Intramolecular Transfer of Excitation from Tryptophan to 1-Dimethylaminonaphthalene-5-sulfonamide in a Series of Model Compounds*

Richard H. Conrad† and Ludwig Brand

ABSTRACT: The Förster theory of resonance energy transfer predicts that the rate of transfer of excited state energy between chromophores should be extremely sensitive to interchromophoric distances. The present study was undertaken in order to explore the potential of energy transfer measurement as a means of calculating distances between fluorescent residues on polypeptides and proteins in solution. For the purpose of obtaining a flexible model system in which the transfer of excitation could be studied, 1-dimethylaminonaphthalene-5-sulfonyl chloride has been coupled to the amino end of a homologous series of peptides with the structure $NH_2(CH_2)_nC(=O)$-L-Trp, where n ranges from 1 to 10. The efficiency of intramolecular excitation transfer from tryptophan to the 1-dimethylaminonaphthalene-5-sulfonamido group in each model has been measured in water, glycerol, ethanol, and *p*-dioxane. The applicability of Förster's theory to the transfer data is discussed, and intramolecular distances are calculated by means of the Förster equation. These distances are found to increase consistently with the number of methylenes in the hydrocarbon chain separating the chromophores. In each case the distance calculated is less than the theoretical distance of maximum extension, and is within range of the average separation expected for that model. Whereas the calculated interchromophoric separations in the shorter models are practically independent of solvent,in the longer models they increase significantly with decreasing solvent polarity. This may be indicative of a hydrophobic interaction within the methylene chain.

Electronic excitation energy may be transferred from a donor to an acceptor chromophore through an induced resonance interaction if the emission spectrum of the donor overlaps the absorption spectrum of the acceptor. This transfer can occur without direct contact between the chromophores and in the absence of the emission and reabsorption of quanta. The effect of such a transfer is to decrease the fluorescence of the donor and to increase the fluorescence of the acceptor. A theoretical basis for the resonance transfer of energy was first derived by Perrin (1925) and was carried further by Förster (1948, 1951). The rate of this tranfer was predicted to be inversely proportional to the sixth power of the distance between the interacting chromophores, and thus very sensitive to this distance. Förster's results have been confirmed theoretically by Dexter (1953), Galanin (1955), and Rozman (1958). By application of Förster's theory to experimental resonance transfer data, Bowen and Livingston (1954) and Latt *et al.* (1965) have obtained values of interchromophoric distance which closely approximate known distances. Weber and Teale (1959), Bennett (1964), Ellis and Solomon (1967), Stryer and Haugland (1967), and others (see Förster, 1959) have also provided experimental evidence which supports Förster's theory. This theory is of potential importance to protein studies since it makes feasible the calculation of distances and changes in distance between chromophoric residues on proteins in solution.

Shore and Pardee (1956) and Stryer (1959) have reported a transfer of energy from tryptophan to DNS[1] in DNS-protein conjugates. Weber (1961) measured the transfer of excitation from tryptophan to DNS in DNS-BSA conjugates, and calculated an average interchromophoric distance from Förster's theory. He found that this distance increased in urea, indicating an expansion of the protein. In the work described here sensitized fluorescence is used to measure intramolecular energy transfer from tryptophan to DNS in a homologous series of model compounds of the structure $DNS\text{-}NH(CH_2)_nC(=O)$-Trp. These compounds are flexible and might be expected to undergo solvent induced conformational changes. Spectroscopic evidence indicates that Förster-type calculations are appropriate for this system. The use of Förster's theory to estimate intramolecular distances appears to provide direct evidence for structural changes.

* From the Department of Biology and the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218. *Received May 22, 1967.* Contribution 521 from the McCollum-Pratt Institute. Supported by a Shell fellowship to R. H. C. and by U. S. Public Health Service Grants GM 11632 and 5T01-GM-57-09. A preliminary report of these investigations was presented at the 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1965.

† The material presented here was taken in part from a thesis to be submitted to the Graduate School of the Johns Hopkins University by R. H. C. in partial fulfillment of the Ph.D. degree in Biology.

[1] Abbreviations used: DNS, the 1-dimethylaminonaphthalene-5-sulfonate moiety; BSA, bovine serum albumin.

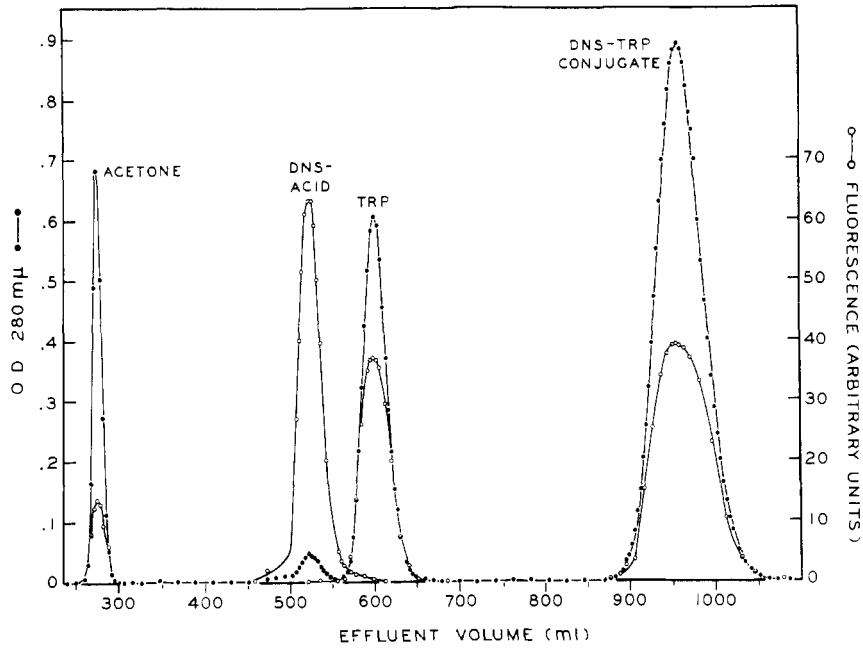
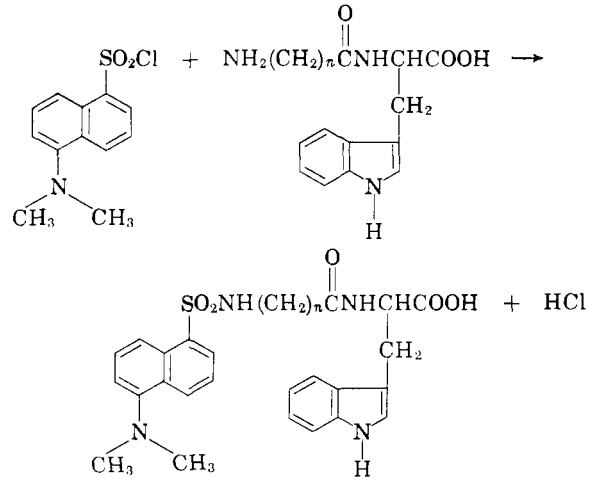


FIGURE 1: Elution of a DNS-Trp reaction mixture from a 2.5 × 40 cm column of fine Sephadex G-25. The column was equilibrated and eluted with 0.1 N sodium phosphate buffer (pH 7.4).

