtained by setting $\delta_i = -1$ in eq (59) and solving the resulting quadratic for δ_j . This defines the set of residuals of greatest asymmetry compatible with a given σ . The other limit is the ellipsoid of revolution, characterized by $\delta_i = \delta_j = -\sigma$, $\delta_k = 2\sigma$. The values thus obtained for $\sigma = 0.6$ to 0.9 are compared in Table I

Table I

Ratios of the rotational rates, $R_1:R_2:R_3$ for ellipsoids of inertia of given σ .

σ	Extreme Asymmetric ellipsoid.	Ellipsoid of revolution
0.6	2.24 : 1 : $\ll 1$	5.5 : 1 : 1
0.65	2.8 : 1 : $\ll 1$	6.6 : 1 : 1
0.70	3.5 : 1 : $\ll 1$	8 : 1 : 1
0.75	4.6 : 1 : $\ll 1$	10 : 1 : 1
0.8	6.1 : 1 : $\ll 1$	13 : 1 : 1
0.9	13.3 : 1 : $\ll 1$	28 : 1 : 1

The table values indicate that the experimental σ fixes the ratio of the two faster rates within a factor of two, independently of the ellipsoid of inertia considered.

Rotational Depolarization of the fluorescence of aromatic molecules.

Aromatic molecules are of particular interest, not only because they are, by far, the most commonly employed fluorophores but because the spatial disposition of the transition moments of absorption and emission permit to draw some particularly simple relations between the limiting anisotropy and the molecular motions responsible for the fluorescence depolarization. We have already commented on the geometrical degeneracy that arises in the electronic oscillations of these molecules and results in transition moments, which, at least for the spectral regions of strong absorption, are confined to the plane of the aromatic rings. This plane may be taken as the equator of the ellipsoid of inertia of these molecules. Rotations about the two principal axes contained in this plane will bring the molecule out of its original plane and are therefore axes of out-of-plane rotation. The normal to the aromatic ring will provide the third principal axis, or axis of in-plane rotation. These simple assignments will be rigorously valid for the unsubstituted aromatic hydrocarbons, but may be considered as approximately correct for molecules in which one or two of the aromatic protons are substituted.

by groups of a few atoms. Moreover in many aromatic molecules the two dimensions of the ring surface are not very different so that the inertial ellipsoid may be taken, without sensible error, as an ellipsoid of revolution with transition moments in the equatorial plane. The phenomenological depolarization ellipsoid is therefore a particularly good approximation for the description of the motions of these molecules.

The stationary fluorescence depolarization adopts the simple two-term form,

$$A = \frac{3}{10} \left[\frac{F+G}{1+x(1-\sigma)} + \frac{F-G}{1+x(1+\sigma)} \right] = \frac{3}{10} \left[\frac{2F+2x(F+G\sigma)}{1+2x+x^2(1-\sigma^2)} \right]$$

where $x = 6R\tau$. Since $A_0 = \frac{3F}{5}$ (77)

$$A_0/A = \frac{1+2x+x^2(1-\sigma^2)}{1+x\left(1+\frac{G\sigma}{F}\right)} \quad (78)$$

and the slope in the Perrin plot for $6R\tau \ll 1$ is given by

$$A_0/A = 1 + 6R\tau \left(1 - \frac{G\sigma}{F}\right) \quad (79)$$

For the ellipsoid of revolution with equatorial oscillations

$$F = \frac{1}{3}(3\cos^2\epsilon - 1) ; G\sigma = \frac{1}{3} \left(\frac{R_{eq}}{R} \cos^2\epsilon + \frac{R_{ax}}{R} \sin^2\epsilon - 1 \right) \quad (80)$$

where we have set $R_1 = R_2 = R_{eq}$, the rates of rotation about the equatorial axis and $R_3 = R_{ax}$ the rate of rotation about the axis of revolution.

After a few elementary rearrangements, employing the equivalence.

$$3\cos^2\theta - 1 = 5A_0. \quad (81)$$

we obtain

$$\frac{A_0}{A} = 1 + 2\tau \langle R \rangle. \quad (82)$$

$$\text{with } \langle R \rangle = R_{eq} \left(1 + \frac{1}{5A_0}\right) + R_{ax} \left(2 - \frac{1}{5A_0}\right). \quad (83)$$

The last equations indicate a strong dependence of the apparent rotational rates, calculated from Perrin plots, upon the limiting anisotropy. If $A_0 = 2/5$, or $P_0 = 1$, $\langle R \rangle = \frac{3}{2}(R_{eq} + R_{ax})$ that is the rotations in-plane and out-of-plane have equivalent weights when the oscillations of absorption and emission are colinear. When $A_0 = 0.1$ or $P_0 = 1/7$, the second term drops out: the depolarization is then produced exclusively by out-of-plane motions. This is in fact to be expected: when $A_0 = 1/10$, or $P_0 = 1/7$, the angle between oscillations is $\pi/4$ which equals the random average produced by motion about the axis of revolution. Rotations about this axis cannot introduce any additional depolarization; and this must be exclusively due to rotation about the equatorial axes. When $A_0 = -2/5$, or $P_0 = -1/3$, it is the first term that disappears: the depolarizing rotations are then exclusively in-plane rotations.

Most aromatic fluorophores have a spectral region, generally in the $S_0 \rightarrow S_1$ transition, where limiting polarizations of 0.4 or higher are observed and in the majority of them a wavelength of excitation for which $P_0 = 1/7$ is found in an easily accessible region of the spectrum. Appreciably negative polarizations are observed in only a few aromatic fluorophores and the values seldom exceed -0.2. Thus the region in which $1/7 < P_0 < 1/2$ is the one usually available for the detection of anisotropic rotations by excitation at different wavelengths. A standard deviation of $\pm 5\%$ in the Perrin slope can be achieved with appreciably efficient fluorophores. If the largest limiting polarization employed is 0.4 ($A_0 \approx 0.31$) a ratio of the Perrin slopes significantly different from 1 would be 1.15, a value that implies $R_{ax}/R_{eq} \approx 2$. A detection of such small value by real-time methods (anisotropy decay or differential phase) does not appear feasible with today's techniques. Larger degrees of rotational anisotropy are very easy to detect. Fig shows Perrin plots for Perylene in propylene glycol. In this case the three methods: stationary polarization, real-time anisotropy decay and differential phase can detect and estimate the anisotropic rotations.

Repolarization of the fluorescence by rotations.

Perrin predicted already on the basis of his general theory that the Brownian rotations, if anisotropic, could lead to an increase rather than decrease of the polarization, a phenomenon that would be best observed on excitation at a wavelength such that $A_0 = P_0 = 0$. Setting $\dot{F} = 0$ in equation (77) gives

$$A = \frac{3x}{5} \frac{x G \sigma}{1 + 2x + x^2(1 - \sigma^2)} \quad (84)$$

If $\sigma \neq 0$ the polarization cannot remain null as rotation proceeds. The equation predicts that as the rate of rotation increases, A must rise towards a maximum value and then decline and become vanishingly small again for much larger values of the rate of rotation. The condition of maximum, obtained by setting $dA/dx = 0$, is $x^2 = 1 - \sigma^2$. Introducing this into the last equation gives

$$A_{\max} = \frac{3}{10} \left(\frac{G \sigma}{1 + \sqrt{1 - \sigma^2}} \right) \quad (85)$$

For a prolate ellipsoid $G = 1/3$ and for an oblate ellipsoid $G = -1/3$, so that

$$A_{\max} = \pm \frac{1}{10} \left(\frac{\sigma}{1 + \sqrt{1 - \sigma^2}} \right) \quad (86)$$

For the prolate ellipsoid the anisotropies observed on excitation at wavelengths for which $A_0 = 0$ are positive

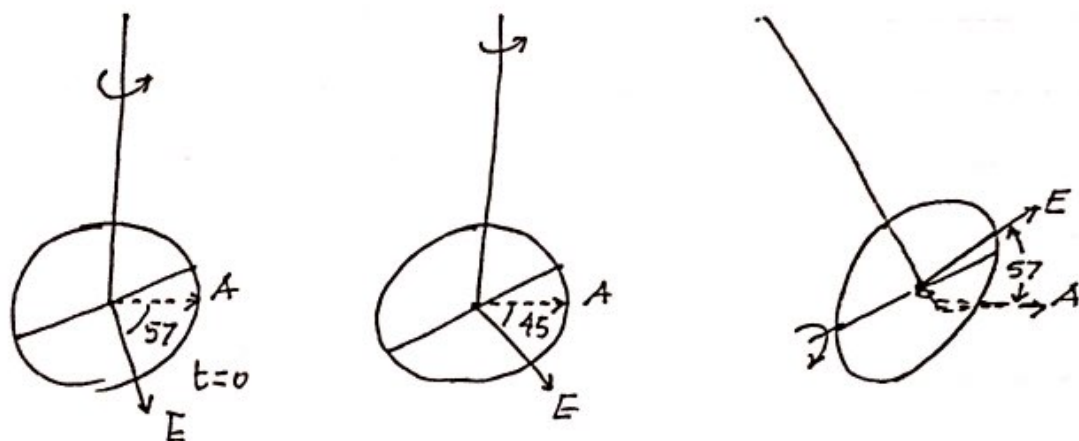
and may reach the limiting value, of 0.1 if σ comes very close to unity, or equivalently $R_{ax}/R_{eq} \gg 1$.

For the oblate ellipsoid the anisotropies are always negative and if $R_{ax} \ll R_{eq}$, or $\sigma \rightarrow 0.5$, A_{max} has the limiting value -0.0268 , or $P_{max} = -0.0396 \left(-\frac{0.1}{2+13} \right)$. The following table gives some values of A_{max} (P_{max}) for increasing σ .

R_{ax}/R_{eq}	σ	A_{max}	P_{max}
2	0.25	0.013	0.019
3	0.40	0.021	0.031
4	0.50	0.027	0.040
5.5	0.60	0.033	0.049
8	0.70	0.041	0.060
13	0.80	0.050	0.073
28	0.90	0.063	0.091
298	0.99	0.0867	0.125
2998	0.999	0.0957	0.137

The table shows that small degrees of anisotropy, of the order of $R_{ax}/R_{eq} \sim 3$ to 4 could be detected by this method. It is of practical interest that in these cases we are able to distinguish whether the first rotations are the in-plane or the out-of-plane motions and obtain a ratio of the axial and equatorial rates by simply the sign and value of A_{max} without reference to the viscosity of the medium.

The repolarizing effect of the rotations in these cases may be intuitively understood by considering the case of an ellipsoid with $R_{ax} \gg R_{eq}$, the stages of motion of which are depicted in figure.



The condition $A_0 = P_0 = 0$ implies that at time zero the angle between oscillators is 57° . As R_{ax} is much larger than R_{eq} , the directions of the oscillator of emission in the equatorial plane will become randomized before any appreciable out-of-plane motions can occur. This reduces the average angle between oscillators to 45° , from the initial 57° ; corresponding to $A = 1/10$ or $P = 1/7$, the maximum possible value. At a latter time the sluggish out-of-plane rotations increase again the angle between the oscillators from the in-plane random value of 45° to the space random value of 57° , reducing the polarization to zero. In the oblate ellipsoid the repolarizing rotations are out-of-plane rotations and increase the average angle between oscillators beyond the initial 57° producing therefore a negative polarization. The more sluggish.

in-plane motion reduces the angle between the oscillators in the plane from 57° to 45° and thus helps to reduce the average angle in space to the random value once more.

Figure shows the plots of the stationary polarization of perylene (excitation at) and 9:10 dimethyl anthracene (excitation at). σ against the temperature of the solvent. From the absolute value and sign of the maximum one calculates $\sigma =$ for perylene and $\sigma =$ for 9:10 dimethylanthracene. values in good agreement with those deduced from the other two methods of determination of anisotropic rotations.

Ellipsoids of inertia of macroscopic bodies and of molecules. Molecular shape and anisotropic rotations of aromatics. The importance of Hydrogen bonds

The ellipsoid of inertia deducible from the rotational motion of a body and characterized by the rotational rates about its three principal axes is an operationally defined figure which need not be directly related to the real shape of the body. In the original works of Einstein, Debye and Perrin the rotational diffusion coefficient D_r is ^{defined as a quantity} directly related to the molecular shape. In general it is given by the equation

$$D_r = \frac{kT}{f} \quad (87)$$

where k is the Boltzmann constant, T the absolute temperature and f the frictional coefficient for the rotational motion envisioned. In the computation of f the assumption ^{is made} that the solute molecule moves without slip in the solvent, experiencing from it a resistance as if it were a macroscopic body of the same shape. The frictional coefficient is therefore proportional to the solvent viscosity η and to a constant dependent only upon the shape of the solute molecule. For a sphere there is a unique f , owing to rotation about a diameter, given by the Kirchhoff equation

$$f = 8\pi\eta a^3 = 6\eta v \quad (88)$$

where a is the radius, or v the volume of the sphere.

For macroscopic ellipsoids of revolution the values of f for rotation about the principal axes have been derived by

True and apparent exceptions to the rules regarding the fluorescence emission.

Because of the rapid thermalization the observation of strong dependence of the fluorescence spectrum upon the exciting wavelength may be taken as a proof of the existence of more than one emitting species in solution. Fluorescence impurities may be often detected by this method. Less trivial is the case due to the presence of forms differing by the dissociation of a proton. For example the naphthylamines associate a proton to the aromatic NH_2 below pH (4.5) ^{at pH value} ~~(actual pK value)~~. *

← The unprotonated ^{naphthylamine} species emits fluorescence in the region of 400-600 nm while the protonated species emits fluorescence not too different from methyl naphthalene with a maximum at 320 nm and appreciable vibrational structure. ~~Evidently~~ At pH values close to the pK, practically within one unit of it, it will be possible to detect a change in the fluorescence spectrum with wavelength of excitation, as well as the presence of two fluorescent bands corresponding to the two ground state species if the excitation falls upon a wavelength at which both the protonated and unprotonated species absorb to appreciable extents.

In yet other cases there may be a single ground state species but two ^{distinct} fluorescence ^{spectra} may be emitted if proton association or dissociation occurs in the excited state. Such is the case of beta-naphthol in water solutions, which we will examine later in more detail. A simple example is that of naphthionic acid which had a blue fluorescence in neutral solution and a green fluorescence in alkaline solution (0.1 M NaOH). By adjusting the concentration of alkali from 10^{-5} to 10^{-1} M it is possible to observe the transition from the blue

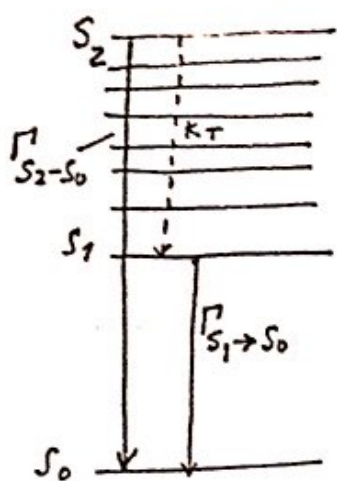
() The absorption by unprotonated naphthylamine extends to nearly 400 nm, with a maximum of the $S_0 \rightarrow S_1$ transition at 340 nm, and the fluorescence occupies a broad band in the visible spectrum with maximum at 440 nm. The protonated form - naphthyl ammonium - has absorption and emission that resemble those of methyl-naphthylamine. The $S_0 \rightarrow S_1$ transition has a maximum at 320 nm. and the emission is entirely in the ultraviolet. At pH values close to the pK , practically within two units of it a change in the fluorescence spectrum with exciting wavelength will be ~~seen~~ detected, as well as the presence of two fluorescent bands corresponding to the two species, if the excitation falls upon a wavelength absorbed by both.

