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Use of Nonspecific Dye Labeling for Singlet Energy-Transfer Measurements in Complex Systems. A Simple Model†

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ABSTRACT: Techniques are described which will permit the semiquantitative interpretation of singlet–singlet energy-transfer measurements on multiply labeled single proteins and protein complexes. The two critical assumptions are that fluorescent chromophores can be placed anywhere at random on a protein surface and that the stoichiometry of labeling is governed by a Poisson distribution. In calculations on protein complexes it is further assumed that one can exclusively localize donors on the surface of one protein, and acceptors on another. Calculations were initially carried out to allow for the occurrence of donor–donor energy transfer. These show that in most cases of interest one can neglect this process. Singlet energy-transfer measurements on a double-labeled protein will permit measurement of the anhydrous radius. The results are quite insensitive to variations in axial ratio if the volume is kept constant. In a complex of two or more proteins, singlet energy-transfer measurements will enable fairly accurate determination of the distances between pairs of proteins. For most accurate results in both cases one should attempt to work at ratios of several acceptor molecules per protein. This minimizes errors due to uncertainties in the extinction of bound dyes. The average number of donor molecules per protein is not important. Examples of the application of these calculations are given in the accompanying paper.

Fluorescence techniques have yielded much useful information about the structure of biopolymers (Cantor and Tao, 1971; Stryer, 1968). Both intrinsic protein fluorescence (from aromatic amino acids) and extrinsic fluorescence (from dye–protein conjugates or prosthetic groups) have been valuable. A number of enzymes bind dyes specifically at the active site (Glazer, 1970). This fact has been fully exploited in the numerous experiments using fluorescent dyes to probe the nature of the active site (or binding site), its polarity, and the disposition of aromatic amino acids nearby (see for example Stryer, 1965; DeLuca, 1969; Chen and Kernohan, 1967; Brand *et al.*, 1967; McClure and Edelman, 1967; Turner and Brand, 1968). Energy-transfer experiments have generally, though not exclusively, been restricted to these situations where the transfer from tryptophan to a fluorescent probe at a specific site is observed.

It is our purpose to demonstrate the feasibility of obtaining useful information on proteins randomly labeled with fluorescent donor and acceptors. Somewhat analogous calculations have been reported in the past by Teale and his coworkers (Badley and Teale, 1969, 1971; Dale and Teale, 1970). However, they were aimed at different kinds of chromophore distributions. We shall make three assumptions. The stoichiometry of dye labeling is governed by the Poisson distribution. The spatial location of dyes is anywhere at random on the protein surface. If more than one protein is present one can control which protein has which dye by separate labeling and subsequent reconstitution. The success of this approach is demonstrated by the experimental results given in the accompanying paper (Gennis *et al.*, 1972). The procedures we describe should find general use in studies on such diverse and complicated systems as ribosomes, membranes, multiprotein complexes, and the cell surface.

Theory

Förster transfer has been shown to apply to the systems we will be discussing (Latt *et al.*, 1965; Stryer and Haugland, 1967). The energy from an excited donor is transferred *via* a dipole–dipole nonradiative mechanism to an acceptor nearby (Förster, 1965). The distance over which this occurs may be as much as 60 Å or more, making it a valuable tool for the study of macromolecules. Förster's equation relates the absolute rate of transfer, k_t, to the spectral characteristics of the donor and acceptor, their relative orientation, and the distance between them.

$$k_t = \frac{1}{\tau} \times \text{constant} \times \frac{\kappa^2 \phi_f J}{n^4 r^6} \text{ sec}^{-1} \quad (1)$$

$$k_t = \frac{1}{\tau}(R_0/r)^6 \quad (2)$$

where R_0 = (constant $\times$ $\kappa^2 \phi_f J/n^4)^{1/6}$, κ^2 is the dipole–dipole orientation factor, ϕ_f is the fluorescence quantum yield of the donor in the absence of transfer, n is the refractive index of