Experimental Section

Synthesis of Model Compounds. A homologous series of compounds with the structure $NH_2(CH_2)_nC(=O)$-L-Trp, where n equals 1, 2, 3, 4, 5, 6, 8, and 10, had been prepared by Edelhoch *et al.* (1967). For the present investigation, DNS was conjugated to each of the eight "parent" tryptophanyl compounds above and to Gly-Trp, and Gly-Gly-Trp through the reaction



All reagents used were analytical grade, and all solvents were of spectral quality. Parent compound (0.01 mmole) was dissolved in 1.0 ml of 0.2 N sodium carbonate–bicarbonate buffer at pH 10.0. The compounds where n equaled 6, 8, and 10 required a few drops of *N,N*-dimethylformamide for complete solvation. About 0.02 mmole of DNS Cl (Mann Research Laboratories) was dissolved in 0.3 ml of *N,N*-dimethylformamide or acetone. As this DNS-Cl solution was added to the buffered solution of parent compound, the DNS-Cl came out of solution as a very fine yellow precipitate. After 24-hr stirring at room temperature, all of the DNS-Cl either had hydrolyzed to DNS-acid, or had reacted with the free amino group of the parent compound to form the DNS conjugate.

Purification of Model Compounds. Each DNS conjugate was isolated from the other reaction components by chromatography on a 2.5 × 40 cm column of G-25 Sephadex equilibrated and eluted with either 0.1 N sodium phosphate buffer (pH 7.4) or 0.5 N NH_4HCO_3. A typical elution pattern is shown in Figure 1. Since the molecular weights of the reaction components were well below the exclusion limit of G-25 Sephadex, this separation was effected by a differential absorption mechanism, possibly dependent on charge and aromaticity. The conjugates were purified further on DEAE Sephadex. The volume of the DNS conjugate eluate fraction was decreased on a rotary evaporator at 30° under reduced pressure. The conjugate, or model compound, was extracted from the buffer salts with absolute ethanol (in the case of the NH_4HCO_3 buffer, most of the NH_4HCO_3 had been volatilized by repeatedly dissolving in water and evaporating to dryness). Yields were on the order of 50%.

In addition to running as single bands on G-25 and DEAE Sephadex, each of the model compounds traveled as a single spot during thin-layer chromatography on Eastman silica gel chromagram sheets in absolute ethanol and in 1-butanol saturated with 3 N acetic acid. In these developers, the parent compounds, DNS-acid,

and DNS-amide had R_F values different from those of the conjugates. Additional proof of purity is that the tryptophan fluorescence of the conjugates was not enhanced in the region of pH 9–10 (indicating the absence of free tryptophan amino groups), and that the spectra of each model were consistent with the expected structure.

Methods of Spectrophotometric Measurement. All measurements were carried out in 1-cm, square quartz cuvets, and at 23° unless otherwise noted. The water, glycerol, and ethanol solutions were equilibrated with air. Since the presence of oxygen was found to cause a large (but reversible) increase in the optical density at 280 mμ of *p*-dioxane, solutions in this solvent were equilibrated with nitrogen in stoppered cuvets. The glycerol and *p*-dioxane were Matheson Coleman and Bell, Spectroquality, and the ethanol was USP absolute.

Absorption spectra were measured on a Cary 14 spectrophotometer. Errors due to sample fluorescence were insignificant. Fluorescence measurements were carried out on an Aminco-Keirs spectrophosphorimeter which utilized a 150-w high-pressure xenon light source and a RCA 1P28 photomultiplier. All excitation band widths were 3 mμ. An aqueous solution of $NaNO_2$ was used to filter out scattered exciting light when measuring DNS fluorescence. The optical density of each sample at all wavelengths of measurement was low enough to render inner filter and reabsorption effects negligible. None of the samples exhibited any time-dependent spectral changes while under observation.

Correction of Fluorescence Spectra. All fluorescence values and spectra given in this paper are corrected. Curves describing the change in the number of exciting quanta with wavelength were obtained from a 1-amino-napththalene-3,6,8-trisulfonate quantum counter (Teale and Weber, 1957), and by comparing the fluorescence excitation spectra of tryptophan and of DNS-glycine in water with their respective absorption spectra.[2] The variation of the emission monochromator transmittance and photomultiplier sensitivity with wavelength was determined by applying the excitation correction calculated above to a spectrum recorded by reflecting the exciting light directly into the emission monochromator. In order to correct for the effect of refractive index on fluorescence intensity, the intensities were multiplied by the square of the index of refraction of the solvent (Hermans and Levinson, 1951). Therefore all fluorescence values obtained have been reduced to relative numbers of quanta per second per constant wavelength interval.

Calculations. If all quenching processes are assumed to be competitive, then a transfer efficiency (T) may be defined as

$$T = \frac{k_T}{k_F + k_T + k_Q} \tag{1}$$

where k_F, k_T, and k_Q represent the rate constants of the donor fluorescence, of the observed transfer from donor to acceptor, and of the sum of all other quenching processes, respectively. The rate constant of transfer through a very weakly coupled resonance interaction can be expressed in terms of the Förster equation (Förster, 1965)

$$k_T = \frac{9(\ln 10)K^2}{128\pi^5 N n^4 \tau R^6} \int F_{D(\lambda)} \epsilon_{A(\lambda)} \lambda^4 d\lambda \tag{2}$$

where K = dipole–dipole orientation factor, N = Avogadro's number (mole^{-1}), n = refractive index of medium, τ = natural lifetime of donor, and R = distance between donor and acceptor (centimeters). $\int F_{D(\lambda)} \epsilon_{A(\lambda)} \lambda^4 d\lambda$ = overlap integral (cm^6 mole^{-1}). $F_D(\lambda)$ describes the probablity of donor emission as a function of wavelength where the total probability of emission, $\int F_{D(\lambda)} d\lambda$, equals 1 and is dimensionless. $\epsilon_{A(\lambda)}$ is the molar decadic extinction spectrum of the acceptor (cm^2 mole^{-1}).