(emission (neutral molecule) to the double emission (neutral molecule and NH^- ion both present in solution) to the green emission (molecular ion alone). In these cases there is no ground state difference, since the species $\text{NH}^- \text{-Ar-SO}_3^-$ does not exist at any pH value in the ground state. The fluorescence spectrum is in this case dependent upon the pH of the solution but not upon the exciting wavelength. This characteristic behaviour permits us to deduce that the additional, green fluorescent species is formed ^{only} in the excited state. *) **

In at least one case it has been shown that the fluorescence does not originate in S_1 , the lowest singlet excited state but in S_2 and corresponds therefore to the $S_2 \rightarrow S_0$ transition. Longuet-Higgins and Beer found this to be the case for azulene and other azulene derivatives. It must be stressed that while $S_0 \rightarrow S_2$ in azulene appears very much like an ordinary absorption transition in any aromatic hydrocarbon, the longest wavelength transition $S_0 \rightarrow S_1$ has no counterpart in the series of the aromatic hydrocarbons. : It extends over a large portion of the visible spectrum and has little vibrational detail. No satisfactory explanation has been advanced as to the nature or origin of this exception. / Evidently the second of our rules is violated since the longest wavelength absorption clearly exceeds the fluorescence in this particular case. The mirror image rule is valid, but the emission is the mirror image of the $S_0 \rightarrow S_2$ and not of the $S_0 \rightarrow S_1$ transition.

In ^{many} ~~most~~ cases a very careful study of the dependence of the emission upon the exciting wavelength shows the existence of very small differences, on the order of a few percent. What do these reveal?

The absence of any apparent contribution to the emission from the upper level ($S_2 \dots$ etc) when these are originally reached by the excitation can be understood in terms of the relative rate of the competing processes of radiative transition from the upper levels ($S_2 \rightarrow S_0$, $S_3 \rightarrow S_0$ etc..) and the rate of thermalization. (Fig). Let the rate of emission from



the S_2 level equal to Γ_{S_2} and, the rate of emission from S_1 be Γ_{S_1} and the rate of thermalization be k_T . ~~For the ratio of the fluorescence from these on the assumption that there are similar if we assume, from the comparable oscillators. The~~

the ratio of the fluorescence emission from S_2 to the fluorescence emission from S_1 equals will then equal $\frac{\Gamma_{S_1}}{\Gamma_{S_2} + k_T}$. From the oscillator strengths of the

transitions we can assume that Γ_{S_2} and Γ_{S_1} are approximately equal and both of the order of 10^8 s^{-1} while k_T , from the observations of Kaiser and others on the vibrational deactivation in liquids is at least of order 10^{12} s^{-1} and may be even higher if the process of crossing from S_2 to S_1 is determined only by intramolecular redistribution of the excess vibrational energy. ~~It is in any case we see~~ In any case the ratio of emission from upper to lower transition from S_2 can be not much greater than 10^{-4} , and would be exceedingly difficult to demonstrate in practice.

** In azulene and its derivatives a rather exceptional situation prevails. The lowest energy electronic transition ($S_0 \rightarrow S_2$) is peculiar to substances with this nucleus, nothing similar being observed in other aromatic hydrocarbons: It covers a large portion of the visible spectrum and is devoid of vibrational structure. The shorter wavelength transitions are very much like those of other aromatic hydrocarbons, and the overall spectrum is not unlike that of naphthalene, though displaced to longer wavelengths. ~~Because of~~ The fluorescence is weak, ^{with a} yield of about 10%, and its spectrum is a mirror image of the $S_0 \rightarrow S_2$ absorption. No detectable fluorescence is observed on excitation to the S_1 level.

The very different nature of this level appears responsible for the sluggish crossing to S_1 from upper levels. This rate of crossing does not appear determined, ^{to be} or of the same order as the rate of thermalization but ^{is slow enough to} permits emission of fluorescence from S_2 with a lifetime of the order of 1 nsec.

The experimenter has here a difficult choice. They may be due to the presence of a very small amount of a second ground-state component, perhaps a chemically^{or} modified molecule, different, but not very different from the bulk, or they may be ground state conformers or tautomers, or complexes of the fluorophore with the solvent existing in very small concentration ^{but} absorbing ^{preferentially at certain} ~~at longer wavelengths than the~~ ~~main species~~. If all these possibilities can be discarded by experiment it becomes necessary to accept the existence of two fluorescent levels, and that the probability of populating one or the other depends upon the excitation wavelength. The compounds more amenable to this kind of investigation are undoubtedly the aromatic hydrocarbons: They can be obtained in high purity using zone refining methods, and because of the planar character of the molecule the existence of tautomers, or stable solvent complexes in a fluid solvent is not likely.

* That undergo interconversion in times ^{that are} longer ^{compared to} than the fluorescence lifetime

(Additional points in 'fluorescence spectrum'.

1. Experiments of Incewicz and Ricard on the vibrational deactivation.
2. Experiments of Kautzsch on vibrational crossing in excited levels.
3. Emission from S_2 due to thermal excitation.

Reactions of the excited state.

We consider first the general formulation of modifications of the fluorescence emission by competitive processes operating during the excited state. ~~[A single general treatment applies to processes as diverse as solvent relaxation, proton dissociation and chemical quenching]~~ ^{and} involving an arbitrary number N of potentially interconvertible excitable molecular species. Let the number of excited molecules of the i th species be n_i . The transformations are described by a system of linear differential equations of the form

$$\frac{dn_i}{dt} = -(\Gamma_i + \sigma_i + \sum_{j \neq i}^N k_{ij}) n_i + \sum_{j \neq i}^N n_j k_{ji} + f_i(t) \quad (1)$$

In these equations σ_i ^{is} the rate of radiativeless deactivation Γ_i is the rate of emission and k_{ij} is the rate of conversion of the i th into the j th species. $f_i(t)$ is an arbitrary function of the time, ^{determining} the rate of excitation of the i th species.

After setting $\Gamma_i + \sigma_i + \sum_j k_{ij} = L_i$ and $\frac{d}{dt} = D$, the set of equations (1) may be put into matrix form

$$\begin{vmatrix} D + L_1 & -k_{21} & \dots & -k_{N1} \\ -k_{12} & D + L_2 & \dots & -k_{N2} \\ \vdots & \vdots & \ddots & \vdots \\ -k_{1N} & -k_{2N} & \dots & D + L_N \end{vmatrix} \begin{vmatrix} n_1 \\ n_2 \\ \vdots \\ n_N \end{vmatrix} = \begin{vmatrix} f_1(t) \\ f_2(t) \\ \vdots \\ f_N(t) \end{vmatrix} \quad (2)$$

or more concisely

$$T \{n_i(t)\} = \{f_i(t)\} \quad (3)$$

where T is the matrix of coefficients on the left of (2) and $\{n_i(t)\}$ and $\{f_i(t)\}$ are column vectors. Since the number of elements of each kind in the ground state may be considered virtually constant and excitation is by a unique monochromatic light beam, $f_i(t)$ may be replaced by $E_i \cdot f(t)$ where E_i is the fraction of the ^{species} exciting light absorbed by the i th molecule and $f(t)$ the time-dependent excitation. Equation (3) becomes then

$$T \{n_i(t)\} = \{E_i\} \cdot f(t) \quad (4).$$

According to the form of $f(t)$ there are three main possible cases: stationary illumination, ^{excitation} illumination by a brief light pulse and ^{excitation} illumination by a sinusoidally modulated beam.

Stationary illumination

If $f(t)$ is constant after a brief transient of the order of τ^{-1} the amounts of excited species reach stationary values, that is $dn_i/dt = 0$. Eq (2) becomes then a system of simultaneous algebraic equations.

Pulse excitation

After a brief light pulse, when all excitation has ceased, $f(t) = 0$ and eq (4) is now a set of homogeneous linear equations of the form:

$$T * \{n_i(t)\} = 0 \quad (5)$$

The solution involves N^2 arbitrary constants, which are obtained from the boundary conditions of the particular problem, and provides the impulse response of the system.

Harmonic response

We give a more detailed description of the general case of sinusoidal excitation and describe a method of solution that gives particularly simple equations for the phase and modulation of the excited components. This is based on the representation of the periodic quantities — the excitation and emission intensities — by complex numbers. The sinusoidal excitation is given by:

$$f(t) = F_0 \exp(j\omega t). \quad (6)$$

where ω is the circular modulation frequency and $j = \sqrt{-1}$. The harmonic response of the system involves a further set of complex functions $\phi_i(j\omega)$ containing the information about the dependence of the amplitude and phase upon ω . This is called the transfer function of the system (2), the solution of which has the form:

$$n_i(t) = \phi_i(j\omega) \exp(j\omega t) \quad (7)$$

According to this equation the amounts of the excited components vary periodically with the excitation frequency ($\omega/2\pi$), and have phases and amplitudes

determined by the N transfer functions. If $\phi_i(j\omega)$ is put in the typical complex number form:

$$\phi_i(j\omega) = P_i(\omega) + j Q_i(\omega) \quad (8).$$

the phase and amplitude characteristics are then given by

$$\tan \delta_i = Q_i/P_i$$

$$S_i = (Q_i^2 + P_i^2)^{1/2}. \quad (9).$$

The above equations indicate the relations of the transfer functions to the sine and cosine transforms of the impulse response, previously introduced (37).

To calculate the N transfer functions associated with the system (4) this is formally solved to give:

$$\theta \cdot n_i(t) = M_i \cdot f(t) \quad (10)$$

where θ is the determinant of the T matrix and M_i the determinant obtained on replacing the i th column of T by the vector $\{E_i\}$. θ and M_i are differential operators acting upon the functions of time n_i and f . Explicit evaluation of the determinants in terms of powers of D gives:

$$(a_N D^N + a_{N-1} D^{N-1} + \dots + a_0) n_i(t) = (b_{i,N-1} D^{N-1} + b_{i,N-2} D^{N-2} + \dots + b_{i,0}) f(t) \quad (11)$$

The a coefficients in the left side of (11) are functions of τ_i and k_{ij} , identical for all i , while the b coefficients on the right are functions of the E_i values as well and differ for each i , as indicated by their indexing.

If the powers of D of $f(t)$ and $n_i(t)$ are calculated by means of eqs (6) and (7) respectively, and the results are

introduced into (11), we obtain

$$\phi_i(j\omega) = (A_i + jB_i)/(C + jD) \quad (12)$$

A_i and B_i are functions of the b coefficients

$$A_i = b_{i0} - b_{i2}\omega^2 + b_{i4}\omega^4 - \dots$$

$$B_i = b_{i1}\omega - b_{i3}\omega^3 + b_{i5}\omega^5 - \dots$$

C and D are functions of the a coefficients (13).

$$C = a_0 - a_2\omega^2 + a_4\omega^4 - \dots$$

$$D = a_1\omega - a_3\omega^3 + a_5\omega^5 - \dots$$

Equating the two forms of $\phi_i(j\omega)$ in eqs. (8) and (12) gives

$$P_i = (A_i C + B_i D)/(C^2 + D^2) \quad (14)$$

$$Q_i = (B_i C - A_i D)/(C^2 + D^2)$$

and from this

$$\tan \delta_i = \frac{B_i C - A_i D}{A_i C + B_i D} \quad (15)$$

$$S_i = (A_i^2 + B_i^2)/(C^2 + D^2)$$

For reasons already explained (see under)
we will often be interested in the relative phases and
amplitudes of two given components, k and l ,
rather than in their separate relations to the excitation.
Their differential tangent and modulation ratio
are

$$\tan \Delta_{KZ} = \frac{A_2 B_K - A_K B_2}{A_2 A_K + B_2 B_K} \quad (16)$$

$$M_{KZ} = \left(\frac{I_2}{I_K} \right) \left[\frac{(A_2^2 + B_2^2)}{(A_K^2 + B_K^2)} \right]^{1/2} \quad (17)$$

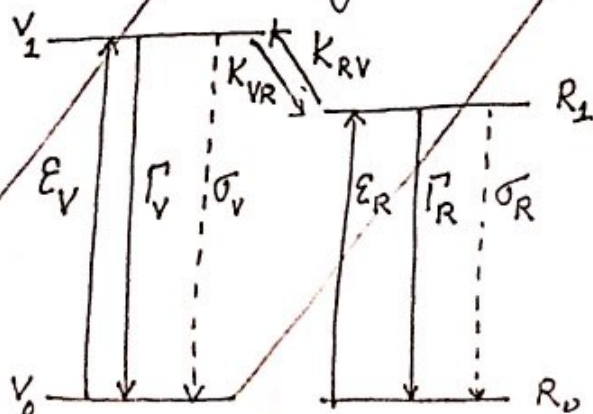
The simplicity of these equations is to be noted. They involve only the b coefficients of eqs. (13) which, particularly in the case of only two components, are easily written down.

Their computation involves no assumption as to the boundary conditions, a necessary step in the calculation of P and Q from the impulse response.

The two state system

We examine in detail a system of only two interconvertible components. Solvent relaxation around an excited fluorophore, proton dissociation in the excited state and chemical quenching can all be formulated, to a very satisfactory first approximation, as systems involving only two states, although they present very different phenomenological aspects.

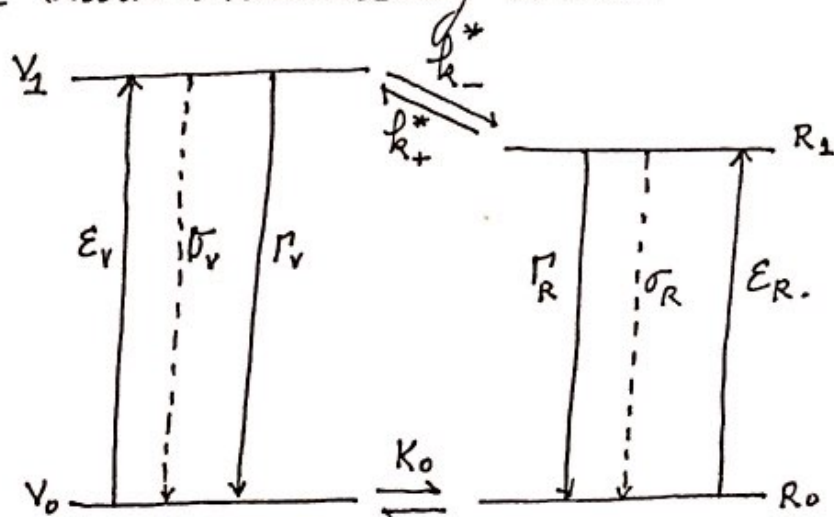
The relevant energy levels of such two-state system is schematically shown in the figure below.



The two-state system.