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the medium between the donor and acceptor, and r is the distance between the centers of the donor and acceptor transition dipoles. The overlap integral, J, is defined as

$$J = \frac{\int [F_D(\nu)\epsilon_A(\nu)/\nu^4]\, d\nu}{\int F_D(\nu)\, d\nu} \quad (3)$$

where $\epsilon_A(\nu)$ is the molar extinction coefficient of the acceptor, and $F_D(\nu)$, the fluorescence of the donor. R_0 is the distance at which half of the excited donors are quenched by energy transfer to the acceptor. It is a characteristic distance for every donor–acceptor pair. The key factors to note are the dependence of the transfer rate, k_t, on the sixth power of the inverse of the distance between donor and acceptor, and the dependence on the overlap integral, J. In order for there to be Förster transfer the donor fluorescence has to overlap the absorption of the acceptor. Many studies over the past several years have demonstrated the validity of the Förster equation (Latt *et al.*, 1965; Haugland *et al.*, 1969; Stryer and Haugland, 1967).

The measured quantity in energy-transfer experiments is the efficiency of transfer. For a single donor–acceptor pair this is given by

$$E = k_t/(k_t + k_f + k_0) = \frac{1}{1 + (r/R_0)^6} \quad (4)$$

where k_t is the rate of transfer to the acceptor, k_f is the rate of fluorescence, and k_0 represents the sum of all the other rates of nonradiative modes of deexcitation of the donor.

Calculations

We wish to calculate the average expected transfer not from a specific donor–acceptor pair but rather from a random distribution of donors and acceptors. To do this several simplifying assumptions are necessary.

Assumptions

Spatial Distribution. In the absence of any other information we assume our labels to be distributed completely at random on the surface of the protein. In all the calculations the dyes are considered points on this surface. We are interested primarily in globular proteins, and most of our calculations assume the protein to be spherical. There are clearly limits to the validity of this assumption. Certain labeling procedures will maximize the probability of random labeling on the surface. There will, of course, be proteins where the structure of the surface precludes a random label distribution (Weber and Teale, 1959; Mihalyi and Albert, 1971). In this case our calculations will not be valid. The Förster transfer mechanism is not valid when the distance between the donor and acceptor is very small. This may be a source of error in our calculations but it is likely to be small.

Number Distributions. It is usually possible to measure only the average number of dyes bound per protein moiety. Again, relying on randomness, we will assume a Poisson distribution for statistical calculations. If μ is the average number of dyes bound per protein, the probability of finding a protein with N dyes bound is then given by

$$P(N,\mu) = \frac{\mu^N}{N!} e^{-\mu} \quad (5)$$

The Poisson distribution is an approximate form of the binomial distribution in the limit of small probability for an event (binding, in our case). The probability of binding may be expressed as μ/M, where M is the number of possible binding sites on the protein. Hence, the Poisson distribution will be most accurate in the limit of low binding, and where the number of binding sites is large. This will frequently be the case; however, we have used a Poisson distribution also in cases where the degree of labeling is not small. The alternative is to use the binomial distribution; this requires a knowledge of the number of binding sites, M. It might be possible to determine this number by exhaustive reaction with a fluorescent label. Care must be taken to avoid opening up the protein and exposing sites normally not on the surface. We have chosen to avoid this problem and use the simpler Poisson distribution, realizing its limitations.

The most important result obtained from the number distribution is the fraction of proteins which is unlabeled ($N = 0$). The average energy-transfer function, $E(N)$, is most sensitive to changes in N when N is small. Very different transfer efficiencies will result from a stoichiometry of 1:1 (one dye per protein), than a Poisson distribution with $\mu = 1$. In the latter case about 37% of the proteins are unlabeled, and those proteins with more than one dye (26%) do not compensate for this. If the degree of labeling, μ, is large, the fraction of proteins with $N = 0$ will be quite small and the errors involved in choosing the correct number distribution consequently will also be small. Both the Poisson and binominal distributions have been applied before to the problem of random fluorescent labeling (Weber and Daniel, 1966; Green, 1964).