The numerical value of the overlap integral is calculated as follows. The relative intensity axis of the donor's emission spectrum is given the units of cm^{-1} and is multiplied by a constant such that the area under the spectrum is equal to 1. The value of F_D at each λ is then multiplied by the molar extinction of the acceptor at that λ and by the fourth power of that λ. These products are plotted against λ, and the area under the resulting curve is the overlap integral.

The rate constant for transfer may be expressed in terms of experimental parameters through the equation

$$k_T = \frac{T}{\phi_D \tau} \tag{3}$$

where $\phi_D = k_F/(k_F + k_T + k_Q)$ = the donor's fluorescence efficiency in the presence of transfer, and $\tau = 1/k_F$. Substituting eq 3 into eq 2 and solving for R in centimeters

$$R = [(8.77 \times 10^{-28} \text{ mole})(\text{overlap integral})(K^2/n^4)(\phi_D/T)]^{1/6} \tag{4}$$

T, the efficiency of transfer from donor to acceptor, is equal to the efficiency with which a quantum absorbed by the donor is emitted by the acceptor, divided by the fluorescence efficiency of the acceptor. In the model compounds described here the donor is tryptophan, whose absorption maximum is at 280 mμ, and the acceptor is DNS. Tryptophan does not absorb at the long-wavelength absorption maximum of DNS (about 335 mμ) nor does it emit near the DNS emission maximum. The DNS moieties in the models have the absorption spectra of DNS-glycine, and therefore at low optical densities

$$T = \frac{(\text{DNS fluorescence of model excited at 280/DNS fluorescence of model excited at 335}) - X}{(\text{optical density of model at 280/optical density of model at 335}) - X} \tag{5}$$

[2] Weber and Teale (1958) found that in water, the corrected excitation spectra of tryptophan and of the sodium salt of DNS-acid are identical with their respective absorption spectra.

where X = optical density of DNS-Gly at 280/optical density of DNS-Gly at 335.

ϕ_D is the tryptophan fluorescence efficiency or ϕ_{Trp}, and was obtained by comparing the relative tryptophan emission of each model compound[3] to that of pure tryptophan, for which an absolute efficiency in water of 0.20 was assumed (Teale and Weber, 1957).

Results

Evidence for Energy Transfer. The absorption and fluorescence excitation spectra in ethanol of DNS-glycine and of a representative model compound (DNS-$NH(CH_2)_6C(=O)$-Trp) are shown in Figure 2a. Emission was observed at 514 mμ, which is near the DNS maximum. The spectra are normalized at 335 mμ. Curve 4 of Figure 2b is the difference between the absorption of the model (curve 1) and of DNS-glycine (curve 3). This difference spectrum is identical with the absorption spectrum of the parent tryptophanyl compound.

There is appreciable overlap between the emission of tryptophan and the absorption of DNS. Energy is transferred between these chromophores, as is shown by the fact that the excitation spectrum of DNS fluorescence (curve 2) is not equal to the excitation spectrum of DNS-glycine (curve 3). The excitation difference spectrum (curve 5) is exactly superimposable with the excitation spectrum of the model's tryptophan fluorescence. Curve 4 corresponds to the excitation difference spectrum to be expected if transfer were 100% efficient. The transfer efficiency at a particular wavelength is equal to the ratio of the heights of curves 5 and 4 at that wavelength, as expressed by eq 5.

The spectral characteristics of the other models[4] in all solvents used are very similar to those of the example discussed above. The transfer efficiencies calculated through eq 5 for each model compound in water, glycerol, ethanol, and *p*-dioxane are presented in Table I.

Fluorescence Efficiencies. The tryptophan fluorescence efficiencies in the presence of transfer (ϕ_{Trp}) are shown in Table I. In order to calculate what ϕ_{Trp} would have been in the absence of transfer, we define

$$\phi_{Trp(k_T=0)} = \frac{k_F}{k_F + k_Q} = \frac{\phi_{Trp}}{1 - T} \qquad (6)$$

The values of ϕ_{Trp} for the parent tryptophanyl compounds and the values of $\phi_{Trp(k_T=0)}$ for the models are given in Table II. The efficiencies of tryptophan flourescence are lower in the models than in the parent compounds. In both the parent and the model series, the efficiencies increase as the length of the methylene chain is increased and as the polarity of the solvent is decreased. The tryptophan fluorescence efficiencies of the models and the transfer efficiencies are independent of oxygen concentration, of temperature from 0 to 40°, and of pH over a range of 5–11.

Mechanism of Transfer. A variety of processes could be involved in the transfer of energy described here. These include radiative or "trivial transfer," ground-state complexing, excimer formation, collision between excited and ground state with transfer but no complex formation, and the nonradiative induced resonance transfer known as Förster transfer. The latter mechanism is used here to calculate interchromophoric distances within each model compound.

Justification for the use of Förster theory is as follows. Radiative transfer could not contribute significantly. The efficiency of intramolecular radiative transfer is equal to $\phi_{Trp(k_T=0)}$ times the probability that a quanta emittted by a tryptophan will be absorbed by the DNS on the same molecule. The latter probability is equal to the average effective cross-sectional area for complete absorption per molecule of DNS (which is equivalent to 3.8×10^{-21} times the molar extinction coefficient (Setlow and Pollard, 1962)) divided by the area of the surface of a sphere whose radius is equal to the distance between chromophores. Using the largest $\phi_{Trp(k_T=0)}$ calculated for any of the models (0.1) and the minimum distance (6 Å), the maximum possible efficiency of intramolecular radiative transfer is calculated to be about 10^{-4}. The greatest possible efficiency of intermolecular radiative transfer is $\phi_{Trp(k_T=0)}$ times the largest fractional absorption of DNS, or (0.1)(0.08) = 0.008. In an aqueous solution which is 1 mM in tryptophan and 1 mM in DNS-glycine, the transfer efficiency is below the limit of detection. Thus intra- and intermolecular radiative transfer together can account for a transfer efficiency of no more than 0.01, whereas the transfer efficiencies observed (Table I) range from 0.35 to 0.79. In conformity with these calculations is the fact that the tryptophan emission spectra of the models are identical with those of the parent tryptophanyl compounds. In water, where the tryptophan emission and DNS absorption spectra overlap but do not coincide, radiative transfer would have distorted (see Förster, 1959) the tryptophan emission spectra of the models.

