Intramolecular Singlet Excitation Transfer. Applications to Polypeptides*

J. Eisinger, B. Feuer, and A. A. Lamola

ABSTRACT: A critical examination of the conditions under which the formalism of the Förster singlet energy-transfer theory may be used to determine the donor-acceptor separation from the experimental energy-transfer efficiency is presented. In particular, the importance of multipole transitions, exchange interaction, “before-relaxation” transfer, and translational and rotational diffusion and the useful range of distances are examined. The overlap integrals and Förster distances for transfer between aromatic amino acids are evaluated from experimental absorption and fluorescence data for various environments and temperatures. The usefulness and limitations of energy-transfer experiments in the determination of intramolecular distances in polypeptides are discussed.

The long-range, radiationless transfer of singlet excitation energy has in recent years been observed in many systems. Its theoretical basis is the dipolar interaction between the transition moments of two chromophores and has been developed and discussed in detail by Förster (1948, 1951, 1966). This theory has been tested experimentally under a variety of conditions (Ermolecov and Sveshnikova, 1963; Bennett, 1964; Bennett et al., 1964; Kellogg, 1964; Stryer and Haugland, 1967; Birks and Georgiou, 1967; Latt et al., 1965; Conrad and Brand, 1968) and the agreement between the calculated and observed transfer rates is excellent.

Many proteins contain several aromatic amino acids which fluoresce and exchange singlet excitation energy. It has been suggested that the measurement of energy-transfer rates among these chromophores offers in principle the possibility of determining a structure-sensitive parameter. While the application of this technique is complicated by the multiplicity of donors and acceptors in most proteins, it appears to be practical for peptide hormones (Eisinger, 1969b). In addition several experiments have been reported in which the transfer is to fluorescent labels bound to the protein (Edelman and McClure, 1968; Teale, 1960; Weber, 1952; Stryer, 1968). In the present paper we wish to evaluate the usefulness and limitations of studies of intramolecular energy transfer, particularly between the aromatic amino acids, as a means of studying molecular conformation, and to evaluate the spectral overlap integrals which are needed to translate experimental values of transfer efficiencies into intramolecular distances between the aromatic residues.

Förster Theory

Several reviews of Förster's theory for singlet energy transfer have appeared in the literature (Förster, 1967; Lamola, 1969). Here we wish to limit our discussion to those aspects of the theory which are our particular concern, i.e., the transfer between Trp, Tyr, and Phe residues in polypeptide chains. Transfer rates between pairs of aromatic amino acids have been previously estimated by Karremann et al. (1957, 1958). These authors made the following two approximations in calculating $R_0$, the Förster critical distance, which is defined below. (1) The fluorescence and absorption spectra of the donor are mirror images when plotted on a wave-number scale. This is justified only if the absorption band arises from a single transition and in the absence of solvent effects or geometrical changes in the excited state (see below). (2) The donor lifetime may be estimated from the absorption strength after correction for the emission quantum yield. This assumption is subject to the same limitations as the previous one.

Additional calculations of $R_0$ values for aromatic amino acids by Perlman et al. (1968) and Konev (1967) made use of the same approximations. Since it is now feasible to measure these energy-transfer rates to a reasonable precision it seemed worthwhile to reevaluate spectral overlap integrals and $R_0$ values using Förster's exact formulas (see below), so that these results might be used in the determination of intramolecular distances in proteins and peptide hormones. The results are, in general, subject to the following conditions. (1) The dipolar coupling between donor and acceptor is assumed to be small compared with the (unresolved) absorption band of the acceptor ("Very Weak Coupling" of Förster, 1951, 1966). (2) The point dipole approximation is assumed to be valid. This is the case if donor–acceptor separations are large compared with the dimensions of the chromophores. This condition also assures that higher multipoles need not be considered and that exchange interactions are negligible (see Appendix II). (3) Relaxation to the lowest vibronic level of the excited donor is fast compared with energy transfer. If this condition is not

* From the Bell Telephone Laboratories, Incorporated, Murray Hill, New Jersey. Received June 5, 1969.
fulfilled, before-relaxation transfer (Guérin et al., 1967) will occur. The efficiency of before-relaxation transfer can be estimated for a given donor-acceptor separation and the results for the aromatic amino acids are given in Appendix I. In principle one can determine experimentally whether such a mechanism needs to be considered since the before-relaxation transfer rates will depend upon the wavelength of excitation of the donor.