More Than One Donor per Protein. In many cases there will be no interaction between donors. The average transfer efficiency observed should then be independent of the number distribution of donor molecules. This assumes that the distributions of donors and acceptors in the system are mutally independent, *i.e.*, the probability of finding a donor on a given protein is not a function of the number of acceptors on that protein. However, in some cases there will be significant donor–donor transfer. For this reason we felt it necessary to perform calculations to see how large an effect such interactions can have. These are included in the next section. We conclude that the effects are small, and, in view of all the other uncertainties, not significant. We have not taken such interactions into account in most of our calculations. The kinetics of a system of many interacting donors and a single acceptor has been discussed by Pearlstein (1968).

More Than One Acceptor. The problem of multiple acceptors is one which has been dealt with previously. It is usual to assume parallel first-order kinetics for the transfer of energy from the donor to the several acceptors (Bay and Pearlstein, 1963; Dale and Teale, 1970). That is, the kinetics is represented as the sum of the pairwise rates. Hence, for N acceptors, the transfer efficiency is given by

$$E(N) = \frac{\sum_{i=1}^{N} \left(\frac{R_0}{r_i}\right)^6}{1 + \sum_{i=1}^{N} \left(\frac{R_0}{r_i}\right)^6} \quad (6)$$

r_i is the distance from the donor to the ith acceptor, and the R_0 value is taken as being the same for all the donor–acceptor pairs. This serious simplification is discussed in the next section.

If all R_0's are the same, one can write assuming parallel first-order reactions that the rate of transfer is

$$k_t(N) = \sum_{i=1}^{N} \left(\frac{R_0}{r_i}\right)^6 = N\left(\frac{R_0}{r}\right)^6 \quad (7)$$

Equation 7 could now be averaged over the donor and acceptor spatial distributions.

However, things are more complex when one wants to calculate the efficiency of transfer. For a rigorous calculation each acceptor in eq 6 would have to be averaged independently. As a mathematical simplification we have applied the approximation that

$$E(N) = N(R_0/r)^6/(1 + N(R_0/r)^6) \quad (8)$$

Physically, we are considering, for example, two acceptors on a protein as being equivalent to a single acceptor with twice the extinction coefficient. For exact calculations each acceptor should be considered independently. We have done efficiency calculations both ways for a representative case of 2 acceptors and one donor on the surface of a small sphere. The calculations differed in this case by 15%. Thus the approximate results will still hold well for semiquantitative measurements. The error will be least in the cases we are interested in; specifically where the distances are substantial as in transfer from one protein to another, and where the degree of labeling is large as in the transfer between donors and acceptors on the same sphere. Should experimental results justify more exact calculations, this approximation could easily be avoided.

Calculation of R_0. The R_0 calculated for a given dye pair depends critically on the spectral characteristics of the dyes. However, frequently the spectral properties of a bound dye will depend on the nature of the binding site on the protein. We calculate an R_0 based upon the average measured spectral characteristics and do not directly consider any such heterogeneity. Thus for the theory to be adequate one should use chromophores that are insensitive to environment and avoid dyes such as ANS. Limiting labeling to surface sites will help to insure a generally uniform environment for all the labels and this further assures uniform spectral properties. This assumption can be checked experimentally by observing how close the fluorescence decay of a single labeled sample is to single exponential. Surface labeling will also help to assure flexible dye attachment and thus permit simple averaged values to be used for orientation factors—see below.

Methods of Calculations. Three methods were used to calculate transfer efficiencies. (1) NUMERICAL INTEGRATION. Most of our calculations of the transfer efficiencies averaged over the surface of a sphere (or an ellipsoid of revolution) were performed using a numerical integration routine on an IBM 360-91 computer. The integration was fairly coarse; the range over which the variables were integrated usually divided into 13 parts. This was found to be sufficient for our purposes. For all calculations the geometrical factor, κ^2, was assigned the value of 0.66. This represents the averaged value for the case of rapid Brownian rotation of both the donor and acceptor (Förster, 1965). Maksimov and Rozman (1961) have determined the value κ^2 to be 0.475 for the case of an ensemble of random but fixed orientations. The best value probably lies somewhere in between the two; the error resulting from an arbitrary choice of this value will be very small, however, since it is the one-sixth root of κ^2 which enters into the calculation.

