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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 (Ermoleav and Sveshnikova, 1963; Bennett, 1964; Bennett *et al.*, 1964; Kellogg, 1964; Stryer and Haugland, 1967; Birks and Georghiou, 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 Karreman *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

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fulfilled, before-relaxation transfer (Guéron *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)

$$k_{AD} = \frac{8.8 \times 10^{-25}\Phi_D\kappa^2 J_{AD}'}{n^4\tau_D r^6} \quad (1)$$

with

$$J_{AD}' = \int_0^\infty F_D(\nu)\epsilon_A(\nu)\nu^{-4}d\nu \quad (2)$$

where τ_D and Φ_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. κ is the dipole–dipole orientation factor and r is the donor–acceptor separation. J_{AD}' is an overlap integral between $\epsilon_A(\nu)$, the decadic molar extinction coefficient of the acceptor, and $F_D(\nu)$, the spectral distribution of the donor emission normalized to unity, modified by the frequency factor ν^{-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)

$$R_0^6 = 8.8 \times 10^{-25}\Phi_D\kappa^2 n^{-4} J_{AD}' \quad (3)$$

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 the wavelength-dependent response of the instrument and were obtained with dilute samples (approximately 10^{-4} 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, $\epsilon(\nu)$, obtained with dilute aqueous solutions. The shape of these curves is relatively insensitive to temperature and solvent. The emission spectra, $F_D(\nu)$, 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.

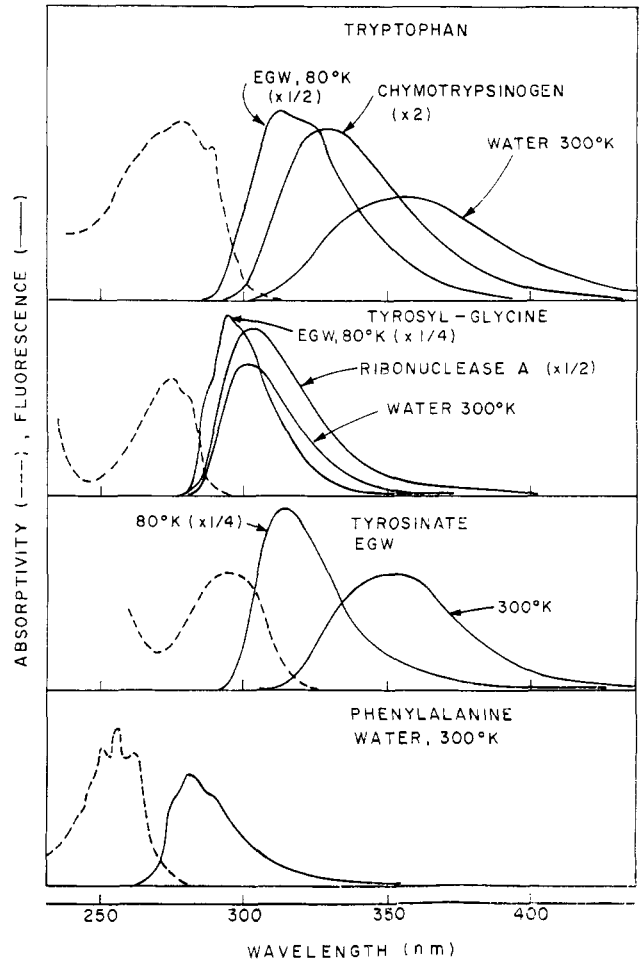

FIGURE 1: The absorption and fluorescence spectra of aromatic amino acid in different environments and at different temperatures. While the fluorescence spectra are uncorrected for the wavelength dependence of the fluoroscope sensitivity, the appropriate correction factor does not exceed 1.5 over the wavelength range covered by these spectra.

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 Φ_D is often not known precisely, we have chosen to give values for J_{AD}' and $R_0\Phi_D^{-1/6}$ as well as for R_0 assuming three different values for Φ_D. R_0 depends not only upon the donor quantum yield but also on 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 Navon, 1969). Such a red shift invariably decreases J_{AD}' and

TABLE I[a]

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

[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 in room temperature. [c] Ethylene glycol–water glass (1:1, v/v). [d] κ^2 is taken to be two-thirds (random orientation); n = 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).

hence leads to a lower value of R_0. Konev (1967) has pointed out that this effect is particularly important in tryptophan whose emission spectrum is very sensitive to the viscosity of the solvent. He used an approximate method to calculate R_0 for Trp → Trp transfer using the fluorescence spectrum of Trp in chymotrypsin for $F(\nu)$ but his estimate for R_0 is about twice as large as ours. Table I shows that if Φ_D is 0.1, R_0 for Trp → Trp transfer is 5.8 Å in water at room temperature, 7.8 Å for Trp in a typical protein environment or in a sucrose matrix at room temperature, and 10.5 Å in an ethylene glycol–water (1:1, v/v) glass at 80°K (see Figure 1).

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-

tiary 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°) to be 0.015 (Cornog and Adams (1963) report 0.01) and found values of 0.06 in water at 4°, 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 somewhat 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 absorptivity 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

$$e = \frac{r^{-6}}{r^{-6} + R_0^{-6}} \tag{4}$$

where R_0 is the appropriate Förster distance for which e becomes $1/2$. It follows therefore that

$$r = (e^{-1} - 1)^{1/6} R_0 \tag{5}$$

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 Δe 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, Φ_D, κ^2, and n^4 must be known in addition to J_{AD}'. These parameters will now be discussed in turn.

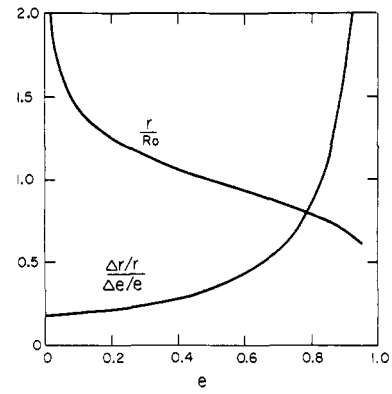

FIGURE 2: The donor acceptor separation, r, as a multiple of the Förster distance, R_0, is given as a function of the energy transfer efficiency, e. Also shown is the uncertainty in r (Δr) resulting from an uncertainty in e (Δe) as a function of e.

a. Φ_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 mm (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 β (1–24).

In cases where this method is not practicable Φ_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.

b. κ^2. If the donor and acceptor transition dipoles have a fixed but unknown orientation, θ_{AD}, with respect to each other and make angles θ_D and θ_A with the line joining them, κ is given by

$$\kappa = \cos\theta_{AD} - 3\cos\theta_A\cos\theta_D \tag{6}$$

κ^2 may therefore vary between 0 and 4. Since the angles θ_A, θ_D, and θ_{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 κ^2 may be used. The appropriate value for $\langle\kappa^2\rangle_{av}$ 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; Børresen, 1967; Eisinger, 1969a) indicate that the quantum yield of Trp is more nearly 0.14.

time) (Maksimov and Rozman, 1962; Steinberg, 1968) and $\langle\kappa^2\rangle_{av}$ 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 κ^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 κ^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_α–C_β and C_β–C_1 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 κ^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 κ^2 exists.

c. n^4. 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, τ_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 Georghiou, 1967; Elkana *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 x, y, and z directions is considered equally likely ($L_x = L_y = L_z$). 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

$$\langle r^{-6}\rangle_{av}^{-1/6} \approx r - L_x \approx r - \frac{1}{3}L$$

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) 0.7×10^{-5} cm^2 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 0.3×10^{-5} cm^2 sec^{-1} at 25°. With a donor lifetime of 0.5×10^{-9} sec, one therefore obtains $L = 4$ Å. The value of r obtained from an energy transfer experiment is therefore likely to be low by about 1 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 Φ_D and κ^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 Φ_D and κ^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 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 (Φ_D 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^{13} sec^{-1} and might well compete with the vibronic relaxation rate which has been estimated to be of the order of 10^{11}–10^{12} sec^{-1} in the condensed phase.

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

$$P_b \approx 1 - \exp(-k_{AD}{}^b \tau_v) \qquad (A1)$$

and

$$P_a \approx 1 - \exp(-k_{AD}\tau_s) \qquad (A2)$$

where τ_v and τ_s are the vibrational relaxation lifetime and singlet lifetime of the donor, respectively. The after-relaxation transfer rate k_{AD} is given by eq 1; $k_{AD}{}^b$ 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 ν_{ex} and ν_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, $k_{AD}{}^b$ will not exceed k_{AD} by more than an order of magnitude. Since τ_s is usually several orders of magnitude larger than τ_v this means that $k_{AD}{}^b \tau_v < k_{AD}\tau_s$ 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 $k_{AD}{}^b$ may be appreciable so that P_b will exceed P_a.

TABLE II[a]

Donor[b]	Acceptor	J_{AD}'' (10^{-16} M^{-1} cm^6)[c]	$k_{AD}{}^b$ (r = 5 Å)[d] (10^{10} sec^{-1})
Tyr	Tyr	4.2	1.5
Tyr	Trp	21.0	7.5
Tyr	Phe	0.037	0.01
Trp	Tyr	0.46	0.2
Trp	Trp	3.8	1.3

[a] 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). [b] In water at room temperature. [c] Assuming excitation at the wavelength of the donor absorption maximum. [d] Assuming $\Phi_D/\tau_D = 5 \times 10^7$ sec^{-1}; $\kappa^2 = {}^2/_3$.

We have estimated some before-relaxation spectral overlap integrals, J_{AD}'', according to the method outlined above for the case when the donor is excited at the wavelength corresponding to its absorption maximum. The results are presented in Table II. The J_{AD}'' values are seen to exceed the J_{AD}' values for after-relaxation transfer given in Table I by one to four orders of magnitude. Table II also gives numerical values for $k_{AD}{}^b$ for donor–acceptor separation of r = 5 Å with $\kappa^2 = {}^2/_3$ and Φ_D/τ_D equal to 5×10^7 which is an adequate approximation for both Tyr and Trp donors. These rates are of course much smaller when the separation is greater than 5 Å, but it is seen that even with this small separation $k_{AD}{}^b$ is in most cases negligible compared with $1/\tau_v$ which was recently measured to be of the order of 1.3×10^{11} sec^{-1} for azulene (Rentzepis, 1968).

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)

$$k_{AD} \approx \frac{32|U|^2}{h}\int \langle X_D|X_D^*\rangle \rho_D \langle X_A|X_A^*\rangle \rho_A dE \qquad (A1)$$

where U is the interaction energy between the donor and the acceptor. $\langle X_D|X_D^*\rangle$ and $\langle X_A|X_A^*\rangle$ are the Franck–Condon overlap factors for donor emission and acceptor absorption, respectively, and ρ_D and ρ_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

$$\frac{k_{ex}}{k_{dd}} = \frac{|U_{ex}|^2}{|U_{dd}|^2} \qquad (A2)$$

Several calculations (Jortner *et al.*, 1965; Sommer and

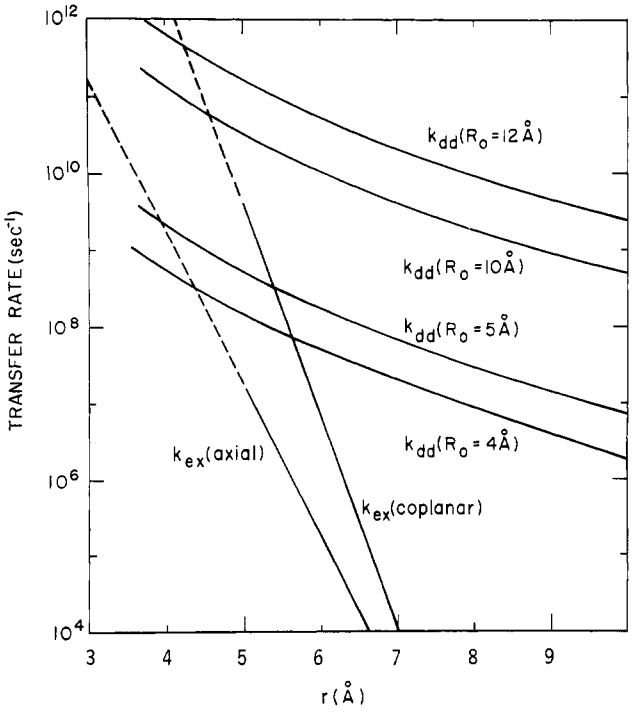

FIGURE 3: A comparison of the energy transfer rates resulting from the dipole–dipole interaction, k_{dd}, on the one hand, and from an exchange interaction, k_{ex}, on the other hand. Two extreme cases for the donor–acceptor geometry are considered: these are the axial and coplanar approaches which are discussed in the text. The donor lifetime is taken to be 2 nsec.

Jortner, 1968) and some experimental evidence (Nieman and Robinson, 1963) indicate that U_{ex} is of the order of a few tens of wave numbers for aromatic donors and acceptors which are nearest neighbors. For adenine molecules in the relative positions which obtain for neighboring stacked bases in double-stranded DNA Sommer and Jortner (1968) find $U_{ex} \approx 20\ cm^{-1}$ with $r = 4$ Å. We assume that the exchange interaction between two stacked aromatic amino acids at $r = 4$ Å is similar.

The distance dependence of k_{dd} was seen to be r^{-6}. That of k_{ex} is expected to be an even stronger function of the distance since $|U_{ex}|^2$ will decrease with the fourth power of the overlap of the donor and acceptor wave functions, or approximately like $\exp(-4r)$.

In order to obtain a better estimate of the relative magnitude of dipolar and exchange interactions as a function of r, the separation between the transition dipoles of the interacting chromophores, U_{ex}, was calculated for two aromatic ring molecules each of which contain a single conjugated electron and which are considered to approach each other in one of two extreme ways. In the first case (axial approach) the planes of the two molecules are assumed to be parallel (stacked) and their symmetry axes coincident. The exchange interaction arises from the σ overlap between the 2p wave-function lobes. In the second case (planar approach) the molecules are considered to be coplanar and the exchange interaction arises from the π overlap of the 2p orbitals. If these 2p orbitals are centered about nuclei separated by a distance a, r is assumed to be given by $(a + 3)$ Å in the latter case.

L. C. Snyder has kindly put at our disposal the results of the 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_{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_{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_{ex}, was calculated using $U_{ex} = 20\ cm^{-1}$ 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 $\tau_D = 2$ 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.

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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[1] 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 $\lesssim 10^{-5}$ 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

* From the Biochemistry Division, Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois. *Received May 12, 1969.* Enquiries should be sent to G. W. at this address. This work was supported by a grant (U. S. Public Health Service GM 11223) from the National Institutes of Health and also partly by a grant from the Swedish Atomic Research Council.

† Present address: Radiobiology Division, Department of Biochemistry, Royal University of Stockholm, Stockholm Va, Sweden.

[1] Abbreviations used are: ASN, 1-anilino-8-naphthalenesulfonate; BSA, bovine serum albumin; DNS, dimethylaminonaphthalenesulfonate.