The rate of Förster-type energy transfer from an excited donor $D$ to an acceptor $A$ is given by (Förster, 1948, 1951, 1966)

$$k_{ad} = \frac{8.8 \times 10^{-20} \Phi_D \kappa^3 J_{ad}'}{n^4 r_D^6}$$

with

$$J_{ad}' = \int_0^\infty F_D(v) e_A(v) v^{-4} dv$$

where $\tau_D$ and $\Phi_D$ are the donor emission lifetime and quantum yield, respectively, and $n$ is the index of refraction of the medium intervening between the donor and acceptor at a wavelength in the region of their spectral overlap. $\kappa$ is the dipole-dipole orientation factor and $r$ is the donor-acceptor separation. $J_{ad}'$ is an overlap integral between $e_A(v)$, the decadic molar extinction coefficient of the acceptor, and $F_D(v)$, the spectral distribution of the donor emission normalized to unity, modified by the frequency factor $v^{-4}$.

The distance $R_0$ at which the rate of energy transfer is equal to the sum of the rates of all other modes of deexcitation of the donor is usually called the Förster critical distance and is given by (Förster, 1948, 1951, 1966)

$$R_0 = 8.8 \times 10^{-20} \Phi_D \kappa^3 n^{-4} J_{ad}'.$$

Experimental Section

The aromatic amino acids and their derivatives were reagent grade and were used without further purification. Chymotrypsinogen A and ribonuclease A were supplied by Worthington.

Tyrosinate solutions were prepared by dissolving tyrosine in 0.1 M NaOH. All other sample solutions were maintained at neutral pH.

Absorption spectra were obtained by means of a Cary Model 15 spectrophotometer.

Fluorescence spectra were recorded by the use of a spectrofluorimeter which has been described previously (Eisinger, 1969). The emission spectra were corrected for wavelength-dependent response of the instrument and were obtained with dilute samples (approximately $10^{-1}$ M) to prevent self-absorption of the fluorescence light which could distort the emission spectrum in the overlap region to which $J_{ad}'$ is particularly sensitive.

Samples used in the determination of quantum yields were optically thick at the excitation wavelength (Eisinger, 1969a).

Results

$J_{ad}'$ was calculated for most pairs of Trp, Tyr, Tyr$, and Phe using absorption curves, $e(v)$, obtained with dilute aqueous solutions. The shape of these curves is relatively insensitive to temperature and solvent. The emission spectra, $F_D(v)$, on the other hand, vary with environment and temperature and representative spectra obtained under different conditions were used to evaluate $J_{ad}'$. The uncorrected fluorescence spectra are shown together with the absorption spectra of Trp, Tyr, Tyr$, and Phe in Figure 1.

The computer program used in the calculation of $J_{ad}'$ was written by R. Povinelli and was modified for the GE 610 computer by W. E. Blumberg.

Table I presents the results of the calculations of $J_{ad}'$ and of the Förster distances $R_0$ for various pairs of aromatic amino acids. Since $\Phi_D$ is often not known precisely, we have chosen to give values for $J_{ad}'$ and $R_0 \Phi_D^{-1/4}$ as well as for $R_0$ assuming three different values for $\Phi_D$. $R_0$ depends not only upon the donor quantum yield but also upon the donor emission spectrum which may be red shifted if solvent reorientation or exciplex formation precedes fluorescence emission (Bowen, 1959; Hercules and Rogers, 1966; Walker et al., 1966; Eisinger and Nannon, 1969). Such a red shift invariably decreases $J_{ad}'$ and
TABLE I

| Donor | Donor Environment (°K) | Acceptor | $J_{AB}'$ ($10^{-16}$ M$^{-1}$ cm$^3$) | $R_0 \Phi_B^{-1/6}$ (Å) | $R_0$ (Å) with $\Phi_D = 0.05$ | $\Phi_D = 0.10$ | $\Phi_D = 0.20$
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr</td>
<td>EGW (80)</td>
<td>Tyr</td>
<td>1.02</td>
<td>14.8</td>
<td>9.0</td>
<td>10.1</td>
<td>11.3</td>
</tr>
<tr>
<td>Tyr</td>
<td>Ribonuclease A</td>
<td>Tyr</td>
<td>0.16</td>
<td>10.8</td>
<td>6.6</td>
<td>7.4</td>
<td>8.3</td>
</tr>
<tr>
<td>Tyr</td>
<td>EGW (300)</td>
<td>Tyr</td>
<td>0.23</td>
<td>11.6</td>
<td>7.0</td>
<td>7.9</td>
<td>8.8 (8.3)</td>
</tr>
<tr>
<td>Tyr</td>
<td>EGW (80)</td>
<td>Trp</td>
<td>11.5</td>
<td>22.2</td>
<td>13.5</td>
<td>15.1</td>
<td>17.0 (15.2)</td>
</tr>
<tr>
<td>Tyr</td>
<td>Ribonuclease A</td>
<td>Trp</td>
<td>4.3</td>
<td>18.8</td>
<td>11.4</td>
<td>12.8</td>
<td>14.4</td>
</tr>
<tr>
<td>Tyr</td>
<td>Water (300)</td>
<td>Trp</td>
<td>4.8</td>
<td>19.2</td>
<td>11.9</td>
<td>13.1</td>
<td>14.7</td>
</tr>
<tr>
<td>Tyr</td>
<td>EGW (300)</td>
<td>Phe</td>
<td>$6.8 \times 10^{-3}$</td>
<td>2.9</td>
<td>1.8$^e$</td>
<td>2.0$^e$</td>
<td>2.2</td>
</tr>
<tr>
<td>Tyr</td>
<td>Ribonuclease A</td>
<td>Tyr$^-$</td>
<td>8.5</td>
<td>21.1</td>
<td>12.8</td>
<td>14.4</td>
<td>16.1</td>
</tr>
<tr>
<td>Tyr$^-$</td>
<td>EGW (300)</td>
<td>Tyr$^-$</td>
<td>0.10</td>
<td>10.1</td>
<td>6.3</td>
<td>6.9</td>
<td>7.7</td>
</tr>
<tr>
<td>Tyr$^-$</td>
<td>EGW (80)</td>
<td>Tyr$^-$</td>
<td>3.6</td>
<td>18.3</td>
<td>11.1</td>
<td>12.5</td>
<td>14.0</td>
</tr>
<tr>
<td>Tyr$^-$</td>
<td>EGW (300)</td>
<td>Trp</td>
<td>0.0057</td>
<td>6.3</td>
<td>3.8$^e$</td>
<td>4.3$^e$</td>
<td>4.8$^e$</td>
</tr>
<tr>
<td>Tyr$^-$</td>
<td>EGW (80)</td>
<td>Trp</td>
<td>0.55</td>
<td>13.4</td>
<td>8.1</td>
<td>9.1</td>
<td>10.2</td>
</tr>
<tr>
<td>Trp</td>
<td>EGW (80)</td>
<td>Trp</td>
<td>1.3</td>
<td>15.5</td>
<td>9.6</td>
<td>10.5</td>
<td>11.8</td>
</tr>
<tr>
<td>Trp</td>
<td>Chymotrypsinogen</td>
<td>Trp</td>
<td>0.21</td>
<td>11.5</td>
<td>6.9</td>
<td>7.8</td>
<td>8.7 (16.0)</td>
</tr>
<tr>
<td>Trp</td>
<td>Water (300)</td>
<td>Trp</td>
<td>0.04</td>
<td>8.6</td>
<td>5.3</td>
<td>5.8</td>
<td>6.6 (6.3)</td>
</tr>
<tr>
<td>Trp</td>
<td>Chymotrypsinogen</td>
<td>Tyr</td>
<td>$2.0 \times 10^{-4}$</td>
<td>3.5</td>
<td>2.2$^e$</td>
<td>2.4$^e$</td>
<td>2.7$^e$</td>
</tr>
<tr>
<td>Trp</td>
<td>EGW (80)</td>
<td>Tyr</td>
<td>0.0032</td>
<td>5.7</td>
<td>3.5$^e$</td>
<td>3.9$^e$</td>
<td>4.4$^e$</td>
</tr>
<tr>
<td>Trp</td>
<td>Water (300)</td>
<td>Trp$^-$</td>
<td>$8.7 \times 10^{-5}$</td>
<td>3.1</td>
<td>1.9$^e$</td>
<td>2.1$^e$</td>
<td>2.4$^e$</td>
</tr>
<tr>
<td>Trp</td>
<td>Water (300)</td>
<td>Trp$^-$</td>
<td>0.35</td>
<td>12.4</td>
<td>7.7</td>
<td>8.4</td>
<td>9.5</td>
</tr>
<tr>
<td>Trp</td>
<td>Chymotrypsinogen</td>
<td>Trp$^-$</td>
<td>1.9</td>
<td>16.4</td>
<td>10.0</td>
<td>11.2</td>
<td>12.6</td>
</tr>
<tr>
<td>Phe</td>
<td>Water (300)</td>
<td>Phe</td>
<td>0.064</td>
<td>9.4</td>
<td>5.8</td>
<td>6.4</td>
<td>7.2 (5.6)</td>
</tr>
<tr>
<td>Phe</td>
<td>Water (300)</td>
<td>Tyr</td>
<td>4.0</td>
<td>18.6</td>
<td>11.6</td>
<td>12.7</td>
<td>14.2 (12.0)</td>
</tr>
<tr>
<td>Phe</td>
<td>Water (300)</td>
<td>Trp</td>
<td>21.8</td>
<td>24.7</td>
<td>15.3</td>
<td>16.8</td>
<td>18.9 (16.0)</td>
</tr>
<tr>
<td>Phe</td>
<td>Water (300)</td>
<td>Tyr$^-$</td>
<td>9.0</td>
<td>21.3</td>
<td>13.2</td>
<td>14.5</td>
<td>6.9</td>
</tr>
</tbody>
</table>

a The overlap integrals and Förster distance, $R_0$, for singlet excitation transfer between pairs of aromatic amino acids. $R_0$ is given assuming three donor fluorescence yields. b Since the acceptor absorption spectra, unlike the donor emission spectra, change only slightly with environment, all calculations used the absorption properties of acceptors in water at room temperature. c Ethylene glycol–water glass (1 : 1, v/v). d $x^2$ is taken to be two-thirds (random orientation); $\eta = 1.5$. e In view of the assumption under which $R_0$ is calculated, values of less than 5 Å are probably meaningless but indicate very low transfer rates. f Karreman et al. (1958). g Konev (1967).

Discussion

Apart from the sensitivity of the calculated values of $R_0$ on the donor environment, Table I shows several other interesting results. First of all, the Förster distances of several pairs of aromatic amino acids (e.g., Tyr → Trp, Phe → Tyr) are of the same order of magnitude as protein and hormone dimensions. It will be seen below that accurate determinations of distances from transfer efficiencies are only possible for distances which are within about a factor of two of the Förster distances. If the ambiguities resulting from the presence of several donors or acceptors can be avoided the measurement of intramolecular separation from transfer efficiencies is therefore a practical method for biomolecules.

Since Phe absorbs only weakly at wavelengths longer than the absorption band of the peptide bonds, Tyr and Trp are the most convenient chromophores for such experiments. If we restrict ourselves to these amino acids and assume that their fluorescence are typical of those observed for these residues in a protein environment (exemplified here by chymotrypsinogen for Trp and ribonuclease A for Tyr) we may draw the following conclusions from the data given in Table I. (1) If the quantum yields of Tyr and Trp are a few per cent, the Förster distances for transfer between identical amino acids is of the order of 6 Å so that singlet transfer between identical chromophores is usually negligible. (2) Transfer from Tyr to Trp is very efficient occurring over distances of about 11 Å while transfer in the opposite direction is to all intents and purposes negligible. (3) Transfer from Trp to tyrosinate (Tyr$^-$) is not unlikely but transfer in the opposite direction is probably negligible. Since Tyr$^-$ exists only at values of pH greater than 10, where many polypeptides have lost their secondary and ter-
tary structure, the emission spectrum of tyrosinate in proteins probably resembles that of Tyr$^{-}$ in aqueous solution at room temperature.

The fluorescence yield of Tyr$^{-}$ at room temperature has been claimed to be zero (Vladimirov and Chin-Kuo, 1962; Cowgill, 1963) by some and to be finite and small (Cornog and Adams, 1963) by others. We have observed that the fluorescence of Tyr$^{-}$ depends strongly upon the temperature and viscosity of the solvent. We measured the quantum yield in water at room temperature (25$^{\circ}$) to be 0.015 (Cornog and Adams (1963) report 0.01) and found values of 0.06 in water at 4$^{\circ}$, and 0.06 in ethylene glycol-water (1:1, v/v) at room temperature. Tyrosinate therefore undergoes appreciable temperature- and viscosity-dependent fluorescence quenching at temperatures above those at which solvent reorientation in the excited state occurs, similarly to the quenching reported in Trp (Eisinger and Navon, 1969).

The most intriguing possibility presented by these results is that of determining intramolecular separations between aromatic residues in polypeptides and proteins from energy-transfer rates. Such rates may be measured experimentally either from transfer efficiencies or from fluorescence decay rates. Both methods are based on the fact that Tyr, Tyr$^{-}$, Trp, and Phe all have different absorption properties (Eisinger, 1969b; Weber, 1961; Longworth, 1968). In the first technique one makes use of the fact that the fluorescence yield of these chromophores will depend upon their relative absorbivity at the excitation wavelength as well as on the extent of energy transfer among them. The second method is the dynamic equivalent of the first in which energy transfer manifests itself by changes in the fluorescence decay rates (Bennett, 1964; Bennett et al., 1964; Kellogg, 1964) for the chromophores. While this method is capable of providing more detailed information about energy transfer it requires much more sophisticated instrumentation since decay rates of the order of $10^{9}$ sec$^{-1}$ must be measured. The determination of transfer efficiencies from excitation spectra on the other hand is also not without its experimental difficulties. Relative quantum yields at several excitation wavelengths must be determined with a precision of 1-2% in order to measure the efficiency of energy transfer from Tyr to Trp to within 10% (Eisinger, 1969b).

If the distance between a donor and acceptor is $r$ the efficiency of transfer between them is given by

$$ e = \frac{r \epsilon t}{r \epsilon t + R_0 \epsilon t} $$

where $R_0$ is the appropriate Förster distance for which $e$ becomes $\frac{1}{2}$. It follows therefore that

$$ r = (e^{-1} - 1)^{-1/2} R_0 $$

Figure 2 gives $r$ in units of $R_0$ as a function of $e$. The same figure also shows the fractional error in $r$, $\Delta r / r$, which results from an experimental uncertainty $\Delta e$ in $e$. From these results it is clear that the determination of $r$ from $e$ is probably feasible only if $e$ is between 0.1 and 0.8. Values of $e$ below 0.1 cannot generally be measured accurately and when $e$ is larger than 0.8 the error in $r$ becomes intolerable.

In order to evaluate $R_0$, $\Phi_d$, $\kappa^2$, and $n^4$ must be known in addition to $J_{AD}$. These parameters will now be discussed in turn.

$\Phi_d$. The donor fluorescence quantum yield can be measured in certain cases. Thus, if the polypeptide contains only a single Tyr residue and a single Trp residue the spectral contribution of the Tyr may be made observable by comparing the emission spectra obtained when the polypeptide is excited at 275 nm (where both Tyr and Trp absorb) and at 295 nm (where only Trp absorbs). The yield may then be estimated from the known absorption coefficients of Tyr and Trp and the total quantum yield measured when 275-nm excitation is used. It must be borne in mind, of course, that only that fraction of Tyr emission is observed in the difference fluorescence spectrum which is not transferred to Trp. This method has been used (Eisinger, 1969b) to estimate the quantum yields of Tyr-2 and Tyr-23 in ACTH $\beta$ (1-24).

In cases where this method is not practicable $\Phi_d$ may be taken to be an average value for typical yields from the same chromophore in proteins and oligopeptides (Konev, 1967; Cowgill, 1963). These values$^1$ range from 0.02 to 0.07 for Tyr, and from 0.06 to 0.15 for Trp.

$\kappa^2$. If the donor and acceptor transition dipoles have a fixed but unknown orientation, $\theta_{AD}$, with respect to each other and make angles $\theta_D$ and $\theta_A$ with the line joining them, $\kappa$ is given by

$$ \kappa = \cos \theta_{AD} - 3 \cos \theta_A \cos \theta_D $$

$\kappa^2$ may therefore vary between 0 and 4. Since the angles $\theta_A$, $\theta_D$, and $\theta_{AD}$ are generally not known but one often finds several rotatable (single) bonds between the two chromophores one may argue that an average value for the orientation factor $\kappa^2$ may be used. The appropriate value for $(\kappa^2)_{av}$ is 0.475 for random orientation of the donor and acceptor molecules in rigid solutions (i.e., rotation is slow compared with the donor life-

$^1$ These quantum yields are one-third lower than the published values (Konev, 1967; Cowgill, 1963). The original determination used Tyr and Trp in water to calibrate the fluorescence yield and assumed quantum yields 0.21 and 0.20, respectively. Recent redeterminations (Chen, 1967; Borresen, 1967; Eisinger, 1969a) indicate that the quantum yield of Trp is more nearly 0.14.
time) (Maksimov and Rozman, 1962; Steinberg, 1968) and \( (k^2)_{av} \) is two-thirds ( Förster, 1951, 1966) where the molecules are free to rotate at a rate which is much larger than the deexcitation rate of the donor. Anomalously large effective values for \( k^2 \) may occur when rotational correlation times and donor lifetimes are comparable since transfer will be more likely at times when the instantaneous value of \( k^2 \) is large.

A more careful examination of the molecular motion of the aromatic groups shows that these simple ideas which work well in solution may be inadequate in polypeptides. Thus it is known that the \( C_\alpha-C_\beta \) and \( C_\beta-C_\gamma \) bonds in tyrosine, to take an example, are far from being freely rotating bonds but are likely to have potential barriers between rotational minima which may be as large as a few kilocalories per mole (Coulson, 1961). While stochastic motion between these minima can of course occur at a fast rate at room temperature, this does mean that all angles of rotation are not equally likely.

It is clear from these considerations that there is no completely satisfactory way of choosing \( k^2 \) for two chromophores on a polypeptide. If there exists little secondary or tertiary structure the value of two-thirds is probably adequate for room temperature solutions but in the presence of strong steric limitations such as occur in proteins no adequate method of predicting \( k^2 \) exists.

c. \( n^\prime \). The index of refraction that should be used is that of the intervening medium (generally protein) and must obtain in the wavelength region in which the donor and acceptor have their spectral overlap (~300 nm). In water, \( n = 1.5 \) is a value which will not be far off in most cases.

d. Translational Diffusion. If the distance between the donor and acceptor changes as a result of diffusion during the donor lifetime, \( \tau_D \), the value of \( r \) obtained with the assumption of stationary donors and acceptors will be too short as a result of the inverse sixth power dependence of \( k_{AD} (k_{AD} \propto r^{-6}) \) (Birks and Georgiou, 1967; Elkan et al., 1968; Povinelli, 1966). To estimate the magnitude of this effect we consider a fixed donor, D, with an acceptor, A, at distance \( r \) in the \( x \) direction. A is assumed to have a diffusion length \( L = \sqrt{D\tau_D} \) where \( D \) is the diffusion coefficient and where diffusion in the \( y \) and \( z \) directions is considered equally likely \( (L_x = L_y = L_z \text{.} \) Since motion in the \( y \) and \( z \) directions has a negligible effect on \( r \) and diffusion in the negative \( x \) direction is weighed much more heavily than diffusion in the positive \( x \) direction

\[
(r^\prime)_{av}^{-1/6} \approx r - L_x \approx r - \frac{1}{3} L
\]

The effective values of \( D \) of the aromatic amino acids attached to a polypeptide are not known. In aqueous solutions at 25°, \( D \) is approximately (Longsworth, 1953) \( 0.7 \times 10^{-6} \text{ cm}^2 \text{ sec}^{-1} \). If we assume that the "drag" of the polypeptide chain may be approximated by the lowering in \( D \) which is observed in going from an amino acid to a tripeptide, the effective value of \( D \) of the aromatic residues attached to a polypeptide is expected to be about half (Longsworth, 1953) of the value given above or about \( 0.3 \times 10^{-6} \text{ cm}^2 \text{ sec}^{-1} \) at 25°. With a donor lifetime of \( 0.5 \times 10^{-9} \text{ sec, one therefore obtains } L = 4 \text{ Å. The value of } r \text{ obtained from an energy transfer experiment is therefore likely to be low by about } 1 \text{ Å if diffusion effects similar to those described above are present. If the polypeptide has appreciable secondary and/or tertiary structure, as for instance in proteins, this error will be much smaller.}

While the estimates obtained for \( \Phi_0 \) and \( k^2 \) by the methods outlined above may have considerable uncertainties associated with them, it should be noted that these parameters enter into the calculation of \( R_0 \) as sixth roots so that the resultant uncertainty in \( R_0 \) (and \( r \)) is much smaller.

Limits of Applicability of the Method

Apart from the uncertainties in \( \Phi_0 \) and \( k^2 \) which were considered above it is necessary to examine the range of donor–acceptor distances which could be determined by energy transfer experiments.

At sufficiently large distances between D and A, transfer rates are so slow compared to other deexcitation processes of the donor that the experimental uncertainties preclude determining \( r \) when \( r/R_0 \) is greater than 2 (see Figure 2).

At sufficiently small distances the method outlined above cannot be used to determine \( r \) because of the following considerations. (1) Let \( R \) be the spatial extent of the charge distribution of a chromophore. Then the contribution to the transfer rate of the dipole–quadrupole interaction compared with that of the dipole–dipole interaction is of the order \( (R/r)^2 \) (Dexter, 1953). If this term is omitted in the calculation of \( R_0 \), as was done in this paper, the error in \( R_0 \) and hence in \( r \) will be of the order of \( (1/6)(R/r)^2 \). Since \( R \) can be expected to be of the order of 3 Å, the point dipole theory will in general yield a value for \( r \) with an uncertainty of less than 10%, as long as \( r \) exceeds 4 Å. (2) When the donor and acceptor wave functions overlap, the exchange interaction may become comparable to the dipole–dipole interaction. While the intermolecular exchange interaction is difficult to calculate in the absence of reliable molecular wave functions, it has recently been estimated for neighboring adenine molecules in various geometries by Sommer and Jortner (1968) who obtained a value of about 20 cm\(^{-1}\) at a distance of 4 Å. Similar calculations for nearest neighbor anthracene molecules in the crystal gave 44 cm\(^{-1}\) (Jortner et al., 1965). Experimental values for the exchange interaction between neighboring benzene molecules have been reported (Nieman and Robinson, 1963) as 10 cm\(^{-1}\). In Appendix II we have estimated the rate of singlet energy transfer between the aromatic amino acids resulting from the exchange interaction and conclude that if the Förster distance, \( R_0 \), is of the order of 10 Å, the contribution of the exchange mechanism to the transfer rate is negligible at distances greater than about 5 Å.

Summary

We have evaluated the Förster distances corresponding to singlet energy transfer between various pairs of aromatic amino acids, paying particular heed to the dependence of the donor fluorescence spectrum, and hence of \( R_0 \), upon the environment of the donor molecule. Thus \( R_0 \) of Trp → Trp transfer varies between 10.5 Å in low-temperature glasses to 5.8 Å in water at room temperature (\( \Phi_0 \) is assumed 0.1). In proteins an intermediate value may be expected.

Consideration was given to the question over what range of distances the usual point dipole approximation of the Förster theory is applicable, and it was found that for the aromatic amino acids neither the finite extent of the chromophore...
charge distribution nor the possibility of exchange interaction initiated energy transfer is important at distances greater than about 5 Å if \( R_0 \) is on the order of 10 Å.

The determination of transfer efficiencies among the aromatic amino acids, particularly from Tyr to Trp, appears to provide a means for determining certain intramolecular distances between these chromophores in those polypeptides for which the analysis is not complicated by the presence of several donors or acceptors of the same type (Eisinger, 1969b). A simple procedure for carrying out such determinations is given.

Acknowledgments

We wish to thank Dr. L. C. Snyder and Mrs. Z. Wasserman for calculating the exchange integrals.

Appendix I

**Before-Relaxation Energy Transfer.** The need to consider the possibility of before-relaxation energy transfer was first pointed out by Guéron *et al.* (1967) who calculated the corresponding transfer rate for pairs of neighboring bases in DNA at 80°K. For the base separation which obtains in the Watson–Crick structure (3.4 Å) before-relaxation transfer rates were calculated to be of the order of \( 10^{15} \) sec\(^{-1} \) and might very well compete with the vibronic relaxation rate which has been estimated to be of the order of \( 10^{11} \text{ to } 10^{12} \) sec\(^{-1} \) in the condensed phase.

The probability of transfer occurring before relaxation, \( P_0 \), may be compared with the probability after relaxation, \( P_a \), by noting that (Guéron *et al.*, 1967)

\[
P_0 \approx 1 - \exp(-k_{ADb} \tau_s)
\]

and

\[
P_a \approx 1 - \exp(-k_{AD} \tau_s)
\]

where \( \tau_s \) and \( \tau_r \) are the vibrational relaxation lifetime and singlet lifetime of the donor, respectively. The after-relaxation transfer rate \( k_{AD} \) is given by eq 1; \( k_{ADb} \) is the before-relaxation transfer rate which may be calculated in the same way as \( k_{AD} \) except that the appropriate spectral overlap integral, \( J_{AD} \), is computed by using the emission spectrum \( F_D'(\nu) \) instead of \( F_D(\nu) \), where \( F_D(\nu) \) represents the fluorescence spectrum which would be observed if emission preceded vibrational relaxation. Since \( F_D(\nu) \) cannot be determined experimentally it is approximated by shifting \( F_D(\nu) \) to the blue by \( \nu_{ex} - \nu_0 \), where \( \nu_{ex} \) and \( \nu_0 \) are the wave numbers corresponding to the energy of excitation and the energy of the lowest vibrational level of the excited donor, respectively.

If there exists a reasonable overlap between the donor emission and acceptor absorption spectra, \( k_{ADb} \) will not exceed \( k_{AD} \) by more than an order of magnitude. Since \( \tau_r \) is usually several orders of magnitude larger than \( \tau_s \) this means that \( k_{ADb} \tau_r < k_{AD} \tau_s \) and eq A1 and A2 show that after-relaxation transfer will be much more likely than before-relaxation transfer.

If, on the other hand, the donor fluorescence is so far to the red of the acceptor absorption that the overlap between them is negligible then \( k_{AD} \) is vanishingly small while \( k_{ADb} \) may be appreciable so that \( P_0 \) will exceed \( P_a \).

Table I

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>( J_{AD} ) ((10^{-16} \text{ M}^{-1} \text{ cm}^2) )</th>
<th>( k_{AD} ) ((r = 5 \text{ Å}) )</th>
<th>( k_{AD} ) ((10^{10} \text{ sec}^{-1}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr</td>
<td>Tyr</td>
<td>4.2</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Tyr</td>
<td>Trp</td>
<td>21.0</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Tyr</td>
<td>Phe</td>
<td>0.037</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Trp</td>
<td>Tyr</td>
<td>0.46</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Trp</td>
<td>Trp</td>
<td>3.8</td>
<td>1.3</td>
<td>1.3</td>
</tr>
</tbody>
</table>

The overlap integrals and singlet energy transfer rates between pairs of aromatic amino acids, assuming transfer to be fast compared with vibrational relaxation (before-relaxation transfer). In water at room temperature. Assuming excitation at the wavelength of the donor absorption maximum. Assuming \( \Phi_D/\tau_D = 5 \times 10^{10} \text{ sec}^{-1} \); \( k^2 = 1/4 \).

Appendix II

**Exchange Interaction and Transfer Rate.** Energy transfer among aromatic amino acids, as well as among most biological molecules, is governed by the “very weak coupling” limit inasmuch as the interaction energy is small compared to the widths of the absorption bands of the molecules involved. As a result, the rate of after-relaxation energy transfer may be written (Guéron *et al.*, 1967)

\[
k_{AD} \approx \frac{32e^2}{\hbar} \int \langle X_d | X_a^* \rangle \rho_d(X_a) \rho_a dE
\]

where \( U \) is the interaction energy between the donor and the acceptor. \( \langle X_d | X_a^* \rangle \) and \( \langle X_a | X_a^* \rangle \) are the Franck–Condon overlap factors for donor emission and acceptor absorption, respectively, and \( \rho_d \) and \( \rho_a \) are the corresponding level densities. It follows therefore that the rate of energy transfer mitigated by the exchange interaction, \( U_{ex} \), compared with that arising from the dipole–dipole interaction, \( U_{dd} \), is given by

\[
\frac{k_{ex}}{k_{dd}} = \left| \frac{U_{ex}}{U_{dd}} \right|^2
\]

Several calculations (Jortner *et al.*, 1965; Sommer and
necessary computations which were carried out with electronic wave functions which are considered to be particularly useful at large distances from the nuclei. The calculations showed that if \( r = 4 \) Å and the separation between the molecules increases by 1 Å, \( U_{\text{ex}} \) decreases by factors of about 10 and 20 for the axial and coplanar case, respectively. This corresponds to decreases by factors of 100 and 400 in \( k_{\text{ex}} \). At the same time the dipolar transfer rate would drop by about a factor of 3 only.

These results are shown graphically in Figure 3 where the transfer rate due to the exchange interaction, \( k_{\text{ex}} \), was calculated using \( U_{\text{ex}} = 20 \) cm\(^{-1}\) at \( r = 4 \) Å in axial approach and by taking the spectral overlap found for the case of Tyr → Trp. In the same figure is plotted the distance dependence of the dipole–dipole transfer rate, \( k_{dd} \), for various values of \( R_0 \) and taking \( \tau_0 = 2 \) nsec, a typical value for the aromatic amino acids.

While the results of these calculations are little more than order of magnitude estimates it is clear that for pairs of aromatic amino acids for which \( R_0 \) is 10 Å or more the transfer rate can be accounted for almost entirely by the Förster theory as long as \( r \) exceeds 5 Å.

References
Bouw, E. J. (1959), Discussions Faraday Soc. 27, 40.
Eisinger, J. (1969b), Biochemistry 8, 3902.
 Förster, Th. (1948), Physik 2, 55.
 Förster, Th. (1951), Fluoreszenz Organischer Verbindungen, Göttingen, Vandenhoeck and Ruprecht.
Dimer Formation from 1-Anilino-8-naphthalenesulfonate Catalyzed by Bovine Serum Albumin. A New Fluorescent Molecule with Exceptional Binding Properties

Carl-Gustaf Rosen‡ and Gregorio Weber

ABSTRACT: At pH values around 2, nitrite induces a series of chemical changes in 1-anilino-8-naphthalenesulfonate. From the resulting mixture of products a compound has been isolated, the spectroscopic properties of which are very similar to the parent compound, but with an affinity for bovine serum albumin nearly two orders of magnitude greater than that of the latter. The number of strong binding sites displaying fluorescence enhancement is two. If albumin is present during the formation of the compound, the yield, which otherwise amounts to only 1-2%, approaches 100%, i.e., the protein appears to behave in an enzyme-like fashion. Three different preparative procedures are described. Experimental evidence is given to support the conclusion that the new molecule is a dimer of 1-anilino-8-naphthalenesulfonate.

Several aromatic dyes, which are virtually nonfluorescent in water solution, become strongly fluorescent in nonaqueous solvents, or when bound to apparently hydrophobic sites in proteins (Weber and Laurence, 1954). They have for this reason been applied to studies of protein-ligand interactions and binding sites in proteins. In the course of studies by Daniel and Weber (1966) on cooperative effects on binding by BSA ANS was utilized, and it was observed that when a dilute solution of ANS and BSA (concentration \( \sim 10^{-7} \) M) was left at pH 2, the ANS fluorescence increased slowly with time so that doubling of the initial fluorescence intensity took place in about 20 min. Since the quantum yield of fluorescence for the bound dye is initially as high as 0.7, the cause of this effect must obviously be an increase of the binding rather than an increase of the quantum yield, and accordingly there must be a slow process taking place, which affects the binding. This could be either a chemical change in the dye and/or the protein, or a conformation change of the protein modifying the binding sites.

The work presented in this paper was initiated as a search for factors that were responsible for the fluorescence increase. It was found that the process was inhibited by millimolar concentrations of ferrocyanide ions and could be observed only at concentrations of BSA and ANS \( \leq 10^{-5} \) M. It was finally realized that the process required the presence of nitrous ions. These were always present, albeit in small concentrations, because the fluorescence cuvets were cleaned by soaking in...