(2) TWO DONORS AND ONE ACCEPTOR. For models involving two donors and a single acceptor eq 10, derived in the Appendix, was used to calculate the transfer efficiency. The value of k_{QF} (see Appendix) was set equal to 1.0 and all rates were calculated relative to this. When finding the effects of the two donors interacting over a random distribution on a surface (or line) the calculated transfer efficiencies for a fairly large number of different selected positions were simply averaged.

(3) MARKOV CHAIN METHODS (HOARE, 1970; KEMENY AND SNELL, 1960). There is no simple analytic expression for calculating the contribution of donor–donor interaction if there are more than two donors. Matrix methods are ideally suited for this problem, and indeed have been used to calculate the trapping probability in photosynthesis (Robinson, 1967). The key feature is that the probabilities for the various alternatives at each step (transfer to a donor, transfer to an acceptor, fluorescence, or other termination of the excitation) are defined and constant for a given geometry. Consider the case of two donors and one acceptor. We define a column matrix P $(P_1, P_2, P_3, P_4)^{-1}$ where P_1 and P_2 are the probabilities of the excitation residing on donor 1 and donor 2, respectively, P_3 is the probability it has been transferred to the acceptor, and P_4 is the probability it has been lost by fluorescence or other nonradiative quenching. The initial value of P is clearly (0.5, 0.5, 0, 0) if the two donors are equally excited. We now want to follow the evolution of P in "time," to see how the probabilities redistribute. For this purpose we define the 4×4 matrix E; the element E_{ij} represents the probability of the excitation going from P_j to P_i. For instance, E_{12} is the probability of energy transfer from donor 2 to donor 1; and E_{13} is the probability of transfer from the acceptor to donor 1, which we shall take as zero. Both P_3 and P_4 are terminal points and all the E_{13} and E_{14} are zero. The product $P' = E \cdot P$ gives a new column matrix; this represents the redistributed probabilities after one "step." For example, P_3' represents the fraction of energy transferred to the acceptor after this one step. Since the probability matrix E does not change, the distribution after n steps is $(E)^n \cdot P$. This is continued for as many steps as is necessary to remove all the energy from the donors. By keeping track of P_3 at each step, and summing, the total fraction of the initial energy which eventually is transferred to the acceptor can be calculated.

This method is potentially useful in calculating the expected depolarization due to self-transfer from a collection of fluorescent molecules (such as tryptophans). It may be useful in calculating the energy transfer from a set of tryptophans within a protein to an extrinsic acceptor. The results of this method for the case two donors and acceptor are identical with using eq 10 (one is merely an analytical form of the other). This method was used to calculate the transfer expected from a set of three donors to one acceptor in a variety of simple geometric arrangements.

Results

We have performed calculations for two cases: a single protein labeled simultaneously with both donors and acceptors, and two proteins in a complex, one labeled with donors, the other with acceptors.

Single Protein. Consider the case of a donor transferring energy to N acceptors on the same protein. The transfer efficiency will be given by eq 8. This must be averaged over the surface of the protein, which for now we will assume is a