It is unlikely that intermolecular complexing between the chromophores could have been occurring because the fluorescence and transfer efficiencies were independent of concentration in the region of the sample concentration (about 10^{-5} M). Intramolecular complexing between the chromophores in the ground or excited state would have been expected to produce a noticeable spectral change (Rabinowitch and Epstein, 1941; Förster and König, 1957; Birks and Christophorou, 1962; Kasha, 1963). All absorption and fluorescence spectra of the

[3] DNS itself produces some fluorescence in the tryptophan emission region when excited at 280 mμ. This may be due to the presence of a very small fraction of protonated DNS (Lagunoff and Ottolenghi, 1965). The amount of this fluorescence was approximated by measurement of the emission in the region of tryptophan fluorescence of a solution of DNS-glycine whose concentration had been adjusted to give the same intensity of visible DNS fluorescence as the model. This background, which included scattered exciting light, was subtracted from each model's tryptophan emission. The background constituted a significant percentage of the total tryptophan fluorescence only in DNS-Trp in water, glycerol, and ethanol, and in DNS-Gly-Trp in glycerol.

[4] The shape of the tryptophan fluorescence excitation and emission spectra could not be determined accurately in the models DNS-Trp and DNS-Gly-Trp, where the fluorescence yield was very low.

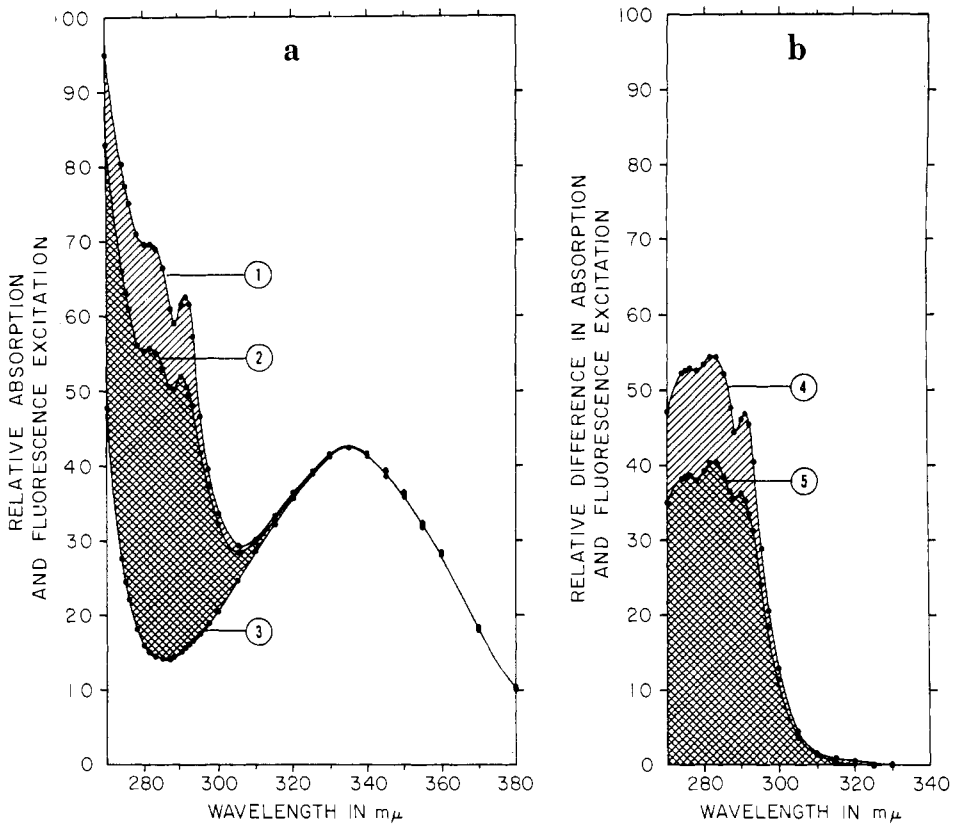


FIGURE 2: (a) DNS-NH$(CH_2)_6$C(=O)-Trp absorption spectrum (curve 1) and fluorescence excitation spectrum (curve 2), DNS-Gly absorption and fluorescence excitation spectra (curve 3). Emission is measured at 514 mμ and the spectra are corrected and are normalized at 335 mμ. (b) Curve 4 is the absorption difference between curves 1 and 3 of a, and curve 5 is the fluorescence excitation difference between curves 2 and 3 of a. The solvent is absolute ethanol.

models were the sum of the spectra of the component chromophores (with the exception of the specific transfer band) to the extent that shifts in extinction maxima of 2 mμ or greater, shifts in excitation and emission maxima of 3 mμ or greater, and changes larger than 3% in relative extinction or band width would have been observable. Absorption spectra of DNS-Gly, DNS-Trp, and DNS-NH$(CH_2)_6$C(=O)-Trp were measured from 700 to 400 mμ in water, glycerol, and ethanol at concentrations such that a new absorption band would have been noticeable if it had an extinction coefficient of greater than 40 (1/mole cm) in water, 20 in glycerol, or 10 in ethanol. Thus there is no spectral evidence for ground- or excited-state complexing between the tryptophan and DNS moieties.

Additional evidence against ground-state complexing is provided by an estimation of the effective rotational volume of DNS in each model compound by means of fluorescence depolarization measurements. Weber (1966) has utilized the Perrin equation, which relates depolarization of fluorescence to the Brownian rotation of a sphere, to detect small differences in the volumes of various flavine derivatives. A similar method was employed here to calculate effective rotational volumes of DNS in glycerol. Fluorescence was excited with polarized light at 355 mμ and the polarization of the emission at 525 mμ was measured with a ratio fluorometer (to be described). P_0 per cent for DNS-BSA was found to be 0.43 ± 0.01 in agreement with Weber (1952). DNS lifetimes were measured with a TRW instrument (Chen *et al.*, 1967) through the kindness of Dr. R. Chen. The results for each model are indicated in Table III.

The effective rotational volume of the DNS moiety of DNS-glycine is very close to the unsolvated van der Waals' volume of DNS-glycine, 123 cm^3/mole. In each solvent the DNS fluorescence efficiencies of the models are equal to or greater than the efficiency of DNS-glycine. It therefore seems that if significant amounts of complexing had been occurring, the complexed DNS moieties would still have been fluorescent and would have contributed to the measured polarization. Thus large amounts of intramolecular complexing would have produced values of rotational volume which were at least twice the volume of DNS-glycine. As can be seen from Table III, this was not found. The volumes calculated for the shorter models and for DNS-Gly-Gly-Trp are somewhat larger than the value for DNS-glycine. This is expected since it is most reasonable to assume that even in the absence of complexing the DNS moiety does not rotate completely independently of the rest of the molecule. Linkage to the tryptophan chromphore and the accompanying rotational restriction on DNS *should* be