We examine in more detail a system of only two interconvertible components. Although they present very different phenomenology, proton dissociation in the excited state, chemical quenching and solvent relaxation about an excited fluorophore, can all be formulated to a very satisfactory first approximation, as systems involving only two interconvertible states.

The relevant energy levels of such two-state systems are shown schematically below.



It is assumed that only a negligible proportion of the molecules are at any given time in the excited state, so that the relative numbers in the states V_0 and R_0 are determined by the 'dark' equilibrium constant K_0 .

V_2 (violet) and R_2 (red) corresponds to excited state of which the latter has the lower energy, so that their fluorescence emission is partially or completely resolved. Γ_V and Γ_R are the ^{rates of} emission from the two states, σ_V and σ_R the corresponding rates of radiationless transitions to their ground states.

The observed fluorescence lifetimes τ_V and τ_R and quantum yields q_V and q_R for the respective emissions are given by:

$$\tau_V = (\Gamma_V + \sigma_V)^{-1} ; \quad \tau_R = (\Gamma_R + \sigma_R)^{-1} .$$

$$q_V = \Gamma_V \tau_V ; \quad q_R = \Gamma_R \tau_R \quad (18) .$$

K_-^* and K_+^* are rates of interconversion in the excited state, and $E_V f(t)$ and $E_R f(t)$ the rates of excitation from the respective ground states. For convenience we take $E_V + E_R = 1$, so that E_V and E_R are the fractions of the monochromatic excitations generating V_2 and R_2 respectively.

It is also assumed that the proportions of ground state component V_0 and R_0 are not appreciably modified by the excitation.

With the substitutions

$$\Gamma_V + \sigma_V = \theta_V ; \quad \Gamma_R + \sigma_R = \theta_R \quad (19)$$

the system (2) becomes,

$$\begin{vmatrix} D + \theta_V + K_-^* & -K_+^* \\ -K_-^* & D + \theta_R + K_+^* \end{vmatrix} \begin{vmatrix} n_V \\ n_R \end{vmatrix} = \begin{vmatrix} E_V f(t) \\ E_R f(t) \end{vmatrix} \quad (20)$$

The stationary state solutions are obtained by setting in eq (20), $\dot{D} = 0$, $f(t) = 1$. Replacing θ and Γ by their equivalents in terms of the observables q and τ by means of eq. (18) these solutions take the form

$$F_V = \Gamma_V n_V = q_V (E_V + \tau_R k_+^*) / (1 + k_+^* \tau_R + k_-^* \tau_V) \quad (21)$$

$$F_R = \Gamma_R n_R = q_R (E_R + \tau_V k_-^*) / (1 + k_+^* \tau_R + k_-^* \tau_V) \quad (22)$$

where F_V and F_R are the fluorescence emissions from the two states. If the rate of interconversion k_+^* , k_-^* are much faster than the corresponding rates of emission so that $\tau_R k_+^* \gg E_V$ $\tau_V k_-^* \gg E_R$.

$$F_V / F_R = (k_+^* / k_-^*) \left(\frac{q_V / \tau_V}{q_R / \tau_R} \right) \quad (23)$$

and if these rates are negligible,

$$F_V / F_R = E_V q_V / E_R q_R \quad (24)$$

Eq (23) gives the emission ratio for strongly coupled excited states and eq (24) the same ratio for virtually independent excited states. In the former case the ratio of the emissions, corrected for yield and lifetime, corresponds to virtual Thermodynamic equilibrium ^{of the components} in the excited state. In the latter case the emission ratio is ^{determined by} the fractional excitation and quantum yield of each state. If lifetimes and yields of the states are known the ratio of the rate of interconversion can be determined only for the limiting case of equation (23).

determined only for the limiting case of equation (23).

Pulse excitation:

The solution of the system of homogeneous differential equations (eq. (20) with $f(t)=0$) has the forms:

$$\begin{aligned} n_V &= V_1 e^{\lambda_1 t} + V_2 e^{\lambda_2 t} \\ n_R &= R_1 e^{\lambda_1 t} + R_2 e^{\lambda_2 t} \end{aligned} \quad (25)$$

with the roots λ_1 and λ_2 given by

$$\lambda_{1,2} = - \left(\frac{\theta_R + \theta_V + K_-^* + K_+^*}{2} \right) \pm \frac{1}{2} \left((\theta_V - \theta_R)^2 + (K_-^* + K_+^*)^2 + 2(\theta_V - \theta_R)(K_-^* - K_+^*) \right)^{\frac{1}{2}} \quad (26)$$

Setting $\frac{\theta_V - \theta_R}{2} + \frac{K_-^* - K_+^*}{2} = u$, $S = (u^2 + K_-^* K_+^*)^{\frac{1}{2}}$

$$\bar{\theta} = (\theta_R + \theta_V)/2 ; \quad \bar{K} = (K_-^* + K_+^*)/2 \quad (27).$$

$$\begin{aligned} \lambda_{1,2} &= -(\bar{\theta} \pm \bar{K}) \pm S \\ \lambda_2 - \lambda_1 &= -2S \end{aligned} \quad (28).$$

The four arbitrary constants $V_1, \dots, R_2$ are obtained from the boundary conditions for $n_R, n_V, dn_R/dt, dn_V/dt$ at $t \rightarrow 0$.

$$V_1 = \frac{K_+^* \cdot E_R - (\theta_V + K_-^* + \lambda_2) E_V}{\lambda_2 - \lambda_1}$$

$$V_2 = \frac{-K_+^* \cdot E_R + (\theta_V + K_-^* + \lambda_1) E_V}{\lambda_2 - \lambda_1}$$

$$R_1 = \frac{K_-^* \cdot E_V - (\theta_R + K_+^* + \lambda_2) E_R}{\lambda_2 - \lambda_1}$$

$$R_2 = \frac{-K_-^* E_V + (\theta_R + K_+^* + \lambda_1) E_R}{\lambda_2 - \lambda_1} \quad (29)$$

with the substitutions (27) these take the simpler forms,

$$V_1 = \left(1 + \frac{u}{S}\right) \frac{E_V}{2} - \frac{K_+^*}{S} \cdot \frac{E_R}{2}$$

$$V_2 = \left(1 - \frac{u}{S}\right) \frac{E_V}{2} + \frac{K_+^*}{S} \cdot \frac{E_R}{2}$$

(30).

$$R_1 = \left(1 - \frac{u}{S}\right) \frac{E_R}{2} - \frac{K_-^*}{S} \cdot \frac{E_V}{2}$$

$$R_2 = \left(1 + \frac{u}{S}\right) \frac{E_R}{2} + \frac{K_-^*}{S} \cdot \frac{E_V}{2}$$

When $\theta_V \sim \theta_R$ the roots approach the simpler values.

$$\lambda = -(\bar{\theta} + 2\bar{K}) ; \lambda = -\bar{\theta} \quad (31)$$

In general,

$$V_1 + V_2 = E_V ; R_1 + R_2 = E_R$$

so that when $E_R = 0$.

$$V_2 = 1 - V_1 ; R_2 = -R_1. \quad (32)$$

Harmonic response of two-state system.

The 6 coefficients for the system of equations in (20) is, from eqs. (13)

$$\begin{aligned} b_{V0} &= \theta_R E_V + K_+^* & ; & & b_{R0} &= \theta_V E_R + K_-^* \\ b_{V1} &= E_V & ; & & b_{R1} &= E_R \end{aligned} \quad (33)$$

The differential phase between the emissions is given by

$$\tan \Delta = \omega \frac{E_R E_V (\theta_V - \theta_R) + E_R K_+^* - E_V K_-^*}{E_R E_V (\theta_R \theta_V + \omega^2) + K_-^* \theta_R E_V + K_+^* \theta_V E_R + K_-^* K_+^*} \quad (34)$$

when one component is negligibly excited, $E_R = 0$ the expression above takes a remarkably simple form:

$$\tan \Delta = \frac{-\omega}{\tau_R^{-1} + K_+^*} \quad (35)$$

It is of practical interest that the highest power of ω entering in the expression for $\tan \Delta$ is determined by the effective number of states. For two states, only one of which is excited, there is a simple linear dependence of the differential tangent upon ω . If more than two states are involved this linear dependence breaks down, so that eq (35) permits to test the degree to which the two-state hypothesis applies in practice to the system.

Proton dissociation in the excited state shows the purest characteristics of a two-state system, as the conjugated forms are a molecule and the ion that results from proton dissociation, both potentially stable, well characterized species.

An additional source of information is present here in that the proportions of the two ground-state components may be varied at will by changing the hydrogen ion concentration, and the yields, lifetimes and polarizations of the . . . emission from each species studied in the virtual absence of the other. The suffixes M and I . . . will be used in reference to the molecule and ion . . .

forms that correspond ^{respectively} to V and R in the general two-state scheme. K^* , k_+^* and k_-^* will designate respectively the proton dissociation constant, rate constant of proton association, and the rate of dissociation ^{of M} of the molecule, in the excited state.

Because the conversion of the ion into the molecule by proton association is a second order process dependent on the hydrogen ion concentration $[H^+]$, the rate of conversion k_+^* of the two state scheme becomes ^{here} $k_+^* [H^+]$. In fluorophores in water solution the entropy change upon dissociation can be neglected in comparison with ΔH° , and consequently the loss of energy of the excited state upon dissociation is directly related to the standard free energy change, namely .

$$\Delta G^\circ - \Delta G^{\circ*} = -2.3RT(\log K^* - \log K) = hc\Delta\bar{\nu}_g \quad (61)$$

where $\Delta\bar{\nu}_g$ is the change in the center of mass of the fluorescence spectrum on dissociation.

For naphthol $\Delta\bar{\nu}_g$ amounts to $4,100 \text{ cm}^{-1}$ or $11.8 \text{ kcal mole}^{-1}$ predicting a change in pK on excitation of 2.3 units, in agreement with the value deduced from the kinetic observations.

E_V and E_R of eqs. (20) et seq. depend upon the wave length of excitation and the proportions of ground state ions and molecules. If we restrict ourselves to excitation at an isosteric point of the spectra,

$$E_V = 1 - \alpha \quad ; \quad E_R = \alpha = \frac{K_0}{K + [H^+]} \quad (36).$$

where α is the degree of ground-state dissociation.

Introduction of the eq gives as

the stationary state solutions;

$$\begin{aligned} F_M &= g_M (1 - \alpha + \tau_I k_+^* [H^+]) / D. \\ F_I &= g_I (\alpha + \tau_M k_-^*) / D. \end{aligned} \quad (37).$$

$$D = 1 + k_-^* \tau_M + k_+^* [H^+] \tau_I$$

At pH values less than $pK - 2$, we can set $\alpha = 0$ obtaining.

$$F_M / F_I = \left(\frac{g_M}{g_I} \right) \frac{1 + \tau_I k_+^* [H^+]}{\tau_M k_-^*} \quad (38).$$

The ratio g_M/g_I can be obtained by observation at low or high pH values in which there is only one emission.

Eq (38) indicates that a plot of $(F_M/F_I) / (g_M/g_I)$ against $[H^+]$ in the pH region in which the spectrum changes rapidly with pH yields a straight line with slope $(\tau_I/\tau_M) (k_+^*/k_-^*)$, and intercept $1/\tau_M k_-^*$. From

these quantities, and the independently determined values of τ_M and τ_I , one can get the dissociation constant in the excited state as well as the rates of association and dissociation. When the ion has negligible fluorescence yield $\tau_I k_+^* [H^+] \rightarrow 0$, and by eq. (37)

$$F_M = q_M \left[\frac{(1-\alpha)}{1 + k_-^* \tau_M} \right] \quad (39)$$

If $k_-^* \tau_M$ is appreciable the dissociation in the excited state appears then as an additional source of radiationless transition of the neutral molecule. This is not easily recognized in practice because the fluorescence emission is then simply proportional to $(1-\alpha)$ and follows closely the proportions of ground state neutral molecules. This behaviour is seen in phenol and phenolic derivative like tyrosine.

Eq (1), applies whenever $\tau_I k_+^* [H^+] \ll 1$, and if $\tau_I \leq 10^{-8} s$ the product $k_+^* [H^+]$ must be at least 10^7 to make a sensible contribution in (38). k_+^* is diffusion controlled and for protons of order $10^{11} s^{-1}$, so that $[H^+] > 10^4$ is required. At pH values higher than 3 or 4 there are not enough collisions of protons with excited molecules to maintain equilibrium and $pK^* > 4$ cannot be directly determined by measuring the concentration of H^+ required to invert the dissociation process.

In the pulse excitation of systems with good fluorescence efficiency ^{both} as the molecule and ion, one can safely neglect the first and third terms under the radical in equation (26), obtaining

$$\begin{aligned}\lambda_1 &= -\frac{1}{2} \left(\frac{1}{\tau_M} + \frac{1}{\tau_I} \right) = \\ \lambda_2 &= -\frac{1}{2} \left(\frac{1}{\tau_M} + \frac{1}{\tau_I} \right) - (k_+^* [H^+] + k_-^*)\end{aligned}\quad (40)$$

Setting $\frac{2}{\tau} = \frac{1}{\tau_I} + \frac{1}{\tau_M}$; $\rho = \left(\frac{1}{k_+^* [H^+] + k_-^*} \right)$ (41.)

the impulse response of the system in the region of pH where $\alpha=0$, is

$$F_M(t) = M_1 \exp(-t/\tau) + M_2 \exp(-t(1/\tau + 1/\rho)) \quad (42)$$

$$F_I(t) = I_1 \exp(-t/\tau) + I_2 \exp(-t(1/\tau + 1/\rho)) \quad ($$

with $M_1 = (k_+^* [H^+] + \tau^{-1})/\rho$; $M_2 = 1 - M_1$
 $I_1 = k_-^* \rho$ $I_2 = -I_1$ (43).

So that the relevant constants may be directly measured, at least in the favourable cases.

The harmonic response can be used best in the region of pH where there is ^{virtually} no ground state dissociation. The equivalent eq. to (35) is

$$\tan \Delta = \frac{\omega \tau_I}{1 + k_+^* [H^+] \tau_I} \quad (44)$$

A plot of $\ln \Delta$ against $[H^+]$ is linear and
permits the determination of $\frac{d \ln \Delta}{d [H^+]}$. Note that the equation
could then be used to check the rate of proton diffusion
as Δ is virtually diffusion controlled.

Spectral relaxation

Figure shows the fluorescence spectra of propylene glycol solutions of two fluorophores, *carbazole* naphthalene sulfonate and propylene glycol naphthalene, typical of the class of compounds that undergo spectral relaxation dependent upon the viscosity of the solvent. Starting from the state in which the solvent is vitrified a rise in temperature produces a red-shift of the emission spectrum, which reaches a limiting value when the viscosity of the solvent falls to a few centipoises. We have already discussed the complex variation of the fluorescence spectrum with temperature and shall make here only some remarks about the phenomenological description of the spectral relaxation, as originating in a two-state system.

The process of spectral relaxation is observed in fluorophores exhibiting strong dipole interactions with the molecules of a polar solvent. It is in these cases that the fluorescence emission ^{spectrum} exhibits minimal resolution consisting usually of a single peak. The spectral distribution can be characterized by the center of mass, $\bar{\nu}_g$ defined in eq. , a characterization that is particularly pertinent in this case as the mean energy of the emission is simply $hc\bar{\nu}_g$, and even very small variations in this energy can be accurately measured. At a temperature sufficiently low

to render negligible the possible reorientation of the solvent molecules during the fluorescence lifetime, the emission is entirely from the state V , which the molecules reach immediately upon excitation.

Setting $E_V = 1$, $E_R = 0$ in eq (21) results in this case in an emission for which it is possible to state

$$\bar{\nu}_g = \bar{\nu}_V \quad (36)$$

characterizing the spectrum of the fluorescence from the V level. At higher temperatures, in which there is appreciable reorientation of solvent molecules between excitation and emission,

$$\bar{\nu}_g = \bar{\nu}_V f_V + \bar{\nu}_R f_R = \bar{\nu}_V - (\bar{\nu}_V - \bar{\nu}_R) f_R \quad (37).$$

where f_R is the fraction of the total emission from the R level.

Equation (22) with $E_V = 1$, $E_R = 0$ gives.

$$f_R = \frac{\Gamma_R K_{VR}}{\Gamma_R K_{VR} + \Gamma_V K_{RV} + \Theta_R \Gamma_V} \quad (38)$$

where $k_-^* = k_{VR}$; $k_+^* = k_{RV}$.

At temperatures at which the relaxation is easily accomplished we expect to find:

$$\Gamma_V \sim \Gamma_R \ll k_{VR}. \quad (39).$$

and

$$f_R \approx \frac{\Gamma_R K_{VR}}{\Gamma_R K_{VR} + \Gamma_V K_{RV}} \quad (40)$$

The fluorescence emitted under these conditions is characterized by a center of spectral mass $\bar{\nu}_\infty$, determined by the equation

$$\frac{\bar{\nu}_V - \bar{\nu}_R}{\bar{\nu}_V - \bar{\nu}_\infty} = 1 + \frac{\Gamma_V K_{RV}}{\Gamma_R K_{VR}} \quad (41)$$

In typical cases we expect $\Gamma_V \sim \Gamma_R$ and K_{VR} considerably larger than K_{RV} . Therefore without too large an error we can set in (41) $\Gamma_V/\Gamma_R = 1$. The ratio K_{RV}/K_{VR} is related to the energy gap between the states ΔE_{VR} by the equation

$$\frac{K_{RV}}{K_{VR}} = \exp(-\Delta E_{VR}/KT) \geq \frac{hc}{KT} (\bar{\nu}_V - \bar{\nu}_\infty) \quad (42)$$

From (41) and (42)

$$\Delta E_{VR} = hc(\bar{\nu}_V - \bar{\nu}_\infty) (1 + \exp(-\Delta E_{VR}/KT)) \quad (43)$$

Equation (43) can be solved by a simple iteration procedure once $\bar{\nu}_V - \bar{\nu}_\infty$ is determined. When the energy gap is large, the second term makes a very small contribution and in these cases one virtually has $\bar{\nu}_R = \bar{\nu}_\infty$.

The assurance of valid estimates of $\bar{\nu}_V$ and $\bar{\nu}_\infty$ rests upon consideration of the extent of the rotational motion of the solvent molecules during the fluorescence lifetime. The "Debye rotational relaxation time" is defined by

$$\rho = 1/2D\tau = 3\eta V/RT \quad (44)$$

with D_R defined by equation (37). For a solvent with molar volume of 30 to 100 ml, a viscosity of 100 poise implies a rotational relaxation time of the order of $\frac{1}{2}$ ps. The solvent molecules may be considered effectively motionless during the fluorescence lifetime and $\bar{\nu}_g$ obtained under these conditions can be equated with $\bar{\nu}_0$. With a viscosity of a few cent poise solvent reorientation can be accomplished in a fraction of the fluorescence lifetime. Based on these simple considerations Bakshiev proposed a description of the spectral relaxation in this temperature range by the equation.

$$\bar{\nu}_g = \bar{\nu}_0 - (\bar{\nu}_0 - \bar{\nu}_\infty) \frac{\tau}{\tau + \rho} \quad (45)$$

where τ is the overall fluorescence lifetime and ρ the characteristic relaxation time of the process. If our $\bar{\nu}_v$ is identified with $\bar{\nu}_0$ in introduction of (41) into (37) after substitution of D_v by Γ_v/q_v , yields

$$\bar{\nu}_g = \bar{\nu}_0 - (\bar{\nu}_0 - \bar{\nu}_\infty) \frac{\Gamma_R K_{VR} + \Gamma_V K_{RV}}{\frac{\Gamma_R \Gamma_V}{q_R} + \Gamma_R K_{VR} + \Gamma_V K_{RV}} \quad (46)$$

This is the Bakshiev equation if

$$\tau = \frac{1}{\sqrt{\Gamma_R \Gamma_V} q_R^{-1}} \quad ; \quad \rho = \frac{\sqrt{\Gamma_R \Gamma_V} q_R^{-1}}{\Gamma_R K_{VR} + \Gamma_V K_{RV}} \quad (47)$$

If $\tau = \tau_1 = \tau_2 = \tau$ these equations have an obvious intuitive meaning, because in such case.

$$\tau = 1/\rho ; \rho = 1/(k_{12} + k_{21}) \quad (41).$$

In summary the observations of the spectral shift of the fluorescence under stationary excitation permits the determination of its characteristic viscosity and temperature for which the emission from the V and R levels are comparable. Without additional hypothesis one cannot extract from the observations the values of the rate of interconversion of the V and R species or even their ratio. To compound the uncertainty observations at a series of temperatures are required conditions under which the rate of radiativeless transitions from the V levels need not remain constant. In spite of these limitations the observations of the spectral shift with temperature are often sufficient to describe the phenomena and to assign limits to the rate of interconversion particularly in cases in which real time observations are difficult or unreliable on technical grounds.

Pulse excitation permits a less equivocal analysis: If the fluorescence emitted at all wavelengths is received by the detector the decay process has the form:

$$F(t) = A_1 e^{\lambda_1 t} + A_2 e^{\lambda_2 t} \quad (49)$$

where

$$A_1 = \tau_V V_1 + \tau_R R_1 ; A_2 = \tau_V V_2 + \tau_R R_2 \quad (50)$$

For unique excitation to the V level

$$A_1 - A_2 = \frac{\Gamma_V}{S} \left[\frac{(\theta_V - \theta_R)}{2} + \frac{(K_{VR} - K_{RV})}{2} \right] - \frac{\Gamma_R}{S} K_{VR}.$$

$$\text{or } A_1 - A_2 = \frac{\Gamma_V (\theta_V - \theta_R)}{2S} + \frac{K_{VR} (\Gamma_V - 2\Gamma_R) - \Gamma_R K_{RV}}{2S} \quad (51).$$

On the assumption that the rates of emission from V and R are equal or almost equal, the difference $\theta_V - \theta_R$ may be neglected in comparison with the sum $K_{VR} + K_{RV}$:

$$A_1 - A_2 \simeq - \frac{K_{VR} + K_{RV}}{2S}; \quad \frac{A_1 - A_2}{\lambda_2 - \lambda_1} \simeq K_{VR} + K_{RV} \quad (52).$$

The energy gap between the emissions corresponding to $\bar{\nu}_V$ and $\bar{\nu}_\infty$ permits estimation of the ratio K_{VR}/K_{RV} (Eq (43)) and together with (52) can be used to assign approximate values to the rates of interconversion.

If the emission from the V level alone is detected the relations

$$\begin{aligned} V_1 - V_2 &= \Gamma_V u / S. \\ \lambda_1 - \lambda_2 &= 2S. \end{aligned} \quad (53)$$

permit the separate determination of u and S if Γ_V is independently determined. This is indeed possible as both the absorption to and emission from the V level can be directly determined by the experiments. The success of this method depends in achieving the spectral resolution required to eliminate emission from the R level without impairing unduly the signal-to-noise ratio necessary

for reliable determination of n and s . This very same restriction applies to the detection of the separate V and R emissions necessary to apply eq. (35) for the determination of K_{RV} by differential phase fluorometry. This latter method presents a favorable aspect in comparison with those involving the stationary fluorescence or the pulse response: Because of the form of eq. (15) separate determination of Γ_V and Γ_R , or an assumption about their equivalence is not required. Besides, one of the rates of interconversion, K_{RV} is directly determined. It is therefore of interest to analyze in some detail the error arising from failure to isolate the V and R emissions. If the phase angle between them is δ , we can set for the photocurrents generated by the two emissions:

$$\tan \phi_V = 0 \quad \tan \phi_R = -\tan \delta \quad (54)$$

by taking as reference the phase of the V component. If a fraction f_R of the fluorescence intensity reaching the detector reserved for the V response is due to emission from R , the tangent of the phase angle with the reference is:

$$\tan \phi'_V = -\frac{f_R \sin \delta}{1 - f_R + f_R \cos \delta} = -\tan \delta \frac{f_R}{f_R + (1 - f_R) \sqrt{1 + \tan^2 \delta}} \quad (55)$$

Similarly,

$$\tan \phi'_R = -\frac{(1 - f_V) \sin \delta}{f_V + (1 - f_V) \cos \delta} = -\tan \delta \frac{(1 - f_V)}{(1 - f_V) + f_V \sqrt{1 + \tan^2 \delta}} \quad (56)$$

where f_V is the fraction of the intensity owing to unwanted V

Component. If $\delta \leq 20^\circ$; $\sqrt{1 + \tan^2 \delta} \leq 1.064$ and without appreciable error

$$\tan \phi'_r = -f_r \tan \delta ; \quad \tan \phi'_R = (1 - f_r) \tan \delta$$

$$\text{and } \tan \delta' = \tan (\phi'_r - \phi'_R) \approx -\tan \delta (1 - f_r - f_R) \quad (58).$$

In the practical application of differential phase fluorometry to solvent relaxation phenomena equation (35) would take the form

$$\tan \Delta (1 - f_r - f_R) = \frac{\omega}{\tau_R^{-1} + K_{RV}} \quad (59).$$

If the differential lifetime between the components is $\Delta\tau = \tan \Delta / \omega$ at any fixed temperature

$$K_{RV} = \frac{1}{\Delta\tau (1 - f_r - f_R)} - \frac{1}{\tau_R}. \quad (60).$$

So long as $\Delta\tau$ is small compared to τ_R , the practical case, the absolute magnitude ^{of K_{RV}} will not be greatly falsified even if f_r and f_R are as large as 0.2 or 0.3. As the temperature is changed the proportions of V and R components emitted change and so will f_r and f_R . However the sum $f_r + f_R$ will remain practically constant since a change in one of the emissions necessarily implies a change of opposite sign in the other emission. There is then every likelihood that the Arrhenius energy of activation for the rate of conversion of R into V will be reliably determined by eq (60).

Perrin and by Grans. The qualitative dependence of these coefficients upon the ratio of the lengths of the principal axes can be guessed by consideration of the moments of inertia of plate-shaped and needle shaped bodies, which are the limiting forms for the oblate and prolate ellipsoids respectively.

- The moments of inertia about the two principal axes of a circular plate ^{are comparable} differ only by a factor of two from an infinitely thin plate ($\sigma = \frac{1}{2}$). On the other hand in a prolate ellipsoid of revolution the ratio of the moments of inertia increases indefinitely with the ratio of the lengths of the principal axes. It follows that the rotational motion of a flat plate cannot differ appreciably from those of a sphere, and ^{that} it will be difficult or impossible to make a distinction between them, the plate behaving simply as a sphere of a much larger volume.

On the other hand the motion of the prolate ellipsoid will permit an easy qualitative distinction with those of a sphere: As the axial ratio increases the resistance to rotation about an equatorial axis increases continuously while the resistance for rotation about the axis of revolution decreases slowly reaching the limiting value of $3/4$ ^{for ellipsoids} of the largest elongation. (fig). In both prolate and oblate ^{ellipsoids} the mean value of the rotational diffusion constant

$$D_r = \frac{1}{3} (2D_{req.} + D_{rax}) \quad (89)$$

will systematically decrease with the axial ratio.

and the Stokes-Einstein volume calculated by means of the equation,

$$v_{SE} = \frac{kT}{6\eta D_r} \quad (90)$$

will exceed the real volume of the object.

If these ideas of classical hydrodynamics were valid for aromatic hydrocarbons dissolved in the glycols, one should be able to derive the real shape of these molecules from the data of the anisotropic diffusion alone. The unsubstituted aromatic hydrocarbons would then be assigned shapes of prolate ellipsoids of revolution with axial ratios of 5-15! Moreover, the volumes calculated by means of the last equation would be expected to exceed the actual volumes of the molecules by a considerable margin. Instead the experimental data yield molar volumes systematically lower than the real ones, sometimes by a factor of five. The reasons for the discrepancy with the hydrodynamic predictions is to be found in the failure of the Stokes-Einstein assumption, that the fluorophores experience a continuous resistance from the medium moving in it without slip, and

simple consideration of the discontinuous motion of the fluorophore among the solvent molecules permits to account readily for the observed effects. The slipping rotations of the fluorophore in its own plane ^{are} facilitated by the absence of strong bonds with the solvent and lead to

angular displacement much larger than those allowed by the Stokes-Einstein assumption (the end averaging). Because of the comparably planar dimensions of molecules like porphyrin and 9:10 dimethylanthracene, in-plane rotation can take place with minimal disturbance of the surroundings but this is not the case with the out-of-plane rotations which require an extensive rearrangement of the solvent molecules in contact with the fluorophore. One would thus expect that out-of-plane rotations to be nearly as predicted by the hydrodynamic model and the in-plane rotations to be much faster leading to the observed decrease of the Stokes-Einstein volume.

This general picture is further validated by the observation that molecules which make 2 or 3 hydrogen bonds with the solvent (e.g. Guanidinoacetic acid and naphthylamine disulfonate respectively) show no detectable rotation at anisotropy and ^{their} Stokes-Einstein volume that approach or somewhat exceed the expected unsolvated molecular volumes. Evidently the presence of strong i.e. fluorophore-solvent bonds, of the same type of those existing among the solvent molecules, rule out the slipping motions and bring about the proportionality with the solvent viscosity predicted by the Stokes-Einstein relation. Multiple hydrogen bonds appear to be more important than single molecular volume in determining the validity of the Stokes-Einstein assumption, as exemplified by liquid water itself.

(Jonas et al.) From these observations one can also conclude that ^{protein} macromolecules, which in water solution are extensively hydrogen bonded to the solvent, would show rotational motions determined by their actual shape and therefore that this shape could be revealed by a study of the fluorescence depolarization of their solutions. To demonstrate this property one requires a protein carrying a fluorophore group oriented with respect to the inertial ellipsoid in a unique manner, and without appreciable independent rotation in the time span of the fluorescence lifetime. This limitations will be discussed in detail in the chapters dealing with the biological applications. At this point we note that not surprisingly - the determination of the absolute shape of a macromolecule by following the anisotropic fluorescence depolarization has not been achieved.

Oscillations in equatorial plane

A. Depolarization by phenomenological ellipsoid.

$$A = \frac{3}{10} \left[\frac{F-G}{1+x(1+\sigma)} + \frac{F+G}{1+x(1-\sigma)} \right] = \frac{3}{10} \left[\frac{2F + 2x(F+G\sigma)}{1+2x+x^2(1-\sigma^2)} \right]$$

$$\frac{A_0}{A} = \frac{3}{10} \cdot 2F \quad x = 6R\tau.$$

$$\frac{A_0}{A} = \frac{1+2x+x^2(1-\sigma^2)}{1+x\left(1+\frac{G\sigma}{F}\right)}$$

Initial slope ($x \ll 1$) is $2 - \left(1 + \frac{G\sigma}{F}\right) = 1 - \frac{G\sigma}{F}$

$$F = \frac{1}{3}(3\cos^2\epsilon - 1); \quad G\sigma = \frac{1}{3}(\delta_1\cos^2\epsilon + \delta_3\sin^2\epsilon)$$

$$G\sigma = \frac{1}{3R}(R_1\cos^2\epsilon + R_3\sin^2\epsilon - R)$$

$$1 - \frac{G\sigma}{F} = 1 - \frac{R_1\cos^2\epsilon + R_3\sin^2\epsilon - R}{3R\cos^2\epsilon - R} = \frac{R_2\cos^2\epsilon + R_3(2\cos^2\epsilon - 1)}{3R\cos^2\epsilon - R}$$

$$\frac{A_0}{A} = 1 + \frac{6R\tau}{3\cos^2\epsilon - 1} \left(\frac{R_2}{R}\cos^2\epsilon + \frac{R_3}{R}(2\cos^2\epsilon - 1) \right)$$

Since $3\cos^2\epsilon - 1 = 5A_0$.

$$\frac{A_0}{A} = 1 + 2\tau \cdot \left(R_2 \left(1 + \frac{1}{5A_0} \right) + R_3 \left(2 - \frac{1}{5A_0} \right) \right)$$

Since $R_2 = R_{eq}$ (out of plane); $R_3 = R_{ax}$ (in-plane).

$$\frac{A_0}{A} = 1 + 2\tau \left(R_{eq} \left(1 + \frac{1}{5A_0} \right) + R_{ax} \left(2 - \frac{1}{5A_0} \right) \right)$$

(Jonas et al.) From these observations one can also conclude that ^{protein} macromolecules, which in water solution are extensively hydrogen bonded to the solvent, would show rotational motions determined by their actual shape and therefore that this shape could be revealed by a study of the fluorescence depolarization of their solutions. To demonstrate this property one requires a protein carrying a fluorophore group oriented with respect to the inertial ellipsoid in a unique manner, and without appreciable independent rotations in the time span of the fluorescence lifetime. This limitations will be discussed in detail in the chapters dealing with the biological applications. At this point we note that not surprisingly - the determination of the absolute shape of a macromolecule by following the anisotropic fluorescence depolarization has not been achieved.

Setting

$$\langle R \rangle = R_{eq} \left(1 + \frac{1}{5A_0} \right) + R_{ax} \left(2 - \frac{1}{5A_0} \right)$$

We obtain

$$\langle R \rangle = \frac{3}{2} (R_{ax} + R_{eq}) \quad \text{for } A_0 = \frac{2}{5}, \text{ or } P_0 = \frac{1}{2}$$

$$\langle R \rangle = 3R_{eq} \quad \text{for } A_0 = \frac{1}{10} \text{ or } P_0 = \frac{1}{7}$$

$$\langle R \rangle = 3R_{ax} \quad \text{for } A_0 = -\frac{1}{5} \text{ or } P_0 = -\frac{1}{3}$$

In terms of P_0 .

$$\langle R \rangle = \frac{3}{10} \left[R_{eq} \left(3 + \frac{1}{P_0} \right) + R_{ax} \left(7 - \frac{1}{P_0} \right) \right]$$

Setting $F=0$ in equation we obtain the equation given the effect of the rotation when $A_0 = P_0 = 0$.
This is

$$A = \frac{3}{10} \left[\frac{2xG\sigma}{1 + 2x + x^2(1 - \sigma^2)} \right]$$

For a prolate ellipsoid of revolution $G = \frac{1}{3}$ and for an oblate ellipsoid ($R_{ax} < R_{eq}$) $G = -\frac{1}{3}$, giving

$$A = \pm \left[\frac{2x\sigma}{1 + 2x + x^2(1 - \sigma^2)} \right]$$

The + corresponding to the ^{prolate} case ($R_{ax} > R_{eq}$) and the negative sign to the oblate case ($R_{ax} < R_{eq}$)

$\alpha(\sigma) = 0$ gives $x = (\sqrt{1-\sigma^2})^{-1}$
 which is introduced in the last equation yields;

$$A_{\min} = \frac{2}{30} \frac{\sigma}{2 + \sqrt{1-\sigma^2}}$$

The maximum for the prolate ellipsoid is $+\frac{1}{10}$, for
 the oblate ellipsoid $-\frac{1}{10} (2+\sqrt{3})^{-1} = -0.02679$. For large
 values of $\sigma \rightarrow 1$ ($b/a \rightarrow \infty$) $A_{\min} \rightarrow \frac{1}{2}$, and for $\sigma = \frac{1}{2}$, $b/a \rightarrow \sqrt{2}$.

Polarization of the fluorescence of solutions.

The existence of polarization in dilute solutions of fluorescein in various solvents was first reported by F. Weigert in 1920. The solution placed at O (Fig) is illuminated by a parallel beam of light polarized along OZ (E vector) and propagating along OX. The observer is along X and makes two measurements of the intensity of the fluorescence after introducing in the beam a polarizer oriented so as to transmit light with its electric vector along OZ (I_0) or along OY (I_1). As Perrin pointed out (1926) the absorbing dipoles are randomly distributed in the solution, and the excited dipole population must have an axis of symmetry coincident with the electric vector of the exciting light. Moreover this axis of symmetry must be preserved afterwards since any random motions of the fluorophores can decrease but never increase the number of symmetry elements present. Then on excitation by light polarized along OZ the fluorescence components I_x , I_y , I_z must obey the relations

$$I_x = I_y \neq I_z. \quad (8)$$

The polarization of the radiation emitted in the three directions will be respectively P_x , P_y , P_z , and the last equation together with (7) gives

$$P_x = P_y > P_z = 0. \quad (9)$$

The important points follow that the radiation emitted in the direction of the electric vector of the excitation has zero polarization, and that the radiation emitted in all directions at right angles to it has the same state of polarization. Failure of this relation

can occur only if the absorbing dipoles are not randomly distributed, and can therefore provide an indication of the existence of preferential orientations, and of an underlying structure that is responsible for them.

Polarization due to excitation with natural light.

The effects of excitation with natural light is simply calculated by using the principle of superposition. Excitation by natural light is entirely equivalent to simultaneous excitation with two beams propagating in the same direction and polarized ^{and} in two directions normal to each other. These may be chosen to be OZ and OX. As the accompanying scheme shows the first beam gives ^{rise to} the polarized components

$$I_z^{OZ} \neq I_x^{OZ} = I_y^{OZ}$$

and the second.

$$I_x^{OX} \neq I_y^{OX} = I_z^{OX}$$

The two ^{exciting} beams being of equal intensity

$$I_x^{OX} = I_z^{OZ} \quad I_y^{OX} = I_y^{OZ} = \dots \quad (11)$$

The polarizations recorded for the fluorescence emitted along

OX, OZ and OY are

$$p_z(\text{nat.}) = p_z(\text{nat.}) = \frac{I_z^{OZ} + I_z^{OX} - I_y^{OZ} - I_y^{OX}}{I_z^{OZ} + I_z^{OX} + I_y^{OZ} + I_y^{OX}} = \frac{p_z}{2 - p_z} \quad (12)$$

$$p_y(\text{nat.}) = 0$$

where p_z refers to excitation with light polarized along OZ.

Notice that in the case of excitation by natural light the axis of symmetry of the excited doublet is the direction of propagation of the exciting beam and the radiation emitted in all directions would have the same state of polarization. The polarization emitted in the direction of the axis of symmetry itself is zero.

Polarization from a heterogeneous population. Selection law of the polarizations. The anisotropy of emission.

If there are several species in solution each of which emits fluorescence characterized by a polarization p_i , the average polarization $\langle p \rangle$ recorded ^{on excitation by polarized light} is given by

$$\langle p \rangle = \frac{\sum_i I_i^{\parallel} - I_i^{\perp}}{\sum_i I_i^{\parallel} + I_i^{\perp}} = \frac{\sum_i (I_i^{\parallel} - I_i^{\perp}) / (I_i^{\parallel} + 2 I_i^{\perp})}{\sum_i \{ (I_i^{\parallel} + I_i^{\perp}) / (I_i^{\parallel} + 2 I_i^{\perp}) \} T_i} \quad (13)$$

where $T_i \equiv I_i^{\parallel} + 2 I_i^{\perp}$ is the total radiation emitted by the i^{th} component upon excitation with light polarized in the parallel direction. Setting $\sum T_i =$ total anisotropy, T the contribution to the total anisotropy by the i^{th} component is $f_i = T_i / T$. Remembering that $I_i^{\parallel} / I_i^{\perp} = (2 + p_i) / (2 - p_i)$ from the definition (1), the last equation simplifies to:

$$\langle p \rangle = \frac{\sum_i \frac{2 f_i}{3 - p_i} \cdot p_i}{\sum_i \frac{2 f_i}{3 - p_i}} \quad (14)$$

Forming the quantity $1/\langle p \rangle - 1/3$ we finally obtain

$$(1/\langle p \rangle - 1/3) = \sum_i (1/p_i - 1/3) \cdot f_i \quad (15)$$

Thus, while the component polarizations p_i are not additive when weighted according to the contribution of the component to the total emitted fluorescence, the quantities $(1/p_i - 1/3)^{-1}$ enjoy this property, as demonstrated by Weber (1953). Because of this advantage Jablonski (1961) proposed to replace the polarization by a related quantity, the anisotropy of emission A . For excitation with polarized light

$$A = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} = \frac{2}{3} \left(\frac{1}{P} - \frac{1}{3} \right)^{-1} = \frac{2P}{3-P} \quad (16)$$

In terms of anisotropy, equation becomes

$$\langle A \rangle = \sum f_i A_i \quad (17)$$

where $\langle A \rangle$ and A_i refer to excitation by polarized light.

In many problems of computation of the fluorescence polarization from an arbitrary distribution of dipoles, the total population may be subdivided into categories for each of which a separate polarization equation may be written, and the overall polarization or anisotropy is then calculated by means of eqs. (15) or (17).

Preserving the definition of P_{nat} as the polarization observed in a direction normal to an unpolarized exciting beam, an equation similar to (15) is derived, namely:

$$\left(\frac{1}{\langle P \rangle_{\text{nat}}} + \frac{1}{3} \right)^{-1} = \sum f_i \left(\frac{1}{P_{i, \text{nat}}} + \frac{1}{3} \right)^{-1} \quad (18)$$

Eq. (17) would be valid for this case if the anisotropy for excitation with unpolarized light is defined as.

$$A_{nat} = \frac{I_0 - I_L}{2 I_0 + I_L} = \frac{1}{2} A \quad (19)$$

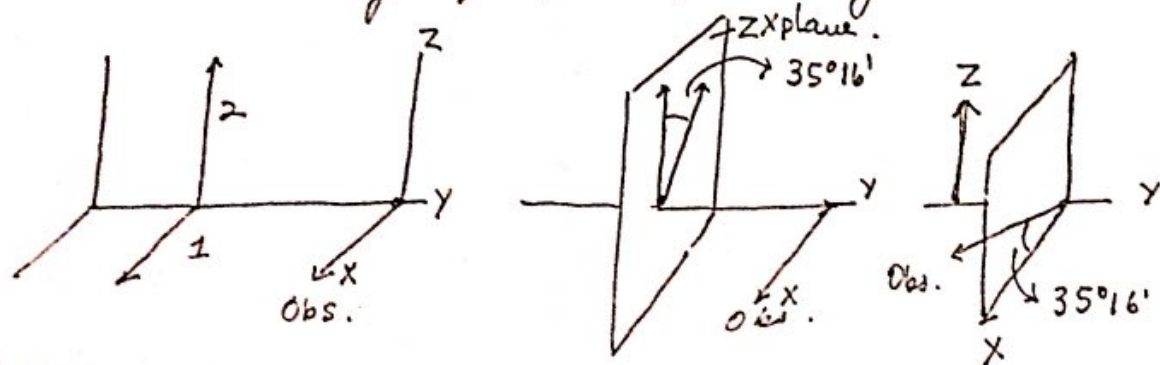
In general if excitation is by light in which the ratio of the intensity ^{polarized} along OZ to the intensity polarized along OX equals P , the fluorescence emitted normal to the direction of excitation follows the addition law

$$\left(\frac{1}{\langle P \rangle} - \frac{1-2P}{3} \right)^{-1} = \sum f_i \left(\frac{1}{P_{ip}} - \frac{1-2P}{3} \right)^{-1} \quad (20)$$

This yields equations 15 and 18 respectively on setting $P=0$ (polarized light) and $P=1$ (natural light). If $P = \frac{1}{2}$, the addition law simplifies to

$$\langle P \rangle = \sum f_i p_i \quad (21)$$

For this case the ordinary polarizations are additive to yield the average polarization, that is p and A are the same, A being then defined by eq. (7). A value of $P = \frac{1}{2}$ represents not only excitation by two beams polarized along OZ and OX and in the ratio of 2:1, but also excitation with polarized light with its electric vector at an angle of $35^\circ 16'$ to OZ, It is also valid for the fluorescence emitted at an angle of $54^\circ 44'$ to OZ on excitation by light polarized along OZ.



Definition of the function f on the interval $[a, b]$
Let f be a function defined on the interval $[a, b]$
and let x be a point in $[a, b]$.

We will consider the value of f at the point x .
 At first we will consider the case when x is a rational
 number. In this case we will consider the value of f at the point x .
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The value of f at the point x is denoted by $f(x)$.
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we have here $p = 1/2$. This is the maximum value that can be observed in the polarization of the fluorescence of solutions. Differences in the orientation of the absorbing and emitting dipoles, as well as a reaction of the molecule between excitation and emission can only effect a decrease in the maximum value. In general a system of molecules with preferential orientation can yield $p > 1/2$. In a crystal in which the fluorescent molecules are related to each other by displacement without rotation p can conceivably reach unity.

The relation

$$I_x = I_y = \frac{(1 - I_z)}{2} \quad (27)$$

and the equivalent

$$I_z = \langle \cos^2 \theta \rangle ; \quad I_x = I_y = \frac{1}{2} \langle \sin^2 \theta \rangle \quad (28)$$

where the mean values refer to the angle θ with the direction of the E vector of the excitation, are general for all cases of random orientation of the molecules at excitation and are not restricted to molecular dipoles, or to the case of orthogonal transition moments of absorption and emission.

Molecular dipoles. Non collinear oscillators.

Consider a coordinate system rigidly attached to the excited molecule and defined by three orthogonal directions 1, 2, 3 of which direction 1 lies along the absorption oscillator.

The matrix Λ below contains the nine cosine squares of these directions with the experimental coordinates of which Z is the direction of the electric vector of the excitation. On account of the additive property of the individual dipole contributions, these cosine squares will be taken as the mean values for the whole population. The matrix is doubly stochastic since the sum of the cosine squares from or to a given direction must equal unity. Therefore

$$\Lambda \equiv \begin{vmatrix} 1Z & 1X & 1Y \\ 2Z & 2X & 2Y \\ 3Z & 3X & 3Y \end{vmatrix} = \frac{1}{5} \begin{vmatrix} 3 & 1 & 1 \\ 1 & 2 & 2 \\ 1 & 2 & 2 \end{vmatrix} \quad (29)$$

The border values are determined by eq. (26) and (28) and the values of the principal minor follow from the doubly stochastic character and the equivalence of the X and Y directions.

An arbitrary direction in the molecule is defined by the direction cosines E_1, E_2, E_3 that it makes with 1, 2 and 3 respectively. The average cosine squares of this direction with the experimental coordinates are given by the product

$$\{E_1^2 \ E_2^2 \ E_3^2\} \cdot \Lambda = \left[\frac{1+2E_1^2}{5} \quad \frac{2-E_1^2}{5} \quad \frac{2-E_1^2}{5} \right] \quad (30)$$

where we have used the property $E_1^2 + E_2^2 + E_3^2 = 1$.

If the direction defined in the molecule is that of the

oscillator of emission, E_1^2 defines the cosine square of the angle between oscillators, and the intensities of the component dipoles are

$$\langle I_z \rangle = \frac{1+2E_1^2}{5}; \quad \langle I_x \rangle = \langle I_y \rangle = \frac{2-E_1^2}{5} \quad (31)$$

which reduce to (28) for the case of collinear oscillators.

The spherical symmetry of the system about z is reflected in the symmetry of Λ and results in the disappearance of E_2 and E_3 in the final result, namely the angle between the oscillators, not their absolute orientation within the molecule determines the polarization. Explicitly this is given by

$$P = \frac{3E_1^2-1}{3-E_1^2} \quad \text{or} \quad A = \frac{3E_1^2-1}{5} \quad (32)$$

For $E=1$ we obtain the collinear oscillator values, $1/2$ and $2/5$. For $E^2 = 1/3$ ($\cos^2 E = 54^\circ 16'$), $P = A = 0$. For $E_1^2 < 1/3$, polarization or anisotropy become negative reaching a maximum: absolute value at $E=0$, for which $P = -1/3$, $A = -1/5$.

The polarization of motionless dipoles, given by (32) is called the limiting or fundamental polarization and is often symbolized by P_0 , or A_0 . In practice it is obtained by observation at low temperature of dilute solutions of the fluorophore in a transparent solvent with a viscosity sufficiently high to render negligible the rotational motion of the molecule during the fluorescence lifetime.

Dependence of the limiting polarization upon the wavelength of excitation and emission.

The spectral distribution of the limiting polarization across the emission spectrum, for a fixed wavelength of excitation is called the 'emission polarization spectrum' and the set of limiting polarization values observed for a fixed waveband of emission as a function of the exciting wavelength is called the 'absorption polarization spectrum'. The variations of P_0 across the emission band are generally minor, but the variations with the excitation wavelength are usually large and in some fluorophores cover a range of values not much smaller than that set by equation (32)

Absorption polarization spectrum.

A case sufficiently simple to permit a straightforward interpretation is that of phenol in propylene glycol at -70°C (Fig) The plots show one region of high positive polarization corresponding to the long wave band with maximum at 275 nm. and one region of high negative polarization; in the range of the short wave band with maximum at 230 nm. Thus, the absorption transition moment for absorption to the S_1 state must be nearly parallel to the transition moment for the emission ($S_1 \rightarrow S_0$ transition) as it may have been expected one would expect. The negative value for excitation at 230 nm shows that the $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ transition moments of absorption must

be at a very large angle to each other, in fact close to 90° .

One can account for the observations on the basis of the geometric degeneracy of the electronic transitions of the aromatic hydrocarbons.

The $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ transitions of phenol correspond respectively to the L_b and L_a bands already discussed.

The fluorescence being essentially the result of L_b emission, it would be expected to have a transition moment direction nearly identical to that of absorption and therefore a high polarization, approaching $1/2$. Excitation of the L_a band would, on the other hand produce P_0 approaching $-1/3$. Neither in this case nor in any other aromatic molecule thus far studied are these values reached. The maximum positive and negative values observed are 0.45 and -0.25 (perylene). Apparently it is not possible in aromatics to obtain absorption in one of the two degenerate transitions without some excitation of the other. Linear conjugated molecules have no geometric degeneracy and it is possible to observe with them values of P_0 slightly above 0.49. $NADH_2$, although non-linear is not a planar aromatic molecule and it shows $P_0 = 0.47$ distinctly higher than the aromatic values.

* The excitation polarization spectrum of 6-propionyl-2-dimethylamino naphthalene is shown in fig . Three distinct transitions can be seen in absorption. Of these $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_3$ are very much stronger than $S_0 \rightarrow S_2$. The polarization spectrum shows an almost constant high value (~ 0.43) except in the region of appreciable $S_0 \rightarrow S_2$ absorption, but even here the minimum is $+0.16$. The ^{relative} strength of $S_0 \rightarrow S_2$ is not large.

enough to generate a negative polarization and the positive values are fixed by the much stronger L_b and B_b transitions.

The presence of appreciable ^{positive} polarization throughout the observable spectrum (250 nm - 800 nm) is often seen when there is a strong overlap of the absorption bands, like in the flavins.

In the substituted aromatic hydrocarbons no evidence is found that the simultaneous vibrational excitation causes any appreciable effects on the absorption polarization spectrum, but in the unsubstituted aromatic hydrocarbons variations in P_0 are observed which are clearly related in position to the vibrational details (Zimmermann and Joop)

However, the appearance of this vibrational structure in the absorption polarization spectrum does not indicate unequivocally that the vibrational excitation itself is responsible for a change in direction of the transition moment. Fig shows the absorption polarization spectrum of indole carried out with an excitation resolution of 1 nm. The variations of P_0 can be explained in this case by the overlap of the two orthogonal transitions L_a and L_b , each with a constant value of P_0 . The apparent vibrational structure of the limiting polarization results from the rapidly changing weight of the transitions owing to the vibrational structure of the absorption strength in one of them, and is evidently not due to an intrinsic effect of the vibrational excitation upon the direction of the transition moment. The inability of the vibrational excitations to generate such effects is made particularly evident in the absorption and

* emission spectrum of the polyeus. Although absorption and emission have pronounced vibrational details there are no evident changes of ρ_0 across the corresponding bands.

Emission polarization spectrum

The limiting polarization ^{of aromatic fluorophores} shows often a small variation across the emission band. This stands in contrast with the polyeus or NADH which show a single value of ρ_0 throughout. One is lead to the conclusion that in all probability the emitted band is influenced by overlap with a nearby

transition with orthogonal polarization. In this way one can explain the existence of the mirror image character observed in the polarization absorption and emission polarization spectra, without recourse to special depolarizing effects of the vibrations. This problem has not received a great deal of attention and this conclusion must remain provisional.

Depolarization due to extrinsic causes.

In opposition to the loss of polarization that results from the different orientation of the transition moments in absorption and emission, and which is intrinsic to the molecule, depolarization may occur due to extrinsic causes. These are the migration of the excited state from the originally excited molecule to an unexcited neighbor molecule, and the Brownian rotational diffusion of the isolated fluorophores. These processes can act concurrently but they are easily isolated for their separate study. In concentrated solutions of fluorophores in very viscous solvents Brownian rotations are negligible and energy migration is the sole extrinsic cause of depolarization; in dilute solution in solvents of low viscosity energy migration is insignificant and the depolarization is solely due to molecular rotations. The following Table summarizes the causes of depolarization.

Conditions	Observed Polarization	Rate of the depolariz. process	Mol. property responsible.
1. Dilute Solutions Very viscous solvents ($\eta > 10$ Poise)	Maxima value. for a given exc. wavelength $ P_0 $. $-\frac{1}{3} < P_0 < \frac{1}{2}$.	$\propto \tau^{-1}$	- Electronic structure of molecule.
2. Conc. Solutions Very viscous solvents ($\eta > 10$ Poise)	$0 \leq P \leq P_0 $	$\sim \tau^{-1}$	Electronic spectrum coupling between oscillators.

3. Dilute solutions

Fluid solvents

$$0 \leq |p| < |p_0| \quad \tau^{-1}$$

Rot. rate of
kinetic unit carrying
fluorophore.

- It is possible to give a unified description of the processes of extensive depolarization, since in both energy migration and brownian rotation the final effect is dependent upon the mean angle between the transition moments responsible for absorption and emission respectively. It is of no consequence, for the purpose of description that this mean angle is achieved in one case by energy migration between molecules, in the other by motion of the originally excited molecule.

Depolarization by rotation of spherical molecules.

If the fluorophores are excited by a z-polarized beam of light the instantaneous polarized intensities depend upon the corresponding dipoles defined by eq (27).

The molecular rotations work to equalize the differences in intensities created at the time of excitation. If the molecules are spherical there will be a unique rate of transfer of intensity from one ^{component} dipole to another, and this will be the rotational rate R of the sphere. The differential equations giving the change in dipole intensities with time are,

$$\frac{dI_i}{dt} = -(\Gamma + 4R) I_i + 2R I_j + 2R I_k + f_i(t). \quad (33)$$

where $i \neq j \neq k \neq i$, $i = x, y, z$.

The factor 2 associated with rotation about an axis arises because it is possible to exchange orientations by clockwise and anticlockwise rotations. There are only two independent equations (33) because $I_x = I_y$, and setting $I_{||} = I_z$, $I_x = I_y = I_{\perp}$, these equations are:

$$\frac{dI_{||}}{dt} = - (r + 4R) I_{||} + 4R I_{\perp} + f_{||}(t). \quad (34)$$

$$\frac{dI_{\perp}}{dt} = 2R I_{||} - (r + 2R) I_{\perp} + f_{\perp}(t).$$

$f_{||}(t)$ and $f_{\perp}(t)$ are arbitrary functions of the time describing the changes in dipole intensity as a result of excitation. We consider the solution of the system for three different types of such functions.

Rotational depolarization under steady illumination.

For this case we have constant values for $f_{||}(t)$ and $f_{\perp}(t)$, namely

$$f_{||}(t) = I_{||}(0) ; f_{\perp}(t) = I_{\perp}(0) \quad (35)$$

After a transient of the order of r^{-1} from the beginning of the illumination $dI_{||}/dt = dI_{\perp}/dt = 0$. Eq (34)

become a pair of simultaneous algebraic equations.

$$\begin{aligned} (r + 4R) I_{||} - 4R I_{\perp} &= I_{||}(0) \\ -2R I_{||} + (r + 2R) I_{\perp} &= I_{\perp}(0) \end{aligned} \quad (36)$$

Solving for $I_{||}$ and $I_{\perp}$ and forming the anisotropy

$$A = \frac{I_{11} - I_{12}}{I_{11} + 2I_{12}} = A_0 \frac{r}{r + 6R}$$

$$\text{or } \frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3} \right) (1 + 6R\tau) \quad (37)$$

The last form is the equation first derived by Perrin in 1926, if R is identified as the rotational diffusion constant

$$R \equiv D_r = \frac{kT}{6\eta v} \quad (38)$$

In eq (38) k is the Boltzmann constant T the absolute temperature, η the viscosity of the solvent and v the molecular volume of the fluorophore. The phenomenological derivation given above is quite different from the one given originally by Perrin and from two others subsequently proposed (Perrin 1936, Weber). It is considerably simpler and shows that the form of equation ⁽³⁷⁾ is dictated by the existence of a time-independent rate of rotation and is not a consequence of the microscopic properties of the rotary diffusion as one would be inclined to believe from the earlier derivations.

The validity of Perrin's equation may be tested by recording the variation of p^{-1} with T/η . An appropriate test must include a range of T/η sufficiently large to result in values of p from p_0 to $p_0/10$, as a minimum. This is attainable with fluorophores of small molar volume (~ 100) in solvents which, like the glycols, permit a change of viscosity of one to two orders of magnitude upon changing the

temperature. Globular ^{macro}molecules, like the proteins, are in principle very appropriate objects ^{for the purpose} as they approach the spherical shape more closely than most molecules. However their molecular volumes are of the order of 10^4 , so that the values of p are seldom less than 0.1. Over a limited range of values of p the linear relation of τ/η to p^{-1} may appear to hold within error with an evidently non-spherical molecule, or even when thermally activated partial rotations are present. A second way of testing Perrin's law consists in shortening systematically the value of τ by increasing concentrations of a collisional quencher. In this case the plot of $1/p$ against τ is linear, as shown in figure

The system (34) is now a set of two homogeneous linear differential equations of first order. It can be solved to give.

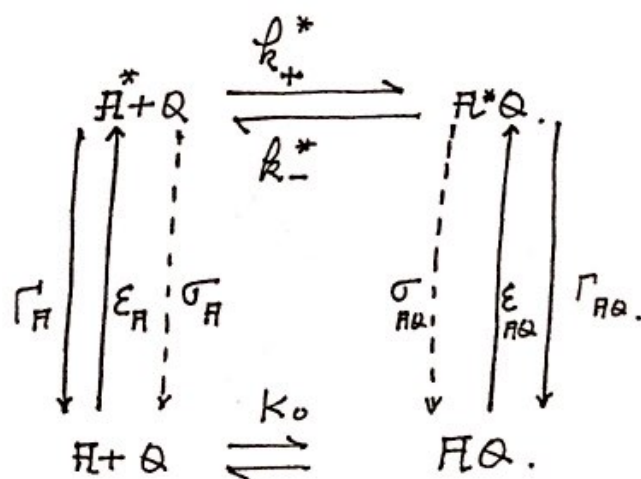
① or in a form including the limiting anisotropy

$$Y_0(t) = \frac{Y_0}{3} \left[\exp -\Gamma t + 2A(0) \exp -(\Gamma + 6R)t \right]$$

$$Y_1(t) = \frac{Y_0}{3} \left[\exp -\Gamma t - A(0) \exp -(\Gamma + 6R)t \right]$$

Chemical quenching.

The interaction of a fluorescent molecule A in its excited state with another molecule Q can lead to changes in the fluorescence yield and spectrum of the former. Various treatments of this problem have been given since the original observations of Stern and Volmer in 1919. The scheme below takes into account the possible existence of a dark complex FQ predating the excitation as well as an excited state complex F^*Q , which can result from either excitation of FQ or encounter of excited fluorophore and quencher. It is assumed that solutions of Q are transparent to the radiation employed to excite F , as well as to the fluorescence. For this reason Q is often called a 'colourless' quencher. The interconverting scheme is shown below:



F and F^* stand here for V_0 and V_1 of the general two-state scheme, FQ and F^*Q for R_0 and R_1 respectively. k_-^* is replaced here by $k_+^*[Q]$ and k_+^* by k_-^* (dissociation rate of the F^*Q complex). Formation of a ground-state or 'dark' complex between F and Q produces usually only a small

contribution of the absorption spectrum. On excitation at an isobestic point of the spectra of F and FS the fractional excitations E_F and E_{FS} will equal respectively α and $1-\alpha$, α being the degree of dissociation of the molecular complex. The fluorescence emission from F^* and FS^* under conditions of stationary excitation equal

$$\begin{aligned} F_F &= \frac{I_0}{I_{00}} (\alpha + k_-^* \tau_{FS}) / D \\ F_{FS} &= \frac{I_0}{I_{00}} (1 - \alpha + k_+^* \tau_F [S]) / D. \end{aligned} \quad (45)$$

$$D = 1 + k_+^* \tau_F [S] + k_-^* \tau_{FS}$$

The typical quench is one which renders the fluorescence efficiency of the fluorophore vanishingly small at sufficiently high concentrations. This requires $\frac{I_0}{I_{00}} \ll 1$ and $k_-^* \tau_{FS} \ll 1$. We shall refer to this type as 'strong quenching'. If these conditions obtain

$$F_F \approx F_F + F_{FS} = \frac{\alpha I_0}{1 + k_+^* \tau_F [S]} \quad (46)$$

From the definition of the equilibrium constant of the complex

$$\alpha = \frac{1}{1 + K_0 [S]}. \quad (47)$$

The ratio F_0/F of the emission in the absence and presence of the 'strong quench' is

$$\frac{F_0}{F} = (1 + K_0 [S]) (1 + k_+^* \tau_F [S]) \quad (48)$$

The first factor in the last equation arises from non fluorescent complexes existing at the moment of the excitation, the second from dynamic quenching of the free excited fluorophores. If either of these two effects is negligible in comparison with the other, the quenching follows the Stern-Volmer law which predicts a linear dependence of F_0/F upon quencher concentration. From such linear dependence it is not possible to infer the character, static or dynamic of the quenching. Moreover when the departure from linearity indicates that both forms are present it is not possible to decide which is the predominant one.

The quenching of fluorescence by dissolved oxygen, $\text{Fig. } 1$, approaches in many cases the ideal collisional case, in which k_+^* approaches the diffusion controlled rate of encounter of fluorophore and quencher. According to Smoluchovsky's theory of coagulation

$$k_+^* = 4\pi N' (a_f + a_q) (D_f + D_q) \quad (49)$$

where D_f, D_q are the diffusion coefficients of fluorophore and quencher, a_f and a_q the radii of fluorophore and quencher respectively and N' the number of quencher molecules in 1 ml. of molar solution (6.03×10^{20}). With $D_f + D_q \sim 4 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ and $a_f + a_q = 5 \times 10^{-8} \text{ cm}$. $k_+^* \approx 1.10^{10} \text{ s}^{-1}$. For a typical lifetime of the excited state of 10^{-8} s , one requires $[Q] = 10^{-2} \text{ M}$ in order to reduce the fluorescence to one half of F_0 . Evidently quenching at concentrations smaller than this

must be due to the formation of molecular complexes that predate the excitation. An equilibrium constant K_0 larger than $10^2 \text{ l} \cdot \text{m}^{-1}$ would be required for such an effect and this corresponds to a ^{standard} free energy of complex formation of nearly 3 kcal/mole. Complexes with these appreciable free energies are found in the interaction of aromatic molecules. But such values are rare and smaller ones are the rule. As a result the common mode of quenching is a mixed one in which neither the dark nor the excited state interactions can be completely neglected.

In cases of "weak quenching" in which F_{AQ} cannot be neglected, and

$$F = (F_A(\alpha + k_-^* \tau_{AQ}) + F_{AQ}(1 - \alpha + k_+^* \tau_A [Q])) / D \quad (50)$$

it is easy to derive that

$$\frac{F}{F - F_0} = \frac{F_{AQ}}{F_A - F_{AQ}} + \frac{F_A}{F_A - F_{AQ}} \frac{(\alpha + k_-^* \tau_{AQ})}{(1 - \alpha + k_+^* \tau_A [Q])} \quad (51)$$

At a sufficiently high concentration of quencher the second term makes a very small contribution and F_A/F_{AQ} may be estimated. The last equation is also applicable to the known cases in which formation of a molecular complex results in an increase in fluorescence efficiency. Some of these cases show appreciable displacements of the emission spectrum which permit further characterization of the process, very much like in the case of protein denaturation with formation of a strongly fluorescent conjugate molecule.

Under conditions of strong quenching eq. (27) adopts a very simple form,

$$u = \frac{1}{\tau_A} + k_+^* [Q] = S. \quad (52)$$

$$V_1 = \frac{\alpha}{2} \quad V_2 = R_1 = R_2 = 0.$$

and the decay of the fluorescence after an instantaneous light pulse is given by

$$F(t) = F(0) \exp(-t/\bar{\tau}). \quad (53)$$

$$\text{where } 1/\bar{\tau} = 1/\tau_A + k_+^* [Q].$$

The ratio $\tau_A/\bar{\tau}$ is given by.

$$\tau_A/\bar{\tau} = 1 + k_+^* [Q] \tau_A. \quad (54).$$

Comparing the last equation with (48) we see that for strong quenching, with $k_0 \ll k_+^* \tau_A$

$$\frac{\tau_A}{\bar{\tau}} = \frac{F_0}{F} \quad (55).$$

Equation (55) is characteristic of perfect collisional quenching. If the ratio $\tau_A/\bar{\tau}$ is measured for a series of quencher concentrations, k_+^* can be independently calculated by eq (54) and the effects of static and dynamic quenching in (48) separately assessed. When quenching is due to complex formation $\tau_A/\bar{\tau}$ remains close to unity at the relevant values of $[Q]$. In general then the quantity

$$\gamma \equiv (F/F_0) (\tau_0/\bar{\tau}). \quad (56)$$

gives a measure of the relative weights of static and dynamic.

quenching approaching unity in the latter case and zero in the former. Measurements of $\bar{\tau}$ and F as a function of quencher concentration permit the separate evaluation and comparison of $k_+^* \tau_F$ and K_0 . We have regarded the latter constant as describing the formation of molecular complexes of fluorophore and quencher. Many cases of predominantly static quenching admit no other interpretation. In the earlier literature on quenching molecular complexes were not recognized and weak but evident static effects, revealed by departure from the Stern-Volmer law, were treated in a different manner. Frank and Wavilov explained the deviations from the Stern-Volmer law by introducing a 'sphere of action' for the quencher. If a fluorophore found itself within such sphere of action at the time of the excitation the probability of quenching was considered to be unity. However the experiments on dynamic quenching show categorically that the maximum quenching rate is that of diffusion controlled encounters with characteristic distances, never larger than the sum of the molecular radii and the assumption of a 'quenching sphere of action' larger than the quencher itself has no physical basis. Predominantly dynamic quenching requires concentrations of 10^{-2} - 10^{-1} M in quencher and the deviations from the Stern-Volmer law are seen at 0.1 M concentration or higher. The free energy of formation of the assumed molecular complexes will be on the order of kT .

or smaller and one cannot in these cases expect formation of a unique complex with rigorous stoichiometry and a single K_0 value independent of quencher concentration. Additionally the complexity of the diffusion process when the mean square displacement of the quencher during the actual lifetime τ becomes comparable to the molecular radius results in further uncertainty. In conclusion when K_0 is only a fraction of $k_+ \tau_R$ it cannot be given a very precise physical significance and is best regarded as resulting from several concurrent causes, of which the predominant one is the formation of weak molecular aggregates of quencher and fluorophore. It must be stressed that the ^{molecular} association and therefore the observation of predominantly static or dynamic effects is strongly dependent upon the solvent. The table below shows that the quenching of 3-methyl lumiflavin by caffeine is predominantly static in water but almost wholly dynamic in chloroform.

Quenching of 3-methyl lumiflavin
by caffeine

In CH_3CH	$[Q]$	F_0/F	τ_0/τ	δ
	0.5	2.0	2.0	1
	1	4.5	4.38	0.97
In H_2O	0.015	2.46	1.36	0.55
	0.040	4.83	1.77	0.37

For $\text{C}_2\text{H}_5\text{CH}_3$ $k_r^* = 5.10^8$ ($\tau_A = 4.3 \times 10^{-9}$)

For H_2O $k_{qT}^* = 4.7 \times 10^8$, $k_0 = 67 \text{ l M}^{-1}$, $\Delta G^\circ = -2.5 \text{ kcal M}^{-1}$.

Differential phase measurements have no application in cases of 'strong quenching' as there is no detectable emission from F^*Q , but phase measurements of the overall lifetime τ , justified by eq (53), constitute an extremely convenient method to study dynamic quenching because of the ease, speed and precision of the measurements of short lifetimes by this method.

Mechanism of action of transparent quenchers

The ^{cause of the} large increase in the rate of radiationless transitions upon interaction of quencher and fluorophore have been much discussed and it is acknowledged that no general rule can be formulated as the processes involved are quasi chemical in nature, and can differ widely from one case to another. Proton transfer, electron transfer, electron exchange, enhanced spin orbit coupling may be major influences in particular instances. The difficulties in the analysis is enhanced because the individual behaviour depends not only on the quencher, but also on the fluorophore. Purine form well defined stacked complexes with many aromatic fluorophores. In some cases (acridine, isoxanthines) they act as strong quenchers. In others (Rhodamine B, Quinacrine carboxylate) they induce a shift in the fluorescence spectrum with some enhancement of the efficiency of emission, and in yet other cases (4-hydro

(N-methyl nicotinamide) no detectable change in the fluorescence is seen upon formation of the complex.

Solvent Effects Upon The Fluorescence Emission

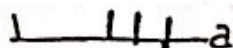
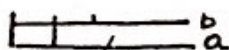
The width of the electronic absorption bands in solution is similar to that observed in the gas phase but the resolution of the energy levels is much less complete. The highest resolution of the spectra is observed in the solutions of the unsubstituted aromatic hydrocarbons in non-polar solvents particularly in these non polar solvents which interact minimally with the fluorophore like the perfluorohydrocarbons (Lipsky). Even in these cases resolution is limited to the vibrational structure. The substituted aromatic hydrocarbons have much less resolved spectra than the unsubstituted ones. When atoms carrying lone pairs are directly attached to the aromatic rings hydrogen bonding with proton donor solvents results in efficient coupling of the electronic and vibrational energies permitting only small ^{differences} ~~changes~~ in the probability of transitions to nearby energy levels. The general result is that a classical analysis of the fluorescence spectrum of substituted aromatic molecules in solution in terms of energy levels is virtually impossible, in all but exceptional cases. The most common observation is of a spectrum consisting of a band of energies closely represented by a skewed Gaussian distribution. (See Schpoliskii) The influences already described are further complicated by the existence of multiple interactions, sometimes quite specific in nature, between solvent and fluorophore molecules. These result from dispersion forces, permanent dipole effects and hydrogen bonds. In this way a whole set of weak molecular complexes of solvent and fluorophore are generated, ^{and} ~~so that~~ the absorption and fluorescence spectra of molecules in solution are best described as belonging to a molecular population rather ^{not} ~~than~~ to a single molecular species. It is not easy to ^{ec} ~~divide~~ in any particular case - let alone in general - the extent of spectral heterogeneity owing to weak complexes of solvent and fluorophore. The energies of interaction between a solvent with an appreciable dipole moment (e.g. water) and a fluorophore

with a permanent dipole moment of 2 - 4 Debye units is as large or larger ~~of the order of~~ ^{than} KT at room temperature. Being ~~of the order of~~ ^{of the order of} the thermal energy these interactions do not give rise to a single ~~species of~~ ^{molecular} complexes with uniquely defined electronic ground states but to a ~~virtually continuous~~ ^{discrete} set of such states.

For dipole-dipole interactions the distribution of the states is prescribed by the Langevin equations: * Upon excitation each of these complexes will generate a Frank-Condon excited state of its own and the interconversion of these within the excited lifetime will depend upon the strength of the molecular interaction as well as the temperature and viscosity of the solvent. It would appear at first examination that because of the Frank-Condon restriction the set of

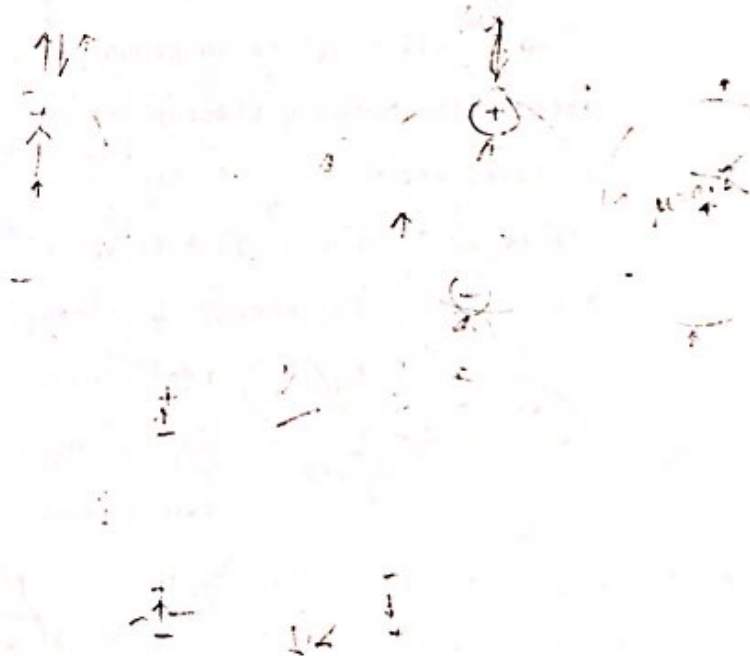
The structure and dynamics of such distributions are not easy to describe; but the average effects of the molecular interactions upon the electronic energy can be estimated much more directly.

Continuum and Dipole-pair models of fluorophore-Solvent interactions



However, owing to changes in the electronic distribution in the fluorescent molecules in the ground and excited states the complexes with the maximum interaction and therefore lowest energy, will be different from the ground and excited states. In over simplified form the change in the electronic distribution may be associated with a difference in both magnitude, and direction of the dipole moments μ_0 and μ^* corresponding to ground and excited state. The smaller energy difference, and therefore the longest wavelength absorption, will correspond to

solvents like cyclohexane and water respectively. If the largest values of $\Delta v'$ do not exceed $5,000 \text{ cm}^{-1}$ and from the molecular dimensions $\mu^* - \mu$ cannot be larger than some 30 D units, the last equation indicates that in the limit the surface density of charge appearing on a sphere of $4 \text{ \AA}$ radius would be close to one electronic charge. This suggests that the interaction of a pair of solvent dipoles with the excited fluorophore would furnish a sufficient approximation and one much more physically realistic than the continuum models commonly employed. Further such dipole pair model would permit straight forward extension to include the effects of viscosity and temperature upon the spectrum. This dipole model can be developed under the following assumptions:

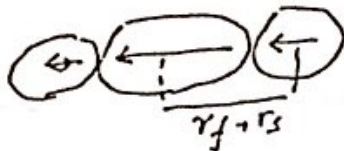


correspond to a less stable state (b) in the above scheme. In a fluid medium excitation at the longest wavelength will still result in a fluorescence spectrum entirely like that observed on excitation at other wavelengths. However, if the viscosity of the solvent is high and the equilibration with appearance of the several possible solvent fluorophore complex is impeded, only the emission from the lowest-energy excited complex is observed. This red shift of the fluorescence has been repeatedly observed in solvents at low temperatures when excited at the red edge of the absorption and is most marked in polar solutions of those fluorophores that differ most in their ground and excited state dipole moments. In agreement with expectations the observed red shifts corresponds to energies of the order of 1 kcal.

The Effect of temperature upon the fluorescence emission

Following in more quantitative fashion the ideas sketched above, the complete process of absorption and emission will take the following course:

1. Absorption takes place from a manifold of states corresponding to discrete solvent-fluorophore complexes. For a quantitative analysis we consider the interactions to be those between point dipoles of strength μ_s (solvent) and μ^0 or μ^* (fluorophore in ground or fluorescent state respectively). Maximal interaction between fluorophore and solvent may be assured as result of anti-parallel arrangement of three dipoles, ^{one belonging to} the fluorophore and two solvent molecules, ^{to be} placed at distances $r_s + r_f$ where r_s and r_f are ^{respectively} the radii of solvent and fluorophore. The energy of interaction between the two solvent dipoles may be



neglected as it would contribute at best a few percent to the total energy, and assuming independence in the two remaining interactions, the energy is

$$u = -\frac{\mu_s \mu^0}{r^3} \quad ; \quad r = r_s + r_f \quad \text{ex (5)}$$

The change in energy of the emission, $\Delta u'$ brought about by interaction of the excited molecule with a polar solvent can be calculated in the manner already explained under 'Influence of the solvent upon absorption'. Assuming for the moment that the interaction between excited fluorophore ~~and~~, with dipole moment μ^* , and the solvent dipoles can reach thermodynamic equilibrium within the fluorescence lifetime, the energy decrease of the lowest singlet state below the energy reached in absorption equals

$$u'^* - u^* = \frac{\mu^*}{a^3} (R_{or}^* - R_{or}) \quad (1)$$

since the electronic contributions cancel out. Similarly the difference between the ground state levels reached after emission and the one before absorption equals,

$$u' - u = \frac{\mu}{a^3} (R_{or}^* - R_{or}). \quad (2)$$

giving for the ^{difference} Stokes shift between a polar solvent and a solvent with $\Delta f = 0$,

$$\Delta u' = hc \Delta \nu' = (\mu^* - \mu) (R_{or}^* - R_{or}) / a^3. \quad (3)$$

If we consider a given fluorophore for which μ and μ^* have fixed values, and an unknown medium with orientational polarizabilities R_{or}^*, R_{or} harboring it we see that

$$\frac{hc \Delta \nu'}{(\mu^* - \mu)} = (R_{or}^* - R_{or}) / a^3. \quad (4)$$

The dimensions of the r.h.s. are evidently charge ^{per} unit area corresponding as they do to the (neutralizing) distribution of neutralizing charge over the surface of the solvent cavity harboring the molecule. The largest Stokes shift difference observed in fluorophores dissolved in

Similarly

$$u^* = -\frac{\mu_s \mu^*}{r^3}$$

(2) 6

The average energy of interaction would be smaller because of the disorienting effect of temperature. The Boltzman average for this case ~~is~~ ^{will be taken as} given by the

Langevin equation

$$\langle u \rangle = u \cdot L(u/KT)$$

$$\langle u^* \rangle = u^* L(u^*/KT)$$

(3) 7

where

$$L(u/KT) = \coth \left(\frac{u}{KT} \right) - \frac{KT}{u}; \quad L(u^*/KT) =$$

(4) 8

The average level from which absorption takes place has an energy

$$\mathcal{E}_a = \mathcal{E}_a^0 - u L(u/KT)$$

(5) 9

where $\mathcal{E}_a^0$ is the zero vibrational level of the ground state in the absence of dipole interactions.

2. Because of Franck-Condon restrictions the average state reached in absorption has an additional energy $- \mu^* L(u/KT)$ since the dipole moment of the fluorophore has instantly changed to μ^* , but the dipole distributions are still those corresponding to μ^0 . The fluorescent state reached has an energy

$$\mathcal{E}_f = \mathcal{E}_f^0 - \mu^* L(u/KT)$$

(6) 10

3. If the temperature and viscosity of the solvent permits the rearrangement of the solvent cage during the fluorescent lifetime, the transition $L(u/KT) \rightarrow L(u^*/KT)$ takes place. The effect of this is to decrease the total energy of the system at the expense of the electronic energy which reaches now a level

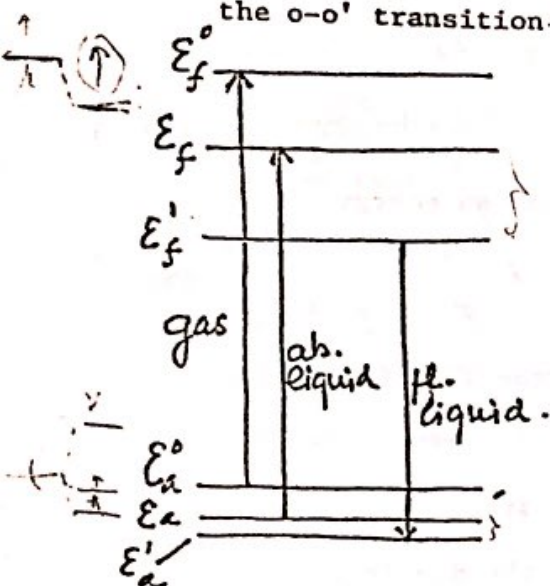
$$\mathcal{E}_f' = \mathcal{E}_f^0 - u^* L(u^*/KT)$$

(7) 11

4. Emission takes place from this state consisting in a transition to a ground state level given by

$$\epsilon_a' = \epsilon_a^0 - u L(u^*/KT)$$

This state lies evidently below the starting ground state, corresponding as it does to a more perfect alignment of the ^{solvent} dipoles. The final step is the conversion of the electronic energy $u(L(u^*/KT) - L(u/KT))$ into entropy with a corresponding rise in the electronic energy level. The general disposition of the levels is shown in the diagram. The energy of the o-o' transition ^{in abs. phase} is given by



$$A = \epsilon_a^0 - \epsilon_f^0 - u L\left(\frac{u}{KT}\right) + u^* L\left(\frac{u^*}{KT}\right)$$

$$A = (\epsilon_a^0 - \epsilon_f^0) + L\left(\frac{u}{KT}\right) * (u^* - u) \quad (9) 13$$

and the fluorescence energy on the assumption of equilibration of the dipole interactions in the excited state is

$$F = (\epsilon_f^0 - \epsilon_a^0) - u^* L\left(\frac{u^*}{KT}\right) + u L\left(\frac{u}{KT}\right)$$

$$F = (\epsilon_f^0 - \epsilon_a^0) - L\left(\frac{u^*}{KT}\right) * (u^* - u) \quad (10) 14$$

and the Stokes shift ^{Stokes} due to the solvent-fluorophore interaction is

$$S = A - F = -(u^* - u) \left[L\left(\frac{u^*}{KT}\right) - L\left(\frac{u}{KT}\right) \right] \quad (12) 16$$

If $u^* > u$ we have $L(u^*/KT) > L(u/KT)$ and the Stokes shift is negative (red shift).

The last equation is valid provided the solvent shell can undergo during the fluorescence lifetime the rearrangement of the dipoles to satisfy the interaction with u^* . Considering such a rearrangement of the fluorophore solvation as a simple two-state process, it will be accomplished during the excited state to a fractional extent given by

$$\frac{\tau}{\tau + \rho_s}$$

where τ is the fluorescence lifetime and ρ_s the correlation time for the

change in the solvent shell. The average state reached at the time of the emission will correspond then to an average energy decrease.

$$L\left(\frac{u}{KT}\right) \frac{\rho_s}{\rho_s + \tau} + L\left(\frac{u^*}{KT}\right) \frac{\tau}{\rho_s + \tau} \quad (13) \quad 17$$

which gives when introduced in eq. (11)

$$F = \varepsilon_f^0 - \varepsilon_a^0 - (u^* - u) \left[L\left(\frac{u}{KT}\right) \frac{\rho_s}{\rho_s + \tau} + L\left(\frac{u^*}{KT}\right) \frac{\tau}{\rho_s + \tau} \right] \quad (14) \quad 18$$

Eq. (14) permits calculation of the fluorescence spectral shift as a function of temperature, rather than the Stokes shift itself. The latter can be difficult to determine experimentally when transitions of higher energy overlap appreciably the first excited state. This difficulty of separating the $S_0 \rightarrow S_1$ transition from others, does not exist in the fluorescence emission which in the vast majority of cases represent the single $S_1 \rightarrow S_0$ transition.

Multiply hydrogen bonded solvents like the glycols forms viscous glasses at low temperatures. Under these conditions $\rho_s \gg \tau$ and equation (14) simplifies to

$$F \equiv F_0 = \varepsilon_a^0 - \varepsilon_e^0 - L\left(\frac{u}{KT}\right) (u^* - u) \quad (15)$$

As the temperature is increased $L\left(\frac{u}{KT}\right)$ is progressively replaced by $L\left(\frac{u^*}{KT}\right)$ and the fluorescence shifts rapidly to the red approaching a limit when $\tau/(\tau + \rho_s) \rightarrow 1$. At still higher temperatures the red shift reverses itself slowly as $L\left(\frac{u}{KT}\right)$ is a decreasing function of the temperature, though it changes much more slowly with temperature than ρ_s , which is proportional to the ratio of solvent viscosity to temperature (η/T). The figure below shows a theoretical calculation for a 'typical' solvent, in which the thermal coefficient of the viscosity depends upon the viscosity itself in the manner,

$$\Delta\eta/\Delta T = -0.02 (1 + \log \eta \cdot n_{cf}) \quad (16)$$

If a fluorophore with dipole moment D^0 and D^* Debye units in the ground and excited state respectively is placed in a solvent with dipole moment D_s , and the distance between the interacting dipoles in Angstrom units is a , equation (18) may be written, after evaluating the numerical constants,

$$\Delta\bar{\nu} = \frac{F-F_0}{hc} = -10,068 \frac{D_s(D^*-D^0)}{a^3} \left[L\left(7,242.7 \frac{D_s D^0}{a^3 T}\right) \frac{P_s}{P_s + \tau} + L\left(7,242.7 \frac{D_s D^*}{a^3 T}\right) \frac{\tau}{P_s + \tau} \right] \quad (19)$$

$\Delta\bar{\nu}$ is the change of fluorescence wave number.

It is easy to recognize three different temperature-dependent regimes. These correspond to regions of temperature where $P_s \gg \tau$, $P_s \ll \tau$ and an intermediate one when neither τ nor P_s is negligible with respect to the other. In the regime where the solvent relaxation time is very long compared to the fluorescence lifetime no change in the solvation shell of the fluorophore can take place during the ^{excited state} $P_s/(P_s + \tau) \rightarrow 1$ and $\tau/(P_s + \tau) \rightarrow 0$. The last equation reduces to:

$$\Delta\bar{\nu} = -10,068 \frac{D_s(D^*-D^0)}{a^3} L\left(7,242.7 \frac{D_s D^0}{a^3 T}\right) \quad (20)$$

At sufficiently high temperatures, when rapid rearrangement of the solvation shell takes place and $\tau \gg P_s$,

$$\Delta\bar{\nu} = -10,068 \frac{D_s(D^*-D^0)}{a^3} L\left(7,242.7 \frac{D_s D^*}{a^3 T}\right) \quad (21)$$

The dependence of P_s upon the temperature is that of the ratio T/η , where η is the viscosity coefficient of the solvent. Over a limited range of temperatures we can set

$$T/\eta = (T_0/\eta_0) \exp -\alpha(T-T_0)$$

where α is thermal coefficient of the ratio T/η over the

* range of temperature centered at T_0 and this

(*)

$$f_5(T_0) = \tau. \quad (1^*)$$

Figure shows a plot of equation (19) for a particular case for which we have chosen.

$$D_1 = 4, \quad D_2 = 2, \quad D^* = 24, \quad a = 5$$

$$\text{and } \alpha = 0.085 \text{ cm}^{-1}$$

The dotted lines are for the asymptotes given by eqs. 18 and 19 respectively, which obtain at $f \ll T_0$ and $f \gg T_0$. The transition region where the regime change from one asymptote to the other spans some 60°C . It is seen that the high-

temperature asymptote ($f \ll T_0$) corresponds to a blue shift with temperature of about 1 cm^{-1} per degree and the low-temperature asymptote ($f \gg T_0$) exhibits a blue shift of ca. 5 cm^{-1} per degree. This follows results from $D^* > D^0$.

In solvents which are very fluid over a very large temperature range (e.g. alcohol or acetone)

observations would virtually cover only the high-temperature regime. Solvents in which it is possible to span a very large viscosity range in an interval of 150° to 200°C , such as the glycols, should permit observation of the three regimes present in the graph. From this one can determine unequivocally the best value of μ^* and the dependence upon μ^0 and μ_0 by spectral observation in a single solvent.

(†) With the condition eq. (24) reads

$$\Delta \bar{\nu} = -12,061 \frac{D_2(D^* - D^0)}{a^2} \left[L \left(7.2427 \frac{D_2 D^0}{a^2 T} \right) \frac{\exp - \alpha(T - T_0)}{1 + \exp - \alpha(T - T_0)} + L \left(7.2427 \frac{D_2 D^*}{a^2 T} \right) \frac{1}{1 + \exp - \alpha(T - T_0)} \right]$$

(19 bis)

The displacement of the fluorescence spectrum with temperature takes place according to three different regimes depending on whether the solvent relaxation is negligible during the fluorescence lifetime ($\rho_s \gg \tau$) or whether commensurate with it ($\rho_s \sim \tau$) or so much shorter that it can permit establishment of the thermodynamic equilibrium of the dipoles in the excited state ($\rho_s \ll \tau$).

At very low temperatures, when $\rho_s \gg \tau$, solvent relaxation during the excited state is absent and the stabilization of the 0 levels of S_0 and S_1 is entirely dictated by the equilibrium dipole orientation in the ground state, the perfection of which is given by the factor $L(u/kT)$. The emission energy shift is $(\mu^* - \mu) L(u/kT)$. The increase in temperature acts by the change in $L(u/kT)$. This decreases as the temperature rises and so a blue shift with increasing temperature takes place, which we shall refer to as the 'low temperature blue shift'.

In the interval of temperatures where ρ_s and τ are commensurate the excited state energy is decreased during the lifetime of the excitation as the equilibrium of the dipole corresponding to $L(u/kT)$ changes into that given by $L(u^*/kT)$. Since $u^* > u$, $L(u^*/kT) > L(u/kT)$ and the (shift in) energy progressively decreases with temperature giving rise to a red shift of the fluorescence spectrum as the temperature rises. At still higher temperatures at which $\rho_s \ll \tau$, the change in energy of the emission is again determined by the progressive disorientation of the dipoles as the temperature rises, but in this high-temperature range, the stabilization of the S_1 and S_0 states are determined by the equilibrium reached in the excited state that is by the factor $L(u^*/kT)$, and a further blue shift with temperature takes place. This 'high temperature blue shift' is smaller than the low temperature blue shift because $dL(u^*/kT)/dT < dL(u/kT)/dT$ if $u^* > u$.