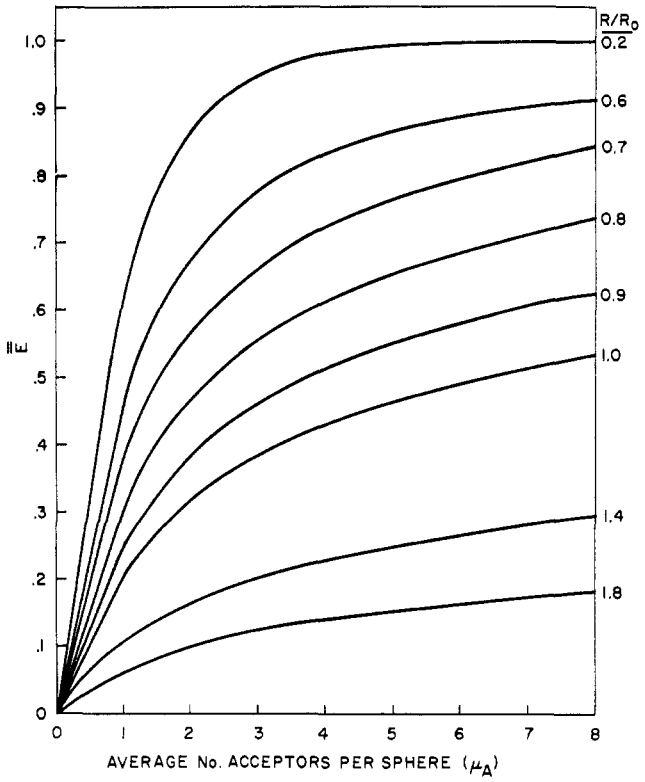

FIGURE 1: Single sphere energy-transfer calculations. The average energy transfer $\bar{E}$, as a function of μ_A, the number of acceptors per sphere for different values of R/R_0. For such an experiment an individual protein would be surface labeled with both donor and acceptor molecules.

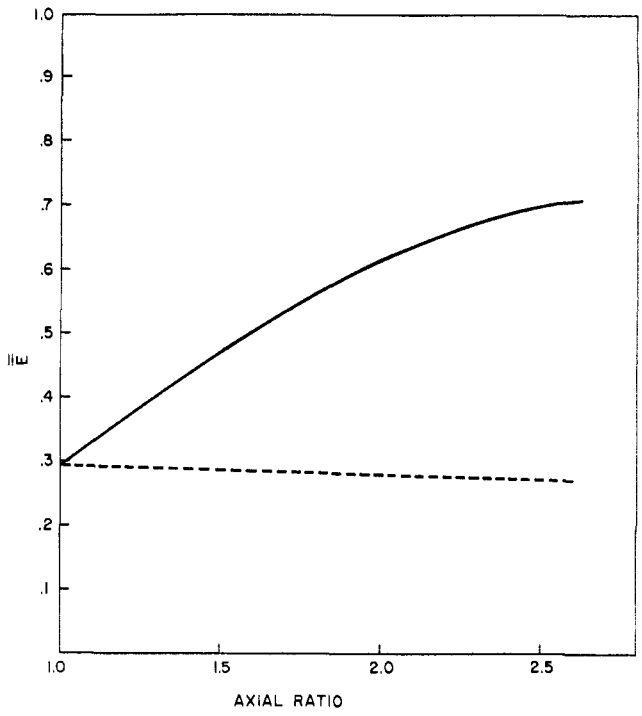

FIGURE 2: Energy-transfer calculations for one donor and one acceptor on the surface of a prolate ellipsoid of revolution. The limiting sphere has a radius equal to R_0. (a) Constant major axis, variable minor axis (———). (b) Variation of axial ratio while maintaining constant volume of the ellipsoid (- - - - - -).

sphere. We numerically integrated this function. The resulting efficiency we will denote $\bar{E}(N)$. In an experiment, what is measured is the weighted average of the $\bar{E}(N)$ where the weighting factors are the Poisson terms. Hence

$$\bar{E} = \sum_{N=0}^{m} P(N,\mu_A)\bar{E}(N) \qquad (9)$$

where m is the point at which the series is truncated, and μ_A is the average degree of labeling of the acceptor. We have calculated E as a function of μ_A and (R/R_0), the ratio of the radius of the sphere R, to the R_0 of the dye pair. Figure 1 shows the results of that calculation. It is possible to determine the anhydrous radius of the spherical protein. By varying the number of acceptors a curve can be generated which will give the ratio R/R_0 (in principle, only one point is necessary). Since R_0 is known, the radius is easily found. For experiments of this type it is interesting to note that to optimize the sensitivity of the method the dye pair should have an R_0 which is close to the radius of the protein. Also, the most reliable results will obviously be obtained at high degrees of labeling where small errors in calculating μ_A (which can be done most easily by spectrophotometric methods) will be less important. An experimental test of these calculated curves is given in the accompanying manuscript (Gennis *et al.*, 1972).