TABLE I: Transfer Efficiencies and Tryptophan Fluorescence Efficiencies in the Presence of Transfer.[a]

	in Water		in Glycerol		in Ethanol		in *p*-Dioxane	
Compd n[b]	T	$\phi_{Trp} \times 10^3$	T	$\phi_{Trp} \times 10^3$	T	$\phi_{Trp} \times 10^3$	T	$\phi_{Trp} \times 10^3$
1	0.35	0.8	0.62	(1.9)	0.55	1.4	0.68	1.0
2	0.39	2.0	0.72	7.8	0.63	2.0	0.62	3.8
3	0.42	2.4	0.73	11.5	0.71	2.5	0.66	2.5
4	0.40	2.7	0.73	8.9	0.69	4.3	0.66	6.9
5	0.42	1.9	0.75	6.7	0.74	3.7	0.65	6.7
6	0.43	3.1	0.68	12.2	0.74	7.7	0.63	13.7
8	0.50	3.8	0.74	17.6	0.79	13.0	0.63	18.9
10	0.63	6.5	0.76	30.6	0.72	16.3	0.65	34.1
DNS-Trp	0.53	(0.1)	0.83	(2.3)	0.54	(0.2)	0.69	0.8
DNS-Gly-Gly-Trp	0.45	2.9	0.71	9.1	0.60	3.7	0.63	10.3

[a] The values given in parentheses are of greater uncertainty[3] than the others. [b] n = number of methylenes in DNS-$NH(CH_2)_nC(=O)$-Trp.

TABLE II: Tryptophan Fluorescence Efficiencies in the Absence of Transfer, $\phi_{Trp(k_T=0)}$.[a]

	in Water		in Ethanol		in Glycerol	in *p*-Dioxane
Compd n[b]	Parent	Model	Parent	Model	Model	Model
1	0.060	0.001	0.305	0.003	(0.005)	0.003
2	0.100	0.003	0.274	0.005	0.028	0.010
3	0.218	0.004	0.281	0.009	0.042	0.008
4	0.246	0.004	0.315	0.014	0.033	0.020
5	0.353	0.003	0.400	0.014	0.027	0.019
6	0.390	0.006	0.392	0.029	0.038	0.037
8	0.445	0.008	0.480	0.063	0.068	0.052
10	0.464	0.017	0.540	0.057	0.127	0.098
DNS-Trp	0.200	(0.0003)	0.205	(0.0004)	(0.014)	0.003
DNS-Gly-Gly-Trp	0.076	(0.005)	0.355	0.009	0.031	0.028

[a] Values of $\phi_{Trp(k_T=0)}$ for the models were calculated with eq 6 from the T and ϕ_{Trp} values listed in Table I. [b] n in $NH_2(CH_2)_n$-C(=O)-Trp (parent compounds) and in DNS-$NH(CH_2)_nC(=O)$-Trp (model compounds).

greatest in the models where the flexibility of the chain between the chromophores is least.

In glycerol the mean free path of diffusional translation of each chromophore during the excited-state lifetime is calculated to be less than 0.5 Å. Since the transfer efficiencies are not decreased in glycerol, collision during the excited-state lifetime, with or without complex formation, cannot be contributing to the observed transfer. As indicated in Table II, the values of $\phi_{Trp(k_T=0)}$ for the models are considerably lower than the values of ϕ_{Trp} for the parent tryptophanyl compounds. The mechanisms responsible for the quenching are not clear at this time. Ground-state interchromophore complexing does not appear to play a major role for the reasons mentioned above. If excited-state complexing between the chromophores had been causing appreciable quenching in the nonviscous solvents, then the models' tryptophan fluorescence efficiencies in glycerol would have been similar to those of the parent compounds. Such correspondence is not observed. The values of $\phi_{Trp(k_T=0)}$ in glycerol do not exceed the values found in the other solvents by more than to be expected due to a decrease in the rate of collisional quenching by the solvent.

Thus radiative or contact mechanisms cannot account for the excitation transfer observed. A conduction

TABLE III: Effective Rotational Volumes of DNS.

Compd n[a]	% Polarizn in Glycerol[b]	Lifetime Measured in Glycerol (nsec)[c]	Calcd Vol (cm^3/mole)
DNS-Gly	0.322	11.7	122
DNS-Trp	0.341	12.0	161
1	0.351	10.9	186
2	0.336	12.7	159
3	0.335	13.9	169
4	0.327	13.5	150
5	0.322	13.9	144
6	0.318	13.4	133
8	0.314	14.4	135
10	0.311	14.5	132
DNS-Gly-Gly-Trp	0.354	11.2	183

[a] n in DNS-NH$(CH_2)_n$C(=O)-Trp. [b] Where RT/η, from eq 8-17 of Weber (1966, p 230) is equal to 4.00×10^9. [c] ±0.7 nsec. These lifetimes were linearly proportional to the DNS fluorescence efficiencies in glycerol.

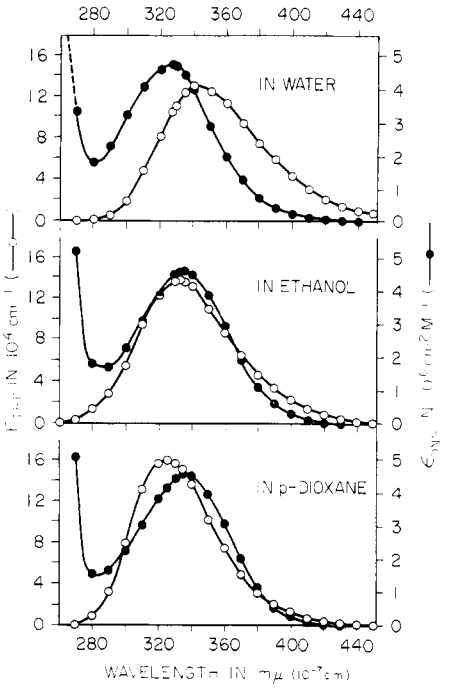


FIGURE 3: Tryptophan emission spectra (—○—) and DNS-Gly extinction spectra (—●—) in water, ethanol, and *p*-dioxane. The emission spectra are excited at 280 mμ, are corrected, and are normalized so that the area under them, $\int F_{Trp(\lambda)}d\lambda$, is equal to 1.

through the saturated methylene chain seems very unlikely. In each model the increase in the excitation spectrum of DNS fluorescence corresponds exactly to the excitation of the tryptophan moiety. The transfer rate constants are independent (●3%) of exciting wavelength from 270 to 293 mμ, the range over which accurate measurements could be made. A transfer from DNS to tryptophan is not observed; a transfer in this direction could not occur at slow rates of transfer since the emission spectrum of DNS does not overlap the absorption of tryptophan. Rate constants of transfer were calculated for the models through eq 3 from the vaues of T and ϕ_{Trp} listed in Table I and a τ_{Trp} of 13 nsec. (This value of τ_{Trp} was computed by dividing the lifetime of tryptophan in water measured by Chen *et al.* (1967), 2.6 nsec, by 0.20, the ϕ determined for tryptophan in water by Teale and Weber, 1957.) The rate constants vary between 1.5×10^9 and 6.3×10^{10} sec^{-1}, and are thus within the range specified by Förster (1960, 1965) for resonance transfer with very weak coupling. The Förster overlap integral for transfer from the first excited singlet of tryptophan to the ground singlet of DNS is of sufficient magnitude to account for these rate constants (triplet → singlet overlap is negligible). Thus the data presented here statisty the criteria for a Förster-type transfer of excitation energy from a tryptophan to a DNS on the same molecule.

TABLE IV: Parameters for Eq 4.

Solvent	Overlap Integral (in cm^6 m^{-1})	K^2/n^4[a]
Water	3.40×10^{-12}	0.200
Glycerol	4.48×10^{-12}	0.089
Ethanol	4.01×10^{-12}	0.185
p-Dioxane	4.18×10^{-12}	0.154

[a] $K^2 = 2/3$. The values of n used are those at the mean overlap wavelength in each case, and were obtained from values given in the International Critical Tables (1930) and Fasman (1963).

Calculation of Distances from Förster's Theory. In each solvent the model compounds have a tryptophan emission spectrum of pure tryptophan and a DNS absorption spectrum of DNS-glycine. The spectra in water, ethanol, and *p*-dioxane are shown in Figure 3. These spectra and similar spectra in glycerol were used to compute the overlap integrals listed in Table IV by means of the method decribed under Experimental Section. K^2 was set equal to two-thirds, the value for random orientations. The values of interchromophoric distance (R) for each model in the various solvents were calculated by means of eq 4 from the values of T and ϕ_{Trp} in Table I and the parameters in Table IV. The results are presented in Table V which also indicates the center-to-center interchromophoric distances of

TABLE V: Interchromophoric Distances (in Å).

Compd n[a]	R (from eq 4) in Water	Glycerol	Ethanol	p-Dioxane	Coulson–Pauling–Koltun Max R[b]
1	10.4	(10.2)	10.9	9.7	12.7
2	12.0	12.5	11.2	12.3	13.9
3	12.2	13.3	11.5	11.4	15.2
4	12.6	12.7	12.6	13.5	16.4
5	11.8	12.1	12.1	13.4	17.7
6	12.7	13.6	13.7	15.2	19.0
8	12.8	14.2	14.8	16.0	21.5
10	13.5	15.5	15.7	17.6	24.0
DNS-Trp	(7.2)	(9.9)	(7.9)	9.4	10.0
DNS-Gly-Gly-Trp	12.5	12.8	12.6	14.5	17.0

[a] n in DNS-NH$(CH_2)_n$C(=O)-Trp. [b] These distances are the maximum possible center-to-center interchromophoric distances (±0.3 Å) as measured on fully extended Coulson–Pauling–Koltun atomic models.

maximum extension as measured on Coulson–Pauling–Koltun atomic models (Ealing Corp., Cambridge, Mass.). The transfer efficiencies remain within the accurately measurable range of 0.35–0.79 for the values of R encountered because the tryptophan fluorescence efficiencies increase with increasing number of methylene groups.

Upper limits of the possible error in each parameter were used to calculate the following standard errors: T, ±12%; overlap integral, ±10%; and ϕ_{Trp}, ±20% in the shorter models, and ±10% in the longer models. From these values, the error in R^6 is found to be ±25% in the shorter, and ±18% in the longer models. In taking the sixth root of the data, the experimental errors are reduced by a factor of five to six. Thus the standard error in the calculated values of R is ±5% for the shorter and ±4% for the longer models. These percentages result in a standard deviation of approximately ±⅔ Å for all of the models. An important feature of the energy transfer technique is that large changes in the experimentally measured parameters T and ϕ_{Trp} are required before the calculated distance is significantly affected, and moderate experimental errors and inaccuracies in the assumption of point dipoles and of values for such factors as the orientation and the index of refraction do not appreciably affect the final result.

Figure 4 illustrates that the values of R calculated from eq 4 are within the allowable range, since the values for each model are from two to four-fifths of the way between the average van der Waals' collision distance (6 Å) and the maximum possible distance as measured on the fully extended Coulson–Pauling–Koltun atomic models. In any one solvent, R increases quite consistently with increasing $(CH_2)_n$. The increase in R is more gradual than the increase in the maximum possible distance; this is expected since folded configurations become more likely as $(CH_2)_n$ increases. In the models of small $(CH_2)_n$, R is independent of the solvent within experimental error (±⅔ Å). As $(CH_2)_n$ becomes larger, R is increasingly sensitive to the solvent, being uniformly greater in ethanol than in water, and in p-dioxane than in ethanol. These consistent differences are well in excess of the experimental uncertainty.

The dielectric constants at 23° of the solvents used are water, 79.2; glycerol, 42.8; ethanol, 24.6; and p-dioxane, 2.2 (from the National Bureau of Standards Circular No. 514, 1951). Thus the lower the polarity of the solvent, the greater is the calculated extension of each of the larger models. Water would not be expected to solvate a methylene chain very well, and thus, in water, configurations which permit considerable methylene–methylene contact may be thermodynamically favored. These configurations would be ones in which the methylene chain loops back upon itself, effectively shortening the interchromophoric distance. Glycerol and ethanol would increase these distances by weakening the hydrophobic interactions, but could not be solvating the chain completely, since the distances increase even more in the less polar p-dioxane. Configurations which allow intrachain interactions are not very probable in the models of small $(CH_2)_n$ since the chain is too short to fold back on itself. Thus the interchromophoric distances in these models should be relatively independent of the solvent. The fact that this independence is observed indicates that changing the solvent itself does not distort the transfer process or measurements in ways which are not compensated for during the calculation of distances from Förster's theory. Therefore these data suggest a significant change in the average configuration of the longer models upon going from polar to nonpolar solvents.

It is of interest that the distances calculated for the

TABLE VI: Comparison of Distances (in Å) Calculated from Energy Transfer with Those Estimated from Moments of Methylene Chains.

Compd n^a	Model $\langle R^{-6}\rangle^{-1/6}$ [b]	Methylene Chain $\langle r\rangle$ [c]	Residual Length $\langle R^{-6}\rangle^{-1/6} - \langle r\rangle$	$\langle r\rangle$ + Av Residual Length (9.5 Å)
5	13.4	4.2	9.2	13.7
6	15.2	5.1	10.1	14.6
8	16.0	6.8	9.2	16.3
10	17.6	8.3	9.3	17.8

[a] n in DNS-NH$(CH_2)_n$(=O)-Trp (second column) and the number of carbon atoms in the methylene chains of the third column. [b] R in p-dioxane from energy transfer calculations (Table V). [c] The first moment average end-to-end distance of the methylene chain, estimated from the second moment of R. L. Jernigan.

model compounds in 8 M urea (not given here) were exactly the same as in water. Whitney and Tanford (1962) and Bruning and Holtzer (1961) have found that urea generally has a much smaller effect on the weakening of hydrophobic bonds in aqueous solution than do organic solvents.

R as an Average. Values of R calculated from experimental data through eq 4 are not simple population averages. The ratio of T to ϕ_{Trp} measured from a population of chromophore pairs is proportional to a rate constant of transfer, k_T (eq 3), which is a population average of the k_T's from the individual pairs. The value of k_T for the i-th pair of chromophores is proportional to R_i^{-6} (eq 4) where R_i is the distance for that pair. Therefore an experimentally determined ratio which is substituted into eq 4 is proportional to the average value $\langle R^{-6}\rangle$, and the R calculated from this equation is actually the value $\langle R^{-6}\rangle^{-1/6}$.

Assuming a gaussian distribution of R_i's around the mean and a range of feasible variances for this distribution, it was estimated that the calculated values of $\langle R^{-6}\rangle^{-1/6}$ must be from 0 to 1 Å less than the actual mean distance $\langle R\rangle$ in the shorter models, and 0.5 to 1.5 Å less than $\langle R\rangle$ in the longer models. These approximations show that changes in variance alone cannot account for the differences in $\langle R^{-6}\rangle^{-1/6}$ of the longest models in the different solvents, but that they might certainly account for a small part of these changes. Intrachain hydrophobic interactions restrict the freedom of the chain, and thus probably alter the distribution of distances to some extent.

The Estimation of Distances from Moments of Methylene Chains. R. L. Jernigan (unpublished data) has very kindly supplied theoretical second moments of the end-to-end distances of methylene chains calculated according to the statistical-mechanical method outlined by

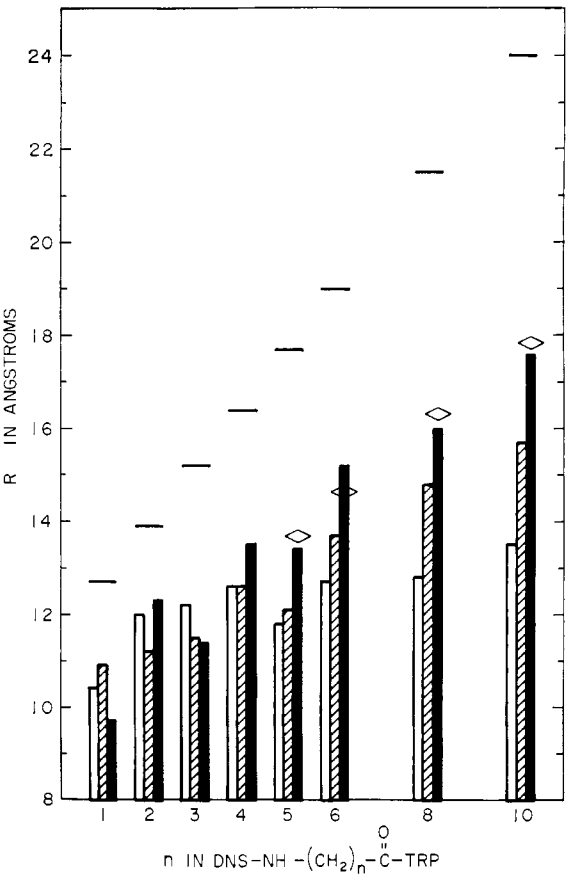


FIGURE 4: Center-to-center interchromophoric distances in angstroms: measured on CPK atomic models at their maximum possible extension (——), calculated through Förster's equation from energy transfer measurements on the models in water (□), in ethanol (▨), and in p-dioxane (■) (standard deviation = $\pm$ 2/3 Å), and estimated from moments of methylene chains (◇).

Flory and Jernigan (1965). These moments are for noninteracting chains in an ideal solvent at 25°. For each chain, a first moment average end-to-end distance $\langle r\rangle$ has been calculated from the second moment through a relation which assumes a gaussian distribution of r's around $\langle r\rangle$. Although the actual distribution is not quite gaussian, these estimates should be accurate to within $\pm$0.5 Å of the real values. Table VI shows the differences between the values of $\langle R^{-6}\rangle^{-1/6}$ obtained from energy transfer for the models in p-dioxane where $(CH_2)_n$ equals 5, 6, 8, and 10, and the values of $\langle r\rangle$ for methylene chains of the same number of carbon atoms. (The p-dioxane values were chosen because this solvent is probably a more ideal one for the models than is water or ethanol.) Since within experimental error all n's give the same difference, or residue, then the rate of increase of the experimental $\langle R^{-6}\rangle^{-1/6}$ with n matches the slope of the values calculated from the theoretical moments.

The differences computed above would be expected to approximate the $\langle R^{-6}\rangle^{-1/6}$ of DNS-Gly-Trp, because this is the residual length which remains when a methyl-

ene chain of n carbons is subtracted from a model of n methylenes. The average residual length (9.5 Å) is very close to the $\langle R^{-6}\rangle^{-1/6}$ calculated from energy transfer for the DNS-Gly-Trp ($n = 1$) model, 9.7 Å. The average residual length was added to each of the distances computed from the moments in order to obtain numbers which could be compared with the experimental values. These sums are listed in the fifth column of Table VI, and the comparison (with the second column) is illustrated in Figure 4. Thus not only do the end-to-end distances calculated from Förster's theory for the models in *p*-dioxane increase with n at the same rate as do the distances computed from the moments, but their absolute values are very similar. In making these comparisons, it must be kept in mind that the experimental $\langle R^{-6}\rangle^{-1/6}$ values have not been corrected to first moment averages, that *p*-dioxane may not be an ideal solvent for the model compounds, and that chromophore end effects have not been taken into account.

Discussion

The spectral data obtained from the the model compounds in various solvents strongly suggest that the transfer observed proceeds through a Förster-type interaction. It has been shown that mechanisms other than resonance transfer are unlikely even though the reason for the quenching of tryptophan fluorescence beyond that which can be accounted for by the observed transfer is not clear. The intramolecular distances and the solvent-induced changes in these distances which have been calculated from Förster's theory are very reasonable in view of the structures of the model compounds. They agree with estimates based on theoretical end-to-end distances of methylene chains.

If the applicability of Förster's theory is accepted for chromophores such as those described here, the results obtained are quite intriguing. Energy transfer studies would provide a very sensitive means of monitoring conformational changes in proteins. The measurements involved require only small amounts of material, and may be carried out over a wide range of temperature, pH, and solvents, since any spectral changes not actually caused by an alteration in distance or orientation are taken into account in the Förster calculation. The transfer of energy from tryptophan to DNS can be measured over greater distances in proteins than in the model compounds because $\phi_{\mathrm{Trp}(kT=0)}$ is higher in proteins. For transfer between one tryptophan and one DNS molecule, where $\phi_{\mathrm{Trp}(kT=0)} = 0.2$ (approximately the value in proteins) and $K^2 = {}^2/_3$, R_0 is about 23 Å, and thus when $T = 0.8$, $R = 18$ Å; $T = 0.5$, $R = R_0 = 23$ Å; $T = 0.2$, $R = 29$ Å. Where transfer can occur between tryptophans or to a number of DNS chromophores from each tryptophan, the calculation of an actual distance becomes difficult. In such cases the efficiency of transfer and of donor emission may be used as a very senstitive but nonspecific indicator of structural change.

In order to obtain accurate measurements of Förster-type transfer, it is necessary that the interchromophoric distance and orientation, the fluorescence efficiency of the donor, the extinction of the acceptor, and the overlap between the emission of the donor and the absorption of the acceptor be of magnitudes which produce a transfer rate constant of less than 10^{11} sec^{-1} and a transfer efficiency which is not too close to 0 or 1. One must be certain that all of the donor excited states from which the observed transfer occurs and all of the acceptor energy levels to which this transfer proceeds, but no others, are included in the calculation of the Förster overlap integral. The possbility of having overlapping electronic transitions within what might appear to be a single band, or mixed polarizations within one transition (Albrecht, 1960; Dörr, 1966) must be kept in mind. This will become especially important when dealing with a population of chromophores whose orientations are not random, such as may be encountered in proteins. It may be possible to obtain some information about the mutual orientation factor in proteins from measurements of the fluorescence polarization of the chromophores, particularly when the population can be homogeneously oriented, or through means suggested by the work of Weber and Daniel (1966). Resonance transfer studies may be of particular value in applications where the sixth moment average which results from such calculations can be compared with other moment averages in order to generate estimates of population distributions.

Acknowledgment

The authors wish to thank Dr. H. H. Seliger for many helpful discussions.

References

Albrecht, A. C. (1960), *J. Am. Chem. Soc. 82*, 3813.

Bennett, R. G. (1964), *J. Chem. Physics 41*, 3037, 3040.

Birks, J. B., and Christophorou, L. G. (1962), *Nature 196*, 33.

Bowen, E. J., and Livingston, R. (1954), *J. Am. Chem. Soc. 76*, 6300.

Bruning, W., and Holtzer, A. (1961), *J. Am. Chem. Soc. 83*, 4865.

Chen, R. F., Vurek, G. G., and Alexander, N. (1967), *Science 156*, 949.

Dexter, D. L. (1953), *J. Chem. Phys. 21*, 836.

Dörr, F. (1966), *Angew. Chem. Intern. Ed. Engl. 5*, 478.

Edelhoch, H., Brand, L., and Wilchek, M. (1967), *Biochemistry 6*, 547.

Ellis, D. W., and Solomon, B. S. (1967), *J. Chem. Phys. 46*, 3497.

Fasman, G. D. (1963), *Methods Enzymol. 6*, 956.

Flory, P. J., and Jernigan, R. L. (1965), *J. Chem. Phys. 42*, 3509.

Förster, T. (1948), *Ann. Physik 2*, 55.

Förster, T. (1951), Fluroeszenz Organischer Verbindungen, Göttingen, Germany, Vandenhoeck and Ruprecht.

Förster, T. (1959), *Discussions Faraday Soc. 27*, 7.
Förster, T. (1960), *in* Comparative Effects of Radiation, Burton, M., Kirby-Smith, J. S., and Magee, J. L., Ed., New York, N. Y., Wiley, p 300.
Förster, T. (1965), *in* Modern Quantum Chemistry, Part III, Sinanoğlu, O., Ed., New York, N. Y., Academic, p 93.
Förster, T., and König, E. (1957), *Z. Elektrochem. 61*, 344.
Galanin, M. D. (1955), *Soviet Phys. JETP Engl. Transl. 1*, 317.
Hermans, J. J., and Levinson, S. (1951), *J. Opt. Soc. Am. 41*, 460.
International Critical Tables (1930), Vol. 7, Washburn, E. W., Ed., New York, N. Y., McGraw-Hill.
Kasha, M. (1963), *Radiation Res. 20*, 55.
Lagunoff, D., and Ottolenghi, P. (1965), *Compt. Rend. Trav. Lab. Carlsberg 35*, 63.
Latt, S. A., Cheung, H. T., and Blout, E. R. (1965), *J. Am. Chem. Soc. 87*, 995.
Perrin, J. (1925), 2^{me} Conseil de Chimie Solvay, Paris, Gauthier and Villar, p 322
Rabinowitch, E., and Epstein, L. F. (1941), *J. Am. Chem. Soc. 63*, 69.
Rozman, I. M. (1958), *Opt. i Spektroskopiya 4*, 536.
Setlow, R. B., and Pollard, E. C. (1962), Molecular Biophysics, Reading, Mass., Addison-Wesley, p 211.
Shore, V. G., and Pardee, A. B. (1956), *Arch. Biochem. Biophys. 62*, 355.
Stryer, L. (1959), *Biochim. Biophys. Acta 35*, 242.
Stryer, L., and Haugland, R. P. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 719.
Teale, F. W. J., and Weber, G. (1957), *Biochem. J. 65*, 476.
Weber, G. (1952), *Biochem. J. 51*, 145, 155.
Weber, G. (1961), *in* Light and Life, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., Johns Hopkins, p 82.
Weber, G. (1966), *in* Fluorescence and Phosphorescence Analysis, Hercules, D. M., Ed., New York, N. Y., Interscience.
Weber, G., and Daniel, E. (1966), *Biochemistry 5*, 1900.
Weber, G., and Teale, F. W. J. (1958), *Trans. Faraday Soc. 54*, 640.
Weber, G., and Teale, F. W. J. (1959), *Discussions Faraday Soc. 27*, 134.
Whitney, P. L., and Tanford, C. (1962), *J. Biol. Chem. 237*, PC1735.