# ENERGY TRANSFER: A SPECTROSCOPIC RULER*

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Electronic excitation energy can be transferred over distances of the order of 30 Å.[1,2] Förster has proposed that the transfer occurs by a dipole-dipole resonance interaction between the energy donor and acceptor chromophores.[3] In his treatment of weak coupling, the rate constant for transfer is proportional to the inverse sixth power of the distance between the groups.[4] The transfer rate is also assumed to depend on the mutual orientation of the groups, the refractive index of the solvent, and the overlap of the emission spectrum of the energy donor and the absorption spectrum of the energy acceptor.

We report here an experimental study of the dependence of energy transfer on distance. Oligomers of poly-L-proline were used as spacers of defined length to separate an energy donor and acceptor by distances ranging from 12 to 46 Å. The energy donor was an α-naphthyl group at the carboxyl end of the polypeptide, while the energy acceptor was a dansyl[5] group at the imino end (I):

(I)

$$n = 1 \text{ to } 12$$
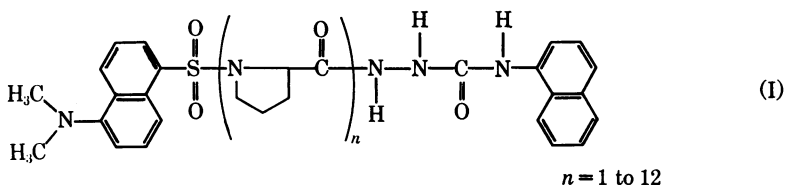
The observed efficiency of energy transfer as a function of distance is in excellent agreement with the r^{-6} dependence proposed by Förster.

Materials and Methods.—*Synthesis of oligomers of poly-L-proline:* The solid-phase peptide synthesis method described by Merrifield[6] was used for the stepwise synthesis of oligomers of poly-L-proline to a degree of polymerization of 12. A chloromethylated copolystyrene-2% divinylbenzene resin (1.20 meq/gm) was refluxed for 2 days with a twofold excess of t-BOC-L-proline and triethylamine in ethyl acetate to yield a prolyl resin containing 0.86 meq proline/gm resin. For each reaction cycle, the t-BOC protecting group was removed with 1 N HCl in acetic acid for 30 min. The resin was washed with acetic acid, ethanol, and methylene chloride, neutralized with 10% triethylamine in methylene chloride, and again washed with methylene chloride. Peptide formation was achieved with dicyclohexylcarbodiimide and a twofold excess of t-BOC-L-proline in methylene chloride. The coupling reaction was left at room temperature for at least 20 hr, filtered, and washed with methylene chloride, ethanol, and acetic acid. The deprotection step for the next reaction cycle was then carried out.

Attachment of the energy acceptor and release of the peptide: The Merrifield method was modified (Fig. 1) to facilitate the attachment of the energy donor and acceptor groups. The energy acceptor was attached to the imino end of the polypeptide before the oligomer was released from the resin. After the neutralization step, about 0.5 gm of deprotected resin was removed from the reaction vessel, reacted for 10 hr with 250 mg of dansyl chloride in methylene chloride containing 1 ml of triethylamine, and washed thoroughly with methylene chloride and ethanol. The dansylated peptide was released by stirring the resin for 20 hr with 20 ml of ethanol containing 4 ml anhydrous (97 + %) hydrazine. The filtrate was evaporated under vacuum to yield a yellow oil.