Globular proteins are not all spherical. For this reason we felt it important to see what effect shape would have on the energy transfer. We have calculated the transfer between a donor and a single acceptor randomly placed on the surface of a prolate ellipsoid of revolution. Figure 2 shows this result of our calculations. A sphere with a radius equal to R_0 will give an average transfer of 29.4%. If the major axis is kept constant and the minor axis reduced the transfer should increase. The effect is large; at axial ratio 2.0 the average transfer efficiency more than doubles. It is more interesting to vary the axial ratio while maintaining constant volume. The results, also shown in Figure 2 are remarkable. In the limit of the very long, thin ellipsoid it is clear that the transfer efficiency should go to zero (the probability of having the donor and acceptor nearby is zero). The rate of decrease, however, in the region of interest is very small. To a good degree of approximation, the energy transfer is independent of the axial ratio for a fixed volume. Hence, this technique is potentially a method for finding the anhydrous molecular volume of any protein.

Two Proteins. The formalism for the two-protein calculation is exactly the same as in the single protein case. Now, however, the donors and acceptors are on different proteins which are either touching or near each other in a fixed arrangement. The distribution of labels is still assumed to be random on the entire protein surface and the Poisson number distribution is again considered valid.

Figure 3 shows the results of the calculation for two spheres in direct contact. The radii of the two spheres are equal to each other. The larger distances require a larger R_0 to get substantial energy transfer. If the proteins have radii of 25 Å and μ_A is a modest 1.0–1.5, one would still need an R_0 of about 40 Å to get 20% transfer. However, an R_0 of 40 Å is attainable experimentally.

Figure 4 demonstrates a general feature of these transfer calculations. In this model the transfer is calculated between two spheres whose centers are separated by R_0. The radii of

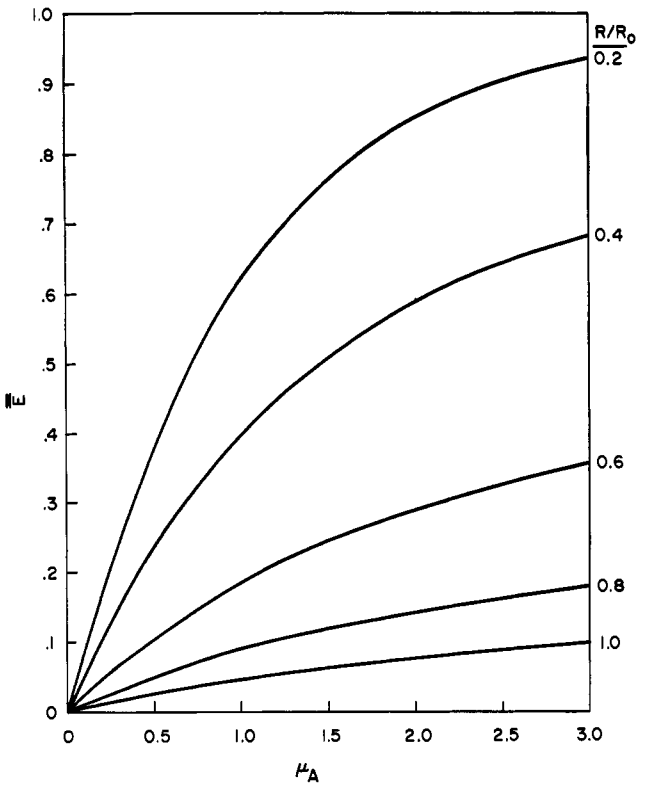

FIGURE 3: Energy transfer between two tangential spheres of equal radii (R) as a function of the number of acceptors per pair for different values of R/R_0. In such an experiment the donors would be localized on one protein while the acceptors would be on the other.

