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Fluorescence-Polarization Spectrum and Electronic-Energy Transfer in Tyrosine, Tryptophan and Related Compounds

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The polarization spectrum of the fluorescence is the set of values of the polarization of the fluorescence emitted upon excitation with light of different wavelengths. Simple symmetry relations exist among the polarizations of the fluorescence emitted in different directions, and by excitation with light of different states of polarization (Perrin, 1929; Soleillet, 1929), so that the values for a single direction of observation and a given state of polarization of the exciting radiation contain all the relevant information. The data recorded in this paper are for the fluorescence emitted at right angles to the direction of the exciting, unpolarized beam.

The absolute value of the polarization of the fluorescence emitted by molecules in solution can decrease as a result of the Brownian molecular rotations (Perrin, 1926), as well as by intermolecular transfer of the excited state (Sveshnikoff & Feofilov, 1940; Weber, 1954). In a dilute solution in which the average distance between molecules is much greater than the distance at which transfer can readily take place (of the order of 30 Å), and in a medium of high viscosity in which the rotational relaxation time of the molecules is much longer

than the lifetime of the excited state, both these causes of depolarization disappear and the polarization reaches a maximum absolute value, the principal polarization. In some well-studied cases this appears still inferior to the value that would be expected from a knowledge of the relative orientation of the absorption and emission oscillators, due to the molecular-torsional vibrations (Jablonski, 1950), and perhaps to other causes at present imperfectly understood. A plot of the principal polarization against wavelength, the principal polarization spectrum, reflects to a first approximation the relative orientations of the transition moments associated with the absorption and emission of light (Jablonski, 1935). These directions of the transition moments are expected to remain constant for each electronic transition. Ideally, the principal polarization spectrum should consist of regions of constant polarization where the excitation falls upon one electronic band alone, joined by regions of rapidly changing polarization where there is overlap of contiguous electronic bands. A study of the principal polarization spectrum should lead to the enumeration of the existing electronic bands, and to the distinction between

vibrational details inside an electronic band and two or more overlapping electronic transitions. In the latter case it should be possible to determine the importance of each of the contributing electronic transitions on changing the environment or in comparing related substances, a possibility that is not offered by a study of the absorption spectrum alone.

THEORETICAL

The intermolecular transfer of the electronic energy has been followed by observations on the polarization of the fluorescence of concentrated solutions in the transfer among like molecules (phenol-phenol and indole-indole), and by observations of the fluorescence spectrum of mixtures in the transfer among different molecules (phenol-indole).

The transfer of electronic energy among molecules in solutions is now well established. The theories developed (Perrin, 1932; Förster, 1947; Oppenheimer & Arnold, 1950) aim at calculating the characteristic distance R between two molecules at which the chances of transfer and of emission without transfer are equal. In Förster's theory R can be calculated explicitly from the maximum molar absorption coefficients E_1 and E_2 of the longest wavelength-absorption band of the donor and acceptor molecules respectively, the refractive index of the medium n , the wave-number maxima of the emission of the donor ν_e and of absorption of the acceptor ν_a , and the overlap integral J_e :

$$J_e = \int_0^\infty \epsilon(\nu) F(\nu) d\nu. \quad (1)$$

In the last equation $\epsilon(\nu)$ is the molar absorption coefficient of the acceptor and $F(\nu)$ the molar emission coefficient of the donor. The molar emission function $F(\nu)$ represents the photon-spectral distribution of the fluorescence in units set by the equality

$$F(\nu_e) = E_2,$$

J_e can be determined by graphical integration, but it is simpler, and sufficiently accurate for our purpose, to calculate it on the assumption that the absorption and emission bands are represented by Gaussian distributions with standard deviations σ_a and σ_e :

$$\epsilon(\nu) = E_1 \exp - \left(\frac{\nu - \nu_a}{\sigma_a} \right)^2, \quad (2)$$

$$F(\nu) = E_2 \exp - \left(\frac{\nu - \nu_e}{\sigma_e} \right)^2. \quad (3)$$

σ_a and σ_e are calculated from the data referring to the overlapping sides of the bands. It is then immaterial whether they represent accurately the

opposite side since the overlap is here identically zero. With the Gaussian approximations,

$$J_e = E_1 E_2 \frac{\sqrt{\pi}}{\left(\frac{1}{\sigma_a^2} + \frac{1}{\sigma_e^2} \right)^{\frac{1}{2}}} \exp - \left[\frac{(\nu_a - \nu_e)^2}{\sigma_a^2 + \sigma_e^2} \right]. \quad (4)$$

According to Förster's theory,

$$R_e = \left[\frac{1660}{n^2} \cdot \frac{\tau J_e}{\left(\frac{\nu_a + \nu_e}{2} \right)^2} \right]^{\frac{1}{3}} 10^{-8} \text{ cm.}, \quad (5)$$

with τ lifetime of the excited state and n refractive index of solvent.

The calculated values given by the last equation, R_e , will be compared with the values R experimentally determined from depolarization or fluorescence spectral data. The theory of concentration depolarization has been developed elsewhere (Weber, 1954). If the reciprocal of the polarization, $1/p$, is plotted against the molar concentration c , from the slope s and the intercept $1/p_\infty$ of the straight line obtained,

$$R = \left[\frac{15s 10^3}{4\pi N \left(\frac{1}{p_\infty} + \frac{1}{3} \right)} \right]^{\frac{1}{3}} (2a)^{\frac{1}{3}}, \quad (6)$$

where N is Avogadro's number and a is the average radius of the molecules. The determination of R of the phenol-indole transfer from the experimental data referring to the fluorescence spectrum of the solutions is done as follows. Let

λ = transition probability of emission of phenol,

μ_{11} = transition probability of transfer from phenol to phenol,

μ_{12} = transition probability of transfer from phenol to indole.

The probability of n transfers among phenol molecules, followed by emission by phenol, W_{n1} , equals

$$W_{n1} = \left(\frac{\mu_{11}}{\lambda + \mu_{11} + \mu_{12}} \right)^n \frac{\lambda}{\lambda + \mu_{11} + \mu_{12}},$$

and the probability of n transfers among phenol molecules followed by transfer to indole, W_{n2}

$$W_{n2} = \left(\frac{\mu_{11}}{\lambda + \mu_{11} + \mu_{12}} \right)^n \frac{\mu_{12}}{\lambda + \mu_{11} + \mu_{12}}.$$

The total emission from the excited phenol molecules is

$$W_1 = \sum_{n=0}^{n=\infty} W_{n1} = \frac{\lambda}{\lambda + \mu_{12}},$$

and the total phenol excitation transferred to indole is

$$W_2 = \sum_{n=0}^{n=\infty} W_{n2} = \frac{\mu_{12}}{\lambda + \mu_{12}}.$$

W_1 and W_2 are the fractions of the original phenol excited states appearing either as emission by the phenol or as excited states of the indole, since $W_1 + W_2 = 1$. Also

$$\frac{W_2}{W_1} = \frac{\mu_{12}}{\lambda}. \quad (7)$$

The observables from which (μ_{12}/λ) are determined are the expected and observed ratios of the phenol fluorescence to the total fluorescence of the sample. These are respectively:

$$A_p = P_e/(P_e + I_e); \quad a_p = P/(P + I),$$

where subscript e refers to the expected fluorescences of indole (I) and phenol (P). If the fluorescence of the phenol appearing as excitation of the indole is Δ , then

$$\begin{aligned} P &= P_e - \Delta, \\ I &= I_e + \Delta(q_I/q_P), \end{aligned}$$

where q_I and q_P are respectively the quantum yields of the fluorescence of indole and phenol and the reasonable assumption is made that both direct and indirect excitation of the indole have identical quantum yield. From the preceding equations,

$$a_p = \frac{P_e - \Delta}{P_e + I_e + \Delta\left(\frac{q_I}{q_P} - 1\right)};$$

$$\frac{\Delta}{P_e + I_e} = \frac{A_p - a_p}{1 + a_p\left(\frac{q_I}{q_P} - 1\right)},$$

so that finally

$$\frac{\Delta}{P_e - \Delta} = \frac{\mu_{12}}{\lambda} = \frac{\frac{A_p - 1}{a_p}}{1 + A_p\left(\frac{q_I}{q_P} - 1\right)}. \quad (8)$$

The last equation permits the calculation of (μ_{12}/λ) from the experimental data. As shown in a previous paper (Weber, 1954)

$$\frac{\mu_{12}}{\lambda} = \frac{4\pi}{15 \times 10^3} cN \frac{R^6}{(2a)^3}, \quad (9)$$

where c refers to the concentration of the acceptor molecules (indole, in our case). If the concentration of phenol is kept constant and that of indole is varied, a plot of $\Delta/P_e - \Delta$ against c should give a straight line from which the value of R may be calculated.

METHODS

The polarization of the fluorescence was measured with the arrangement shown in Fig. 1a. Light from a compact source Xe (375 w, xenon arc) was focused on the entrance slit of the grating monochromator G . The light emerging from the exit slit X is rendered parallel by the quartz lens L

and falls on the solution contained in the quartz cell Q . The fluorescence emerging at right angles to the direction of excitation passes through the Glan-Thompson prism P and reaches the quartz-window photomultiplier (E.M.I. 6255). The photocurrents are measured directly by a Scalamp galvanometer with the polarizing prism in two positions. In one the polarized component normal to the directions of excitation and observation ($I_{\perp}$) is measured. In the other polarizer position the component polarized in the direction of the exciting light is measured ($I_{\parallel}$). The polarization p is given by

$$p \equiv \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}. \quad (10)$$

To reduce the Brownian molecular rotations so that the relaxation time of the rotation becomes very large compared with the lifetime of the excited state, a solvent with a viscosity of a few poises is required. Glycerol, which is currently used for this purpose, has poor transparency in

Fig. 1a. Apparatus for the determination of the polarization spectra. Xe, 375 w xenon arc; G , grating monochromator (dispersion 3.3 m μ /mm. at the slit); L , quartz lens, f 3 cm.; Q , spectrasil cuvette or block; P , Glan-Thompson prism; F , fluorescence filter; E.M.I. 6255, 14-stage, quartz-window photomultiplier; Galv., Scalamp galvanometer.

Fig. 1b. Section of cell container. R , Expanded rubber layer; T , Brass tank with inlet and outlet for circulation; Cu , copper block to position cuvette on block and serve as heat reservoir; Q , spectrasil cuvette or block.

the ultraviolet region, due to impurities which are often strongly fluorescent. Attempts at purification of the glycerol by chromatography or distillation were not successful. Propylene glycol (British Drug Houses Ltd., AnalaR) proved both transparent and free from fluorescent impurities. Its viscosity at room temperature is only about 0.6 poise, but at -70° it forms a stiff glass with a viscosity of many poises (Williams, 1957), in which observations can yield the actual pseudo-principal polarization. To cool the cell or quartz block to this temperature, it was surrounded by a solid copper block which in turn was placed inside a brass jacket through which ethanol cooled by solid CO_2 was rapidly circulated from a Dewar vessel by means of a pump. The jacket was surrounded by a 1 in. thick insulating layer of expanded rubber and fibre glass (Fig. 1b). The metal block, the brass jacket and the insulating coat had channels at the front and side through which the exciting and fluorescent light passed. The lens L was in direct contact with the front opening and the polarizer rim with the side opening, of these channels. In this way circulation of air in contact with the cell was prevented and condensation of moisture on the optical surfaces avoided. A temperature of -69° to -70° could be constantly maintained in the liquid returning from the jacket.

The Glan-Thompson prism used was opaque to wavelengths shorter than $280\text{ m}\mu$. Its transmission above $300\text{ m}\mu$ was very good, allowing the fluorescence of phenol, cresol and tyrosine (λ_{max} $303\text{ m}\mu$) to be satisfactorily measured.

The light emerging from the grating monochromator was found to be partially polarized to a small extent. Calling Ω the depolarization ratio of the exciting light, that is the ratio of its perpendicular to its parallel component, it can be found from the effect of each component independently that the polarization $\bar{p}$ observed is related to p_n , the polarization observable on excitation by natural light, by the equation

$$\bar{p} = \frac{2p_n}{1 + p_n + \Omega(1 - p_n)}. \quad (11)$$

The light emerging from the monochromator was polarized to the extent of less than 10%, the magnitude of this polarization changing along the spectrum. However, as equation (11) shows, partial polarization of 10% makes $\bar{p}$ differ from p_n by about 5%. Since the precision of the polarization measurements was not much better than this no correction for polarization of the exciting light was applied. The smallest exciting band width consistent with reasonable accuracy was employed. Over the region 230–260 $\text{m}\mu$ the band widths employed were 3.0–1.5 $\text{m}\mu$ and 1.5–0.8 $\text{m}\mu$ in the region 270–310 $\text{m}\mu$. A filter (F , Fig. 1) was interposed as usual between fluorescent source and detector to eliminate scattered and parasitic exciting light. With cresol, phenol and tyrosine the filter was a 4 mm. layer of Perspex plastic, which eliminated light of wavelengths shorter than $280\text{ m}\mu$. With indole, tryptophan and derivatives a 3 mm. layer of 0.02M- CuSO_4 in 0.1M-2-amino-2-hydroxymethylpropane-1:3-diol buffer (tris), pH 8.3, in a flat Perspex container was used. This was found to eliminate light of wavelength shorter than $315\text{ m}\mu$ very satisfactorily.

In studying the fluorescence of concentrated solutions it is necessary to eliminate the depolarization that results from absorption of the fluorescence and its secondary

emission. This radiative transfer of the excited state, the importance of which has been overlooked in the past, can lead to serious over-estimation of the non-radiative transfer. Birks & Little (1953), Bailey & Rollefson (1953) and Schmitten (1953) have shown directly the lengthening of the lifetime of the excitation due to radiative transfers in thick layers of fluorescein in solution and in solutions and crystals of anthracene. Schmitten has shown that this lengthening is absent in thin layers in which reabsorption of the fluorescence is negligible. Weber (1956) has shown that the depolarization of the fluorescence observed in thick layers of relatively dilute solutions disappears when thin layers of the same concentration are observed. Thus the observation of the polarization in very thin layers of solution is indispensable if the radiative transfer is to be ruled out. If the fraction of the total fluorescence absorbed inside the layer is β and the quantum yield of the fluorescence is q , a fraction $(1 - \beta)$ of the radiation excited by the external source (primary fluorescence) will reach the detector and fraction β will be absorbed and will excite secondary fluorescence that reaches the detector with intensity $\beta q(1 - \beta)$. In the same way the tertiary fluorescence equals $\beta^2 q^2(1 - \beta)$, etc. The total fluorescence due to reabsorption inside the layer finally reaching the detector equals:

$$(1 - \beta) [\beta q + (\beta q)^2 + (\beta q)^3 + \dots] = (1 - \beta) \left(\frac{\beta q}{1 - \beta q} \right). \quad (12)$$

The ratio of internally excited fluorescence to primary fluorescence is simply $\beta q / (1 - \beta q)$. In the preceding calculation no account has been taken of the geometry of the system. This can be disregarded only when the volumes illuminated and observed are the same and no illuminated volume is excluded from observation. Only in this case do the primary and higher orders of fluorescence have equal chances of reaching the detector. This situation can be realized in practice by using a thin layer of solution. The exciting beam is limited so that the whole illuminated zone falls within the aperture of the photomultiplier which is in turn restricted so as to include the illuminated zone alone.

If equation (12) is used to calculate the proportion of internally to externally excited fluorescence in a $30\text{ m}\mu$ layer of phenol solutions with q 0.2 and where the molar absorption coefficient for its own fluorescence is of the order of 100, it is found that in a 0.5M-solution the internally excited fluorescence amounts to 5% of the total. At lower concentrations, and at all the concentrations of indole employed, the contribution of the internally excited fluorescence was negligible. The actual experimental arrangement consisted of two prisms of spectrasil quartz with optically flat faces. The solution was applied to the hypotenuse faces, which were then pressed firmly together. In this way a layer of very reproducible thickness of $30\text{ m}\mu$ was obtained. All the experiments on concentrated solutions, both of fluorescence polarization and fluorescence spectra, were carried out with these thin layers.

Determination of the contribution of phenol and indole to the fluorescence spectra of mixtures

The fluorescence spectra were determined as previously described (Teale & Weber, 1957). The fluorescent source was a layer of solution of $30\text{ m}\mu$ thickness excited by the light from a 375 w xenon arc, filtered through a Chance OX7 filter. The band-width employed was $1\text{ m}\mu$.

To determine the extent of the energy transfer from phenol to indole in the solutions, it is required to determine the ratio P/I of the photons emitted as phenol and indole fluorescence, and compare this with the ratio $(P/I)_e$ that would be expected in the absence of transfer. If n_p and n_i are the numbers of photons absorbed by the phenol and indole from the exciting beam and q_p and q_i the corresponding quantum yields of the fluorescence,

$$(P/I)_e = n_p q_p / n_i q_i.$$

Moreover,

$$\frac{n_p}{n_i} = \frac{\int_{\lambda_a}^{\lambda_b} I(\lambda) [1 - 10^{-(E_I + E_P)}] \frac{E_P}{E_I + E_P} d\lambda}{\int_{\lambda_a}^{\lambda_b} I(\lambda) [1 - 10^{-(E_I + E_P)}] \frac{E_I}{E_I + E_P} d\lambda}. \quad (13)$$

Of the three factors appearing in the integrals, $I(\lambda)$ is proportional to the photon output of the source after passing through the appropriate excitation filter, in our case a 3 mm. thickness of Chances's OX 7 filter; $1 - 10^{-(E_P + E_I)}$ is the fraction of this radiation of a given wavelength absorbed in the layer, and $E_I/(E_I + E_P)$ and $E_P/(E_I + E_P)$ are respectively the fractions of the total absorption due to indole and phenol respectively. E_I and E_P are the separate extinctions of the indole and phenol layers. The additivity of their contribution is shown clearly in Fig. 2. In practice $I(\lambda)$ is determined as already described (Teale & Weber, 1957) and the E values by spectrophotometry of the thin layers. The integrals were computed graphically, the practical limits of integration being 240 and 305 m μ . The values of q_p 0.20 and q_i 0.42 are already in the literature (Weber & Teale, 1957). The ratio P/I is determined as follows: let $F_1 = P_1 + I_1$ represent the total fluorescence emitted at wavelength λ_1 , at which wavelength we expect a dominant contribution from phenol. Similarly, $F_2 = P_2 + I_2$ is the total fluorescence at wavelength λ_2 at which the con-

tribution from indole is dominant. The coefficients α_{12} and α_{21} obtained from observations of pure indole and phenol solutions are given by:

$$\begin{aligned} I_1 &= \alpha_{21} I_2, \\ P_2 &= \alpha_{12} P_1, \\ \frac{P_1}{I_2} &= \frac{F_1 - \alpha_{21} F_2}{F_2 - \alpha_{12} F_1}. \end{aligned} \quad (14)$$

so that

To relate the fluorescence emission at the given wavelengths to the total fluorescent emission P and I , a correction for the unequal spectral distribution of the separate fluorescences is required. Let the photon-fluorescence emission from each substance be described by two half-Gaussian distributions commencing at the wavelength of maximum emission, with intensity $P_{\max.}$ or $I_{\max.}$ and having standard deviations σ_+ towards the long-wave side and σ_- towards the short-wave side. The total emissions are proportional to $P_{\max.}(\sigma_+ + \sigma_-)_P$ and $I_{\max.}(\sigma_+ + \sigma_-)_I$. Therefore:

$$\frac{P}{I} = \frac{P_1 \alpha_{1m} (\sigma_+ + \sigma_-)_P}{I_2 \alpha_{2m} (\sigma_+ + \sigma_-)_I}. \quad (15)$$

In the last equation the coefficients α_{1m} and α_{2m} relate the fluorescence emitted by pure indole and phenol solutions respectively at the wavelength of maximum emission $P_{\max.}$ and $I_{\max.}$ to the emissions at wavelengths λ_1 and λ_2 , that is to P_1 and I_2 . Finally, if the ratio P/I is to represent the ratio of the numbers of photons leaving the fluorescent solution it has to be corrected by multiplication by factors representing the ratio of the attenuations of the two fluorescences by the grating and the difference in response of the photomultiplier. Because of the vicinity of the spectra these factors are not very different from 1. They have been determined as described by Teale & Weber (1957).

RESULTS

Polarization spectrum of phenol, cresol and tyrosine

As shown in Fig. 3, the spectra are very similar. They consist of two regions, one of positive polarization that reaches a maximum value of 0.21–0.22 in

Fig. 2. Absorption spectra of phenol and indole in propylene glycol in 30 μ layers. ●, 0.02M-Indole; ○, 0.2M-phenol; ⊙, 0.02M-indole + 0.2M-phenol (calc.); ×, 0.02M-indole + 0.2M-phenol (observed).

Fig. 3. Polarization (P) spectra of cresol (×) and tyrosine (●), 0.5 mm in propylene glycol, at -70° .

the 275–290 $m\mu$ region. The negative polarization band has a minimum of -0.065 ± 0.004 at 235 $m\mu$. The increase in the absolute polarization observed at wavelengths shorter than 235 $m\mu$ is due to a contribution to the absorption from a band having its maximum in the vacuum ultraviolet region. The transition with maximum at 275 $m\mu$ in phenol and phenolic derivatives may be expected to have a large contribution by the quinonoid forms to the

excited state, as evidenced by the large increase in absorption and shift to longer wavelengths observed in the phenolate ion. It may be expected that if in this type of transition an electron from the oxygen lone pair is promoted to an excited orbital in the ring ($n \rightarrow \pi^*$ transition) the direction of the transition moment will be normal to the plane of the ring, and therefore at right angles to the direction of the transition moment of the 235 $m\mu$ absorption, which must be expected to be in the ring plane as all $\pi \rightarrow \pi^*$ transitions are. On the other hand, both transitions may be normal to each other while still contained in the plane of the ring. The distinction between the two cases may be made in practice by a study of the dichroism of crystals if the absolute orientation of the benzene ring is known, and should furnish a criterion of the $n \rightarrow \pi^*$ character (as opposed to $\pi \rightarrow \pi^*$ character) of the 275 $m\mu$ absorption. An equation due to Jablonski (1935) permits the calculation of $\cos^2 \beta_i$, where β_i is the angle between the i th oscillator of absorption and the emission oscillator:

$$\cos^2 \beta_i = \frac{7p_i + 1}{3 + p_i}, \quad (16)$$

where p_i is the polarization observed by excitation with natural light. Although the angle δ between the absorption oscillators cannot be calculated directly, if they and the emission oscillator are all contained in the same plane and

$$\delta = \frac{1}{2}\pi, \quad \cos^2 \beta_1 + \cos^2 \beta_2 = 1.$$

From the values of p observed for the two transitions and equation (16),

$$\cos^2 \beta_1 = 0.185; \quad \cos^2 \beta_2 = 0.78;$$

$$\cos^2 \beta_1 + \cos^2 \beta_2 = 0.965.$$

Thus the directions of the transition moments associated with the 235 and 275 $m\mu$ absorption bands of phenol or tyrosine appear to be at right angles to each other. Equation (16) would be expected to be approximately correct even if the direction of the transition moment in emission is not sharp but, as suggested by Jablonski (1950),

Fig. 4. Concentration depolarization in phenol solutions in propylene glycol at -70° : $\times$, 30 μ layers; $\bullet$, 5 mm. layers.

comprises a set of directions determined by the torsional vibrations of the molecule in the excited state.

Concentration depolarization in phenol solutions

The data are shown in Fig. 4, where the reciprocal of the polarization is plotted against the phenol concentrations. The observations refer to thin layers of 30 μ thickness and thick layers of 5 mm. thickness. The linear law is approximately followed by the thin-layer fluorescence. In the thick layers the increase in depolarization at high concentrations is due to radiative transitions, but at the lower concentrations, where radiative transfer may be expected to be absent, the data agree well with those of the thin layers. From equation (6), $R = 17 \text{ \AA}$. On the other hand, from Förster's theory, with $n = 1.5$; $\tau = 8 \mu\text{sec.}$; $\frac{1}{2}(\nu_a + \nu_e) = 34\,700 \text{ cm.}^{-1}$; $\nu_a - \nu_e = 3400 \text{ cm.}^{-1}$; $\sigma_a \approx \sigma_e = 1500 \text{ cm.}^{-1}$; $E_1 = E_2 = 1600 \text{ cm.}^2/\text{m-mole}$; equation (4) gives $J_v = 3.2 \times 10^8 \text{ cm.}^3/\text{m-mole}^2$, and equation (5) gives $R_e = 11 \text{ \AA}$. The value predicted by the theory is apparently considerably smaller than the experimental value, but this may be due, at least partly, to the fact that equation (5) takes into account only the dipole-dipole interaction between the molecules and neglects quadrupole and higher terms.

Polarization spectrum of indole, N-methylindole and tryptophan

These spectra are shown in Fig. 5. The three substances have similar spectra, showing two minima, one at $232 \pm 2 m\mu$ the other at 290 $m\mu$, and two maxima at 265–270 and 300–305 $m\mu$ respectively. The minimum at 232 $m\mu$ coincides with the maximum of one of the absorption bands. The maximum at 270 $m\mu$ does not correspond exactly with the maximum of the longest wavelength-absorption band and the minimum at 290 $m\mu$ does correspond accurately with the

shoulder observed in the absorption spectrum. The maximum at $310\text{ m}\mu$ and a shallow minimum found at longer wavelengths do not have any corresponding features in the absorption spectrum, which at these wavelengths consists only of a weak tail. All the details of the polarization spectrum, up to and including the $310\text{ m}\mu$ maximum, can be explained on the likely assumption that the longest wavelength-absorption band of tryptophan consists of two independent electronic transitions ($G \rightarrow S_1$ and $G \rightarrow S_2$), of which one ($G \rightarrow S_2$) is responsible for the shoulder observed in absorption at $290\text{ m}\mu$. Fig. 6 indicates a possible disposition of the potential-energy surfaces involved in the transitions: r is a co-ordinate or average of co-ordinates which best describes the nuclear-configuration changes taking place in the excited state; V is the potential energy. According to the Franck-Condon principle the absorption takes place from the centre of the zero level of the potential-energy surface of the ground state G , reaching the singlet states S_1 and S_2 placed vertically upwards. At wavelengths at which $V > V_2$, excitation to both S_1 and S_2 , but principally to S_1 , takes place. This corresponds to the region of the absorption spectrum where the polarization increases monotonically. For energies between V_2 and V_1 the excitation to S_2 predominates and shows itself in a decrease in the polarization since the transition moment of the $G \rightarrow S_2$ transition determines a low, perhaps negative, polarization when excited alone.

Fig. 5. Polarization spectra of indole (●), *N*-methylindole (○) and tryptophan (⊙) in propylene glycol at -70° . Concentration 0.2 mm .

At energies $V < V_1$ the Franck-Condon principle determines that the absorption to S_2 must disappear completely, but weak absorption to S_1 may still take place. Since S_1 is excited alone the polarization increases rapidly, well above the $270\text{ m}\mu$ maximum at which both S_1 and S_2 are excited, whereas the absorption drops to a weak tail. The maximum at $305\text{ m}\mu$ is in all probability due to the excitation of S_1 alone, but it is not possible to decide whether S_2 has an actual negative polarization since it is not excited alone at any wavelengths. The transition in fluorescence is the converse of the $G \rightarrow S_1$ transition and takes place therefore from the minimum of the S_1 surface. In agreement with the above scheme the fluorescence bands of indole and tryptophan are found to be unresolved and symmetric and to show no detail on the short-wave side corresponding to the fine-structure band or shoulder in absorption at $290\text{ m}\mu$. The separation of the absorption and fluorescence bands is considerable (3400 cm^{-1} in indole in propylene glycol, 5000 cm^{-1} in tryptophan in water) and the changes in the position of the absorption and fluorescence bands with the dielectric properties of the medium (Lippert, 1957) indicates the loss of energy of the excited state by dipolar interaction with the solvent molecules during the lifetime of the excited state. The fluorescence transition may be described as taking place between S'_1 and G . The energy difference $S_1 - S'_1$ (Fig. 6) is the energy loss by dipolar interaction with the solvent molecules. The transitions $G \rightarrow S_1$ and $G \rightarrow S_2$ may be

Fig. 6. Potential-energy surfaces for the ground state G , the two singlet-excited states S_1 and S_2 and the fluorescent state S'_1 of indole.

provisionally identified with the L_a and L_b transitions in the theory of the absorption spectrum of aromatic hydrocarbons due to Platt (1949). The L_a transition is responsible for the longest wavelength-absorption band. In indole solutions in hexane our $G-S_1$ transition shows five distinct peaks, in correspondence with similar five peaks of vibrational structure present in the L_a transitions of benzene and anthracene. The overlap of the L_a and L_b transitions has also been suggested by Weller (1957) to explain the absorption spectrum of acridine.

Polarization spectrum of *N*-glycyltryptophan

The spectra, shown in Fig. 7, are like those of indole and tryptophan with one important difference: the appearance of a new polarization band, with a band width of a few millimicrons, preceding the 295 $m\mu$ minimum. This band is difficult to reproduce in practice because of the inaccuracy in repeating the setting of the exciting wavelengths. As shown in the following paper this band appears also in the proteins, and since it is not present in the simple indole derivatives it is probably connected with the existence of the peptide bond. The characteristics of the band appear the same in 50% (v/v) propylene glycol-water and in 50%

propylene glycol-water containing 6M-urea. This result appears to indicate that a close external interaction of the indole ring with the peptide bond is not required for its appearance.

Concentration depolarization in indole solutions

If equations (4) and (5) are used to calculate the value of R_c for propylene glycol solutions of indole, for which

$$\sigma_a \approx \sigma_e = 3000 \text{ cm.}^{-1}, E_1 = E_2 = 5600 \text{ cm.}^2/\text{m-mole}, \frac{1}{2}(\nu_a + \nu_e) = 33\,200 \text{ cm.}^{-1}$$

and $\tau \approx 10 \mu\text{sec.}$, it is found that $J_v = 1.8 \times 10^{10} \text{ cm}^3/\text{m-mole}^2$, $R_c = 23 \text{ \AA}$. The polarization spectra of solutions of several concentrations (0.05–0.4M) are recorded in Fig. 8 to show an important property: the polarization decreases rapidly with increasing concentration when the fluorescence is excited with light of wavelength shorter than 300 $m\mu$ but appears hardly changed if the 305–315 $m\mu$ range is used. Thus a 0.4M-solution excited with 305 $m\mu$ shows over 70% of the polarization of the dilute solution, but excited with 270 $m\mu$ the polarization is less than 15% of that of the dilute solution. When $1/p$ is plotted against concentration the value of R calculated from the slope equals 16 $\text{\AA}$, falling well short of the expected 23 $\text{\AA}$. The experimental facts appear to indicate that excitation of the S_2 band is required for the transfer to take place and excitation of S_1 alone is ineffective. It may be argued that this is a case in which a long-wave limit is

Fig. 7. Polarization spectra of 0.2 mm-*N*-glycyltryptophan in propylene glycol at -70° . ●, In 50% propylene glycol-water; x, in 50% propylene glycol-water containing 6M-urea.

Fig. 8. Polarization spectra of 30 μ layers of 0.1–0.4M-indole in propylene glycol at -70° . ■, 0.01M; ▲, 0.05M; x, 0.1M; ○, 0.2M; ●, 0.4M.

found for the transfer because only at wavelengths well above the fluorescent level it is possible to match the given and accepted energies (Moodie & Reid, 1952). In the present case, however, the long-wave limit of the transfer appears sharp and coincident with the limit of an electronic transition.

Fig. 9. Fluorescence spectra of phenol and indole in propylene glycol at room temperature, corrected to equal number of photons absorbed. Areas are thus proportional to the quantum yields of fluorescence. ○, Phenol; ●, indole.

Fig. 10. Fluorescent spectra of 30 μ layers of solutions of phenol and indole in propylene glycol at room temperature. ×, 0.01M-Indole in m-phenol; ○, 0.03M-indole in m-phenol; ●, 0.1M-indole in m-phenol.

Table 1. Fluorescence emitted by indole and phenol in mixtures of fixed phenol concentration (1M) and variable indole concentration (C)

$a_p = P/(P+I)$ and $A_p = P_e/(P_e+I_e)$ are respectively the observed and expected fractions of the total fluorescence emitted as phenol fluorescence. $\Delta/(P_e-\Delta)$ has been calculated by equation 8 with $q_I/q_P = 2.1$.

C (mm)	A_p	a_p	$\Delta/(P_e-\Delta)$
5	0.945	0.78	0.10
10	0.89	0.59	0.26
20	0.70	0.42	0.38
30	0.57	0.29	0.59
50	0.43	0.17	1.04
100	0.26	0.06	2.59

Moreover, as described below, the transfer of the excitation from phenol to indole results in the appearance of a weak negative polarization in the indole fluorescence. This is only possible if the indole transition involved in the transfer would yield a negative polarization when directly excited. From the data for the polarization spectrum of indole it appears that only the $G-S_2$ transition can fulfil this condition. If the S_2 transition must be excited for transfer to occur it follows that use of the value for the total lifetime of the excited state in the calculation of R_e must lead to its overestimation. If the lifetime of the excitation in the S_2 band is 1 μ sec. then $R_e = 15 \text{ \AA}$; if it is 0.1 μ sec. then $R_e = 11 \text{ \AA}$. The latter value for the lifetime is probably an upper limit for the vibrational deactivation that precedes emission (e.g. Weber & Teale, 1958), so that as with phenol, and probably because of similar reasons, R considerably exceeds R_e .

Transfer of the excited state from phenol to indole

This was determined by a study of the fluorescence spectra of molar solutions of phenol in propylene glycol containing increasing concentrations of indole, from 5 mM to 0.1 M. The fluorescence spectra determined are shown in Figs. 9 and 10. The possibility of a ground-state complex of indole and phenol which would invalidate the calculation of R_e based on the random distribution of the indole, with respect to the excited phenol molecules, appears to be excluded by a study of the absorption spectrum of the mixtures, which appeared to be the exact sum of the phenol and indole contributions (Fig. 2). Table 1 gives the experimental values of $A_p = P_e/(P_e+I_e)$ and $a_p = P/(P+I)$. The ratios P_e/I_e were calculated by the use of equation (12). P/I is calculated as already discussed from the total fluorescent emission at the wavelengths, λ_1 300 m μ and λ_2 340 m μ . The data for the fluorescence spectrum of pure indole and phenol in propylene glycol at room temperature (Figs. 8 and 9) required to calculate P and I by the use of equations 14 and 15 are:

	Phenol fluorescence	Indole fluorescence
$\lambda_{\text{max.}}$	303 m μ	335 m μ
σ^+	1750 cm. ⁻¹	3100 cm. ⁻¹
σ^-	1300 cm. ⁻¹	2800 cm. ⁻¹
$\alpha_{12} = 0.155$		$\alpha_{21} = 0.156$
$\alpha_{1m} \approx 1$		$\alpha_{2m} \approx 1$

The corrections for grating transmission and detector response are:

$$\frac{\text{grating transmission for phenol fluorescence}}{\text{grating transmission for indole fluorescence}} = 0.94$$

$$\frac{\text{detector response for phenol fluorescence}}{\text{detector response for indole fluorescence}} = 1.25.$$

Fig. 11. Plot of $\Delta/(P_e - \Delta)$ against indole concentration (c) (data of Table 1). The broken lines correspond to slopes giving respectively R 17.5 Å and 19.5 Å. The continuous line has slope corresponding to 18.5 Å.

Fig. 12. Polarization spectra of mixtures of phenol and indole in 30 μ layers to show the appearance of negative polarizations. ●, 0.01 M-Indole in *m*-phenol; ×, 0.05 M-indole in *m*-phenol.

So that finally $P/I = 0.807 (P_1/I_2)$. Fig. 11 shows a plot of $\Delta/(P_e - \Delta)$ [calculated by means of equation (8)] against c , the indole concentration. The expected linear relation is followed at least up to a concentration of indole of 0.05 M. In comparing the values of R and R_c the probable error on the determined values of these quantities must be considered. If the standard deviation of the straight-line plots from which R is calculated equals $\delta S/S$, the error in R is given by equation (9), as $\delta R/R \simeq \frac{1}{2} \delta S/S$. Similar remarks apply to the calculation of R_c from J_v : $\delta R_c/R_c \simeq \frac{1}{2} \delta J_v/J_v$. Granting the correctness of the assumptions involved in the derivations, the R and R_c values should be accurate to within 1 Å.

With the values already quoted for the spectroscopic constants of the indole absorption and phenol fluorescence, and by the use of equations (4) and (5), we obtain $J_v = 1.04 \times 10^{10}$ cm.³/m-mole², R_c 19 Å, and the slope of the straight line in Fig. 11 gives $R = 18.5$ Å. The rather accidental character of the agreement between the two values must be suspected in view of the disagreement found in other cases (Weber, 1954; Weber & Teale, 1959).

Polarization of the fluorescence of phenol-indole mixtures

The polarization spectrum of the fluorescence of some of the solutions was measured with a copper-tris liquid filter to attenuate preferentially the phenol fluorescence. This filter transmitted 10 % of the phenol fluorescence and 25 % of the indole fluorescence. For this reason the fluorescence observed on excitation by wavelengths shorter than 300 m μ can be given only qualitative significance. The results are given in Fig. 12, which shows a small but doubtless negative polarization, in the region and at the concentrations in which indirect excitation through the phenol is more prominent. Such negative polarization can only be expected if the oscillator active in the transfer on the indole side would give itself a negative polarization when directly excited. Although negative polarization is not observed at any wavelength in the spectrum of indole (Fig. 5), this is probably due to the fact that the S_2 transition cannot be excited alone. The fact that the S_2 transition appears as the active one in the phenol-indole transfer agrees with the sharp long-wave limit observed already in the indole-indole transfer.

SUMMARY

1. The polarization of the fluorescence as a function of the exciting wavelength (polarization spectrum) has been determined from 230 m μ to the long-wave absorption limit for solutions of tryptophan, tyrosine and some related substances, in

propylene glycol solution at -70° , excited with unpolarized light.

2. Phenol, cresol and tyrosine show a simple spectrum, consisting of a negative polarization region with a minimum value of -0.065 at $235\text{ m}\mu$, and a positive region with a maximum of 0.22 at $275\text{ m}\mu$ and longer wavelengths.

3. Indole, *N*-methylinole and tryptophan show no negative polarization at any wavelengths. In the region of the longest wavelength-absorption band two maxima are present at 270 and $305\text{ m}\mu$ and a minimum at $295\text{ m}\mu$. It is shown that at least two electronic transitions are required to describe these effects, one of these transitions corresponding to the spike or shoulder observed in absorption at $295\text{ m}\mu$.

4. *N*-Glycyltryptophan has a spectrum similar to the other indole derivatives but for the existence of an additional sharp polarization band with maximum at $290\text{ m}\mu$, which appears connected with the presence of a peptide bond.

5. Concentration-depolarization measurements show that the distance R at which the probability of transfer of the excitation equals the probability of emission is $17\text{ \AA}$ for phenol molecules.

6. Concentration-depolarization measurements on indole solutions show that $R = 17\text{ \AA}$ if the fluorescence is excited with light of wavelength shorter than $295\text{ m}\mu$, but that excitation with longer wavelengths is not followed by transfer with very much lower efficiency.

7. Measurements of the fluorescence spectrum in

mixtures of phenol (1M) and indole (0.01 – 0.2M) show that phenol can transfer its energy to indole with $R\ 18.5\text{ \AA}$.

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Fluorescence-Polarization Spectrum and Electronic-Energy Transfer in Proteins

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The analysis of the polarization spectrum of the fluorescence of proteins is necessarily more difficult than that of the aromatic amino acids and simple derivatives described in the previous paper (Weber, 1960). The main differences arise because of the various types of Brownian molecular rotations which are possible in the protein, and also the possibility of energy transfer among the aromatic residues. The characteristic distances of transfer R for phenol-phenol, indole-indole and phenol-indole transfer calculated from concentration-depolarization and fluorescence-spectral data (Weber, 1960)

are of the order of the distance between these residues in the protein molecule. Finally, the possibility that interaction of the indole nucleus with the highly polarizable backbone of the protein may lead to changes in the relative positions of the overlapping transitions in tryptophan must be kept in mind.

EXPERIMENTAL

This paper describes the fluorescence-polarization spectrum of two classes of globular proteins.

Class A proteins containing phenylalanine and tyrosine but no tryptophan. These proteins (Teale & Weber, 1959;