Attachment of Energy Donor and Acceptor
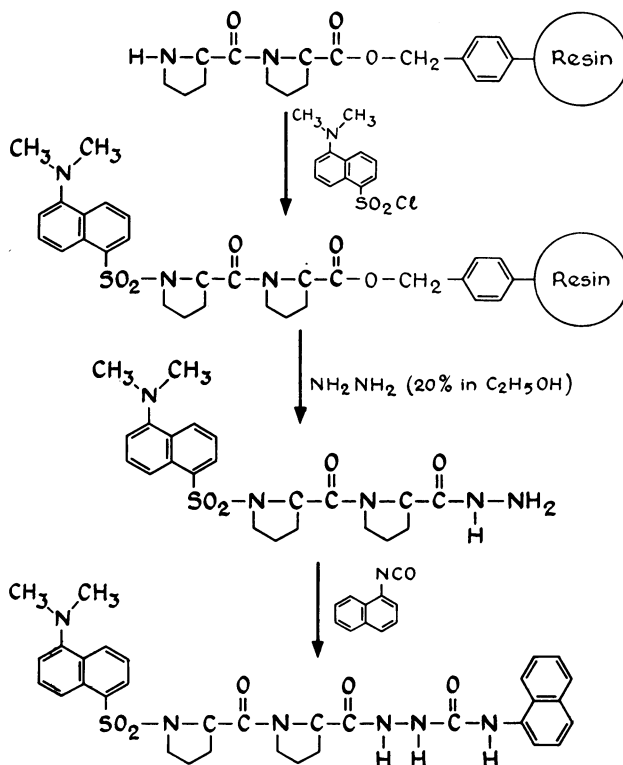
FIG. 1.—Synthesis of dansyl-(L-prolyl)$_2$-α-naphthyl.

Chromatographic purification of the dansylated peptides: The yellow oils were applied to 1 × 10 cm columns of neutral alumina (Woelm) in benzene. An impurity with a blue fluorescence was removed when the columns were washed with ethyl acetate. Elution of the dansylated peptides was conveniently monitored by the greenish-yellow fluorescence of the attached dansyl chromophore. The shorter oligomers were eluted with 10% ethanol in ethyl acetate, while the longer ones required up to 50% ethanol. The purity of these preparations was evaluated by thin-layer chromatography on silica gel (Eastman). The R_f values of the dansyl-(L-prolyl)$_n$-hydrazides in ethyl acetate-ethanol (2:1) were 0.91, 0.58, 0.32, 0.17, and 0.08, for $n = 1$ to 5, respectively; in methanol, 0.84, 0.74, 0.65, 0.56, 0.47, 0.41, 0.30, 0.27, 0.21, and 0.14 for $n = 3$ to 12, respectively. The purity of each of the oligomers was estimated by fluorimetry to be at least 98%.

Attachment of the energy donor: The energy donor was covalently attached to each dansyl-(L-prolyl)$_n$-hydrazide by addition of α-naphthyl isocyanate to a solution of the hydrazide in ethanol. Thin-layer chromatography showed the reaction to be complete within a minute. The dansyl-(L-prolyl)$_n$-α-naphthyl compounds were readily purified since their R_f values are substantially higher than those of the hydrazides.

1-Acetyl-4-(1-naphthyl) semicarbazide was prepared by acetylating solid 4-(1-naphthyl) semicarbazide with excess acetic anhydride. The solution was evaporated under vacuum to an oil and crystallized from ethyl acetate-acetonitrile to yield colorless leaves which decomposed above 230°. Anal. calc. for $C_{13}H_{13}N_3O_2$: C, 64.19; H, 5.38; N, 17.27%. Found: C, 64.10; H, 5.21; N, 17.26.

Spectroscopic measurements: Fluorescence excitation, emission, and polarization spectra were obtained on a recording spectrofluorimeter as described previously.[7] Corrected excitation spectra were recorded directly by use of a ratio amplifier.[7] A Corning 3-70 filter was used to isolate the

emission of the dansyl chromophore. Excitation spectra were obtained at optical densities lower than 0.02, corresponding to chromophore concentrations below 2×10^{-6} M. The excitation spectrum of an equimolar mixture of 2×10^{-6} M dansyl-L-prolyl hydrazide and 1-acetyl-4-(1-naphthyl) semicarbazide in ethanol was the same as that of dansyl-L-prolyl-hydrazide alone. This indicates that there was no energy transfer either by the trivial process of reabsorption of fluorescence emitted by the energy donor or by transfer between chromophores on different molecules. ORD measurements were obtained on a Cary model 60 recording spectropolarimeter. All spectra were taken in ethanol at $25° \pm 1°$, unless otherwise noted.

Results.—Conformation of the oligomers of poly-L-proline: The ultraviolet ORD of dansyl-(L-prolyl)$_8$-hydrazide in ethanol exhibits a large negative Cotton effect centered at 202 mμ. The reduced mean residue rotation at the trough at 215 mμ is $-29,000$ deg cm^2 decimole.$^{-1}$ This large negative Cotton effect was observed for each oligomer from $n = 5$ to 12, as well as for the corresponding α-naphthyl derivatives. An assignment of conformation can be made since the two helical forms of high molecular weight poly-L-proline are known to have contrasting optical rotatory spectra.[8, 9] The type I *cis* helix has a positive Cotton effect centered at 217 mμ,[10] while the type II *trans* helix shows a negative Cotton effect centered at 203 mμ.[11] *Thus, it is evident that the oligomers from $n = 5$ to 12 are in the type II trans helical conformation in ethanol.*
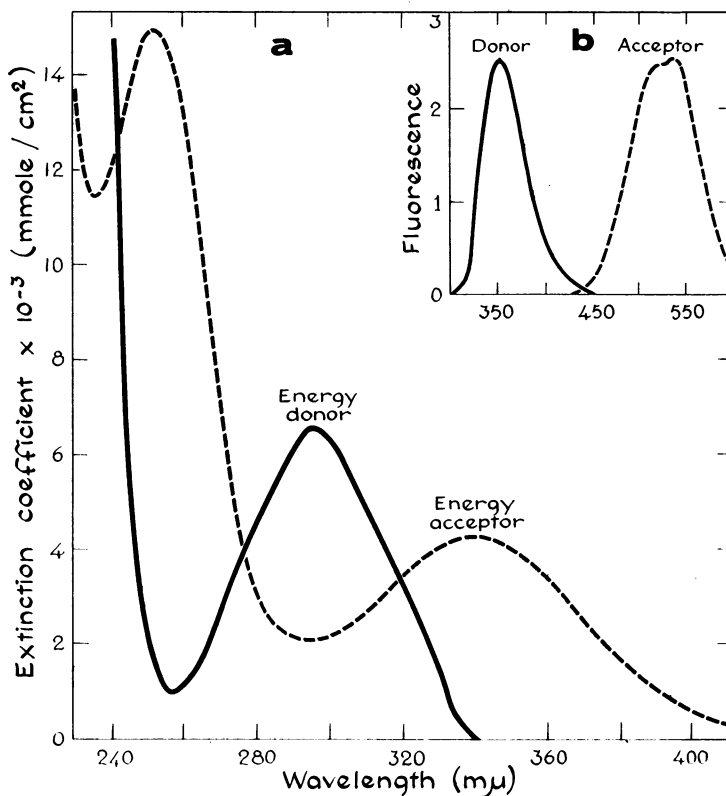

Fig. 2.—(a) Absorption spectrum of the energy donor (1-acetyl-4-(1-naphthyl) semicarbazide, ———) and the energy acceptor (dansyl-L-prolyl-hydrazide, - - - - -) in ethanol; (b) emission spectrum of the energy donor (———) and the energy acceptor (- - - - -) in ethanol.

Absorption and emission spectra of the energy donor and acceptor: The α-naphthyl energy donor and dansyl energy acceptor moieties meet several spectroscopic criteria important in a study of energy transfer: (*a*) their absorption spectra allow selective excitation of either chromophore (Fig. 2*a*); (*b*) their emission spectra are well separated (Fig. 2*b*); (*c*) the emission spectrum of the α-naphthyl group overlaps the absorption spectrum of the dansyl chromophore; (*d*) the energy donor has an appreciable quantum yield, 0.6 for the model compound 1-acetyl-4-(1-naphthyl) semicarbazide; and (*e*) the energy acceptor is sufficiently fluorescent so that the transfer can be readily detected.

Excitation spectra: The efficiency of energy transfer is readily obtained from the excitation spectrum of the energy acceptor.[12]   At a given wavelength, the magnitude of the excitation spectrum of the energy acceptor, A, is related to the transfer efficiency, E, the extinction coefficient of the energy donor, ϵ_D, and of the acceptor, ϵ_A, by the expression $A = \epsilon_A + E\epsilon_D$.   In the absence of energy transfer,
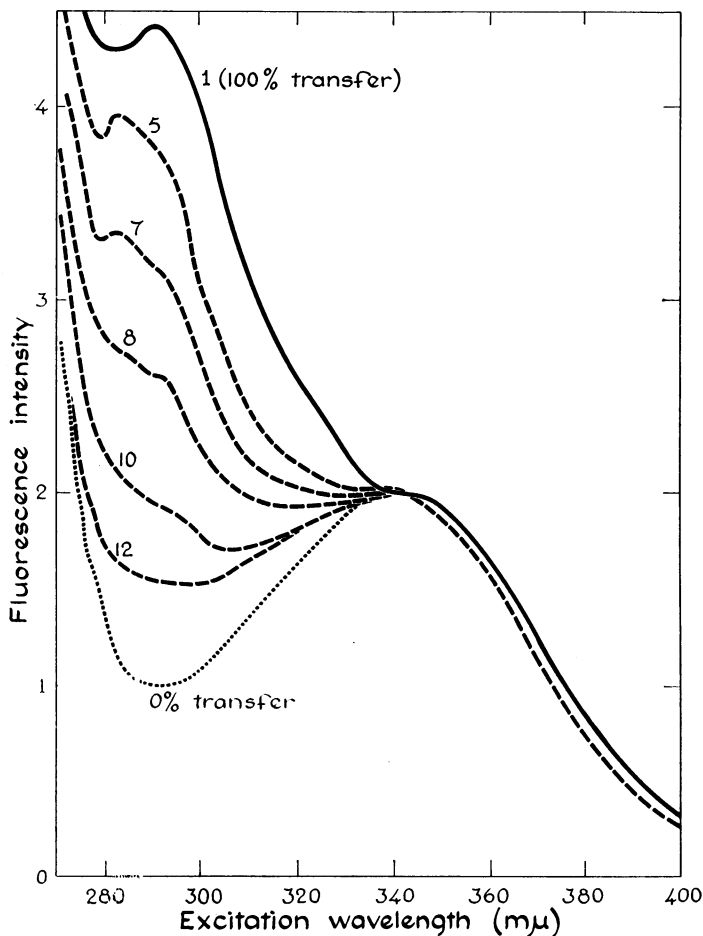

FIG. 3.—Excitation spectrum of dansyl-L-prolyl-hydrazide (· · · · ·, 0% transfer), dansyl-L-prolyl-α-naphthyl (——————, 100% transfer), and dansyl-(L-prolyl)$_n$-α-naphthyl (- - - - -, n = 5, 7, 8, 10, 12) in ethanol.

the excitation spectrum is identical to the absorption spectrum of the energy acceptor. For 100 per cent efficient transfer, the excitation spectrum corresponds to the sum of the absorption spectra of the two chromophores. These limiting cases are illustrated by the excitation spectra (Fig. 3) of dansyl-L-prolyl-hydrazide (0% transfer, donor absent) and dansyl-L-prolyl-α-naphthyl (100% transfer).

Efficiency of energy transfer as a function of distance: The excitation spectra of the dansyl-(L-prolyl)$_n$-α-naphthyl compounds, n = 1 to 12, were measured (Fig. 3). The magnitude of the excitation peak at 295 mμ provides the most sensitive index of the efficiency of transfer from the α-naphthyl to the dansyl chromophore. The transfer efficiency decreases from a value close to 100 per cent for the short oligomers to 16 per cent for the n = 12 oligomer. The distances between the attachment points of the chromophores were assumed to be defined by the Cowan-McGavin coordinates[13] for the poly-L-proline II *trans* helix. A recent theoretical study by Schimmel and Flory[14] shows that the mean square end-to-end distance of the n = 16 oligomer of *trans* poly-L-proline is 94 per cent of the value obtained from the helix coordinates. For shorter oligomers, their calculated agreement is even closer. The distance between the center of each chromophore and its point of attachment to the poly-L-proline oligomer was estimated from molecular models. The uncertainty in these estimated distances between the centers of chromophores is about 3 Å. The efficiency of energy transfer as a function of distance is given in Figure 4.

Relative orientation of the energy donor and acceptor: The mutual orientation of the donor-acceptor pair is important in dipole-dipole energy transfer. Fluorescence polarization studies were carried out to obtain information concerning the
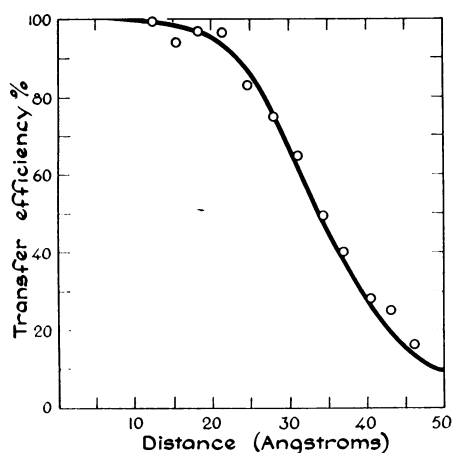

FIG. 4.—Efficiency of energy transfer as a function of distance in dansyl-(L-prolyl)$_n$-α-naphthyl, n = 1 to 12. The α-naphthyl and dansyl groups were separated by defined distances ranging from 12 to 46 Å. The energy transfer is 50% efficient at 34.6 Å. The solid line corresponds to an r^{-6} distance dependence.
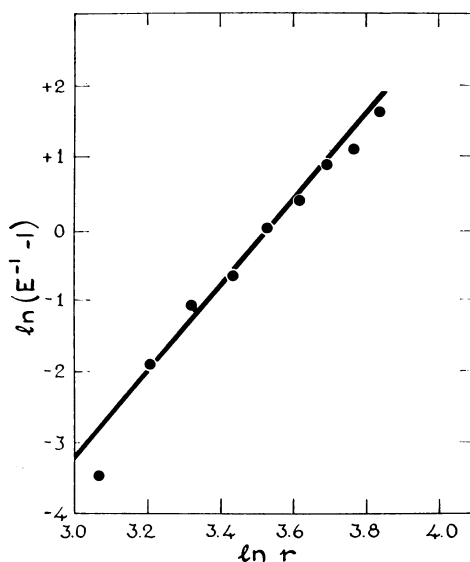
FIG. 5.—The dependence of the efficiency of energy transfer on distance is given by the slope in this plot of ln $(E^{-1} - 1)$ versus ln r. The slope is 5.9, in excellent agreement with the r^{-6} dependence predicted by Förster.

angular relationship of the donor and acceptor. The rotational relaxation time of the dansyl chromophore in dansyl-(L-prolyl)$_{10}$-hydrazide is only 1.8-fold greater than that of dansyl-L-prolyl-hydrazide, under the conditions of the energy transfer measurements. If the dansyl group rotated in common with the poly-prolyl portion, the ratio of the rotational relaxation times would be between 4 and 12, depending on the orientation of the chromophore relative to the helix axis, The low observed ratio indicates that the dansyl group has rotational mobility independent of the poly-L-proline helix. Additional support for the absence of a preferred orientation comes from fluorescence polarization measurements of dansyl-L-prolyl-α-naphthyl in a rigid propylene glycol glass. The sensitized dansyl fluorescence excited between 280 and 300 mμ was completely depolarized, suggesting that there are numerous relative orientations of the donor-acceptor pair. These fluorescence polarization measurements indicate that *the angular relationship between the dansyl and α-naphthyl transition moments is substantially randomized during the excited state lifetime.*

Discussion.—The efficiency of energy transfer as a function of distance is given by $E = (R_0/r)^j / [(R_0/r)^j + 1]$, where R_0 is the distance corresponding to 50 per cent transfer and j is the exponent of the distance dependence. R_0 and j can be obtained readily from the experimental data by plotting log $(E^{-1} - 1)$ versus log r. The slope of the line is j, while R_0 is given by the value of r at $E = 0.5$. A linear relationship is in fact found when the experimental data are plotted in this way (Fig. 5). A least-squares fit to a straight line gives $j = 5.9 \pm 0.3$. *Thus, the experimentally observed distance dependence of the transfer efficiency is in excellent agreement with the r^{-6} dependence predicted by Förster.*

It is also of interest to compare the observed R_0 distance with the value (in angstroms) calculated from Förster's equation,[15] $R_0 = 9.79 \times 10^3 (K^2 QJn^{-4})^{1/6}$ where K is the dipole-dipole orientation factor, Q is the quantum yield of the energy donor in the absence of transfer, J is the spectral overlap integral, and n is the refractive index of the solvent. For the α-naphthyl-dansyl pair, $Q = 0.6$, $J = 4.4 \times 10^{-15}$ cm^6/mmole, and $n = 1.4$. K^2 is assumed to be $^2/_3$ since the fluorescence polarization measurements indicate that the angular relationship between the dansyl and α-naphthyl group is substantially randomized during the excited state lifetime. *For these parameters, R_0 is calculated to be 27.2 Å, while the observed value is 34.6 Å* (Figs. 4 and 5). A rigorous comparison of the observed and calculated R_0 distances should be deferred until the value of K^2 is better defined. It would also be desirable to have independent confirmation of the estimated distances between the energy donor and acceptor groups.

The work of Latt, Cheung, and Blout[2] on energy transfer between chromophores separated by a rigid steroid and the present study provide unequivocal evidence that electronic excitation energy can be transferred over distances of the order of 30 Å. Since the transfer has been shown here to depend on the inverse sixth power of the distance between the chromophores, it seems likely that *the energy transfer process can serve as a spectroscopic ruler.* A particularly interesting possibility is that energy donor-acceptor pairs might be used to reveal proximity relationships in biological macromolecules. However, a number of conditions would have to be fulfilled for the method to be of value in complex systems. Application of the method would depend on (a) the prior calibration of a series of donor-acceptor

pairs; (*b*) the selective attachment of a single donor and a single acceptor to the macromolecule; and (*c*) the availability of information concerning the relative orientation of the donor-acceptor pair. It is possible to suggest some experimental approaches for meeting these requirements. The R_0 values of donor-acceptor pairs could be varied by altering the quantum yield of the donor or the absorption spectrum of the acceptor. A series of donor-acceptor pairs with R_0 values between 15 and 45 Å would be useful in measuring distances between about 10 and 60 Å. The need for a single donor and a single acceptor poses a considerable challenge. We have synthesized a number of specific fluorescent labeling reagents which may prove useful in this regard. One of them specifically labels the active site of α-chymotrypsin, with a fluorescent anthraniloyl group,[16] while another reagent preferentially labels sulfhydryl groups in proteins. Some information concerning the relative orientation of the donor-acceptor pair is likely to be obtained from an analysis of their fluorescence polarization spectra. The use of alternative energy acceptors with different geometrical modes of attachment to the same side-chain of the protein might be another way of determining whether a low transfer efficiency is due to a long distance or an unfavorable orientation.

Summary.—The efficiency of electronic excitation energy transfer was measured as a function of distance. An α-naphthyl energy donor group and a dansyl energy acceptor group were attached to the ends of oligomers of poly-L-proline ($n = 1$ to 12), which served as spacers of defined length ranging from 12 to 46 Å. The oligomers from $n = 5$ to 12 were shown by optical rotatory dispersion to be in the type II *trans* helical conformation. The efficiency of energy transfer decreased from 100 per cent at a distance of 12 Å to 16 per cent at 46 Å. The transfer was 50 per cent efficient at 34.6 Å. The dependence of the transfer efficiency on distance is in excellent agreement with the r^{-6} dependence predicted by Förster for weak dipole-dipole coupling. These results suggest that under suitable conditions the energy transfer process can serve as a spectroscopic ruler in the 10-to-60-Å range.

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[5] Abbreviations: dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; n, degree of polymerization; ORD, optical rotatory dispersion; R_0, distance corresponding to 50% efficient energy transfer; t-BOC, tert-butyloxycarbonyl.

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