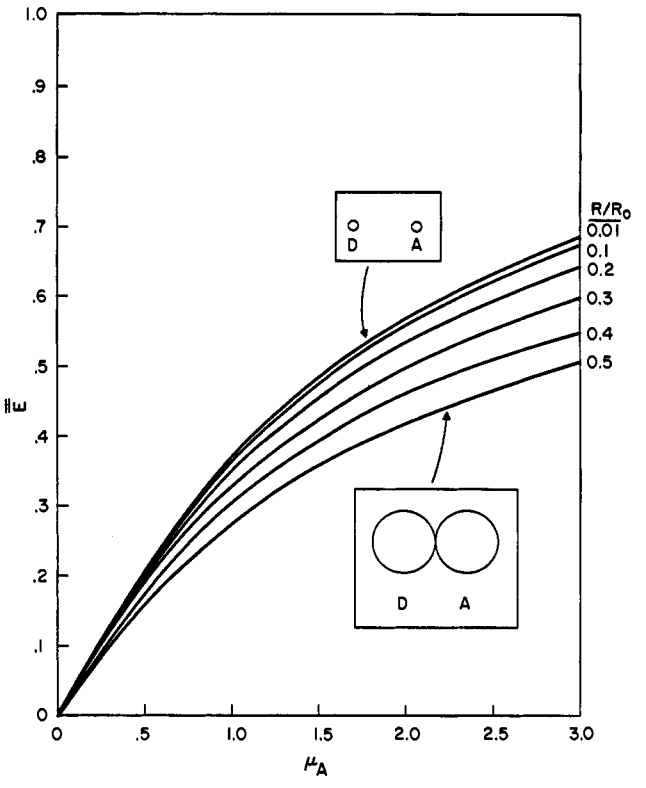

FIGURE 4: Energy transfer between two spheres whose centers are R_0 apart as a function of the average number of acceptors per pair. The radii of the spheres, R, are varied.

the spheres are varied. At one extreme the radii are very small; at the other the spheres extend to touch each other. As they get larger the energy transfer decreases. This results primarily from a simple increase in the distances between points on the two spheres. This becomes apparent if one considers the points on rings formed by the intersection of the spheres with planes passing through their centers. A secondary cause is due to a saturation effect. If a donor and acceptor are close to each other, decreasing the distance between them even further will have only a small effect on the energy transfer. This saturation is not balanced symmetrically as we go to distances greater than R_0. Consequently, moving some dye pairs further apart will not be exactly compensated by moving others closer. Hence, the effect is also to lower the transfer efficiency as the spheres get larger.

If two proteins are in direct contact, it is clear that the areas of contact must be free from bound dye. So far we have not taken this into account. It is important to know how sensitive these calculations are to this parameter. Figure 5 shows the results of eliminating the dyes from the surfaces areas on both spheres beginning with the region of contact. As can be seen the effect is substantial; eliminating surface area from these regions has the effect of removing those donor–acceptor pairs which are the largest contributors to the total transfer. Hence, if just 10% of the total surface of each sphere is eliminated in this manner the transfer drops from 26 to 19% in this particular case.

We next consider two spheres which are not touching, but are rather embedded in some complex and separated by a distance. Such a model is realistic for the proteins within the ribosome, for instance. Once again we are increasing the distances so we can again expect to see a decrease in the actual transfer observed unless a larger R_0 is used. Figure 6 demonstrates the sensitivity to increasing the distance between two spheres. In this case the radii of the two spheres

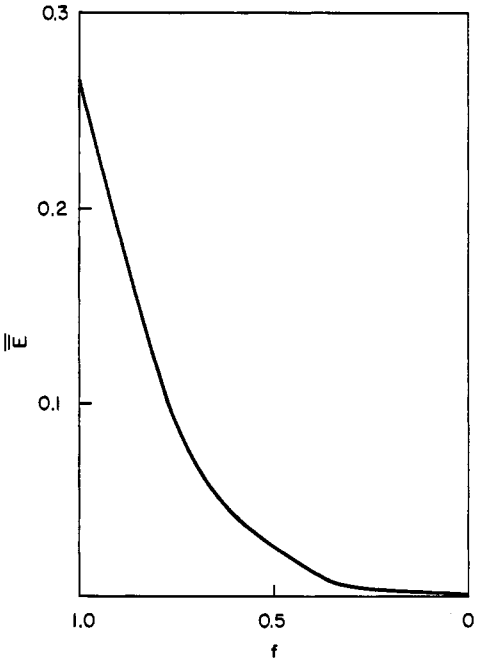

FIGURE 5: Energy transfer between two tangential spheres as a function of the fraction of surface integrated over f. In this case the two spheres have equal radii, $R = 0.6R_0$, and area is eliminated from both spheres beginning at the point of contact.

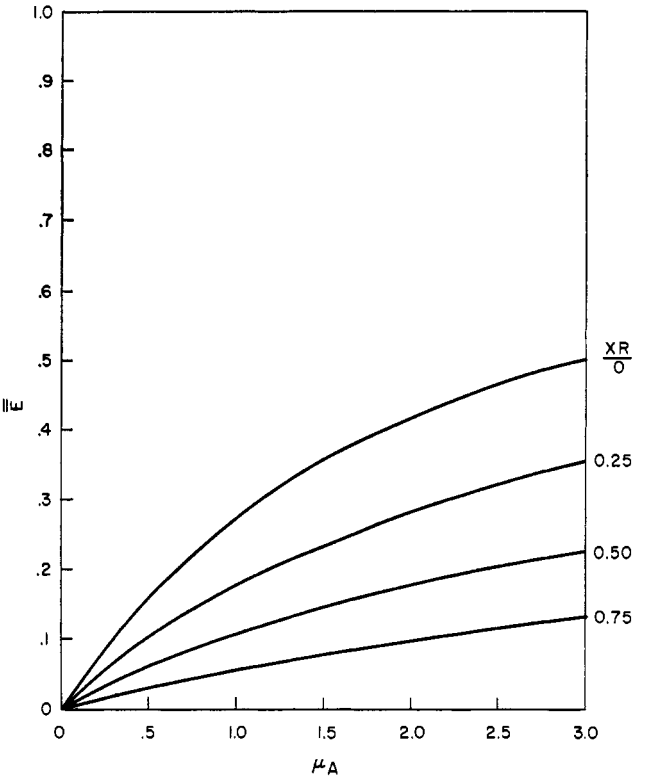

FIGURE 6: Energy transfer between two spheres as a function of the average number of acceptors per pair. Both spheres have radii of 0.5, R_0 = 1.0. XR is the distance of closest approach of the two spheres.

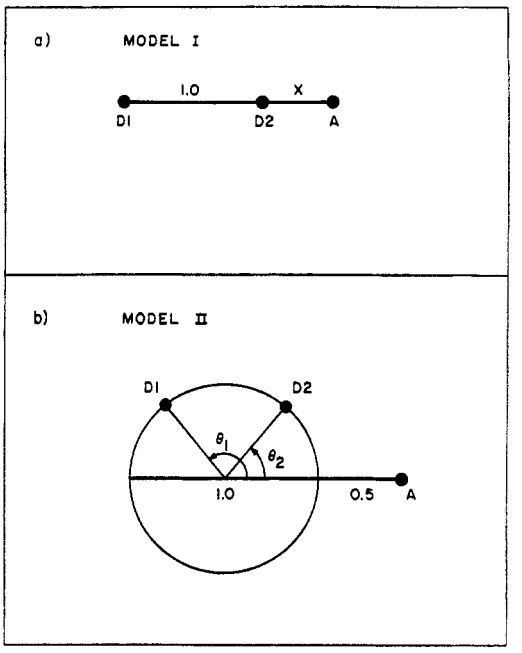

FIGURE 7: Two simple models used for calculating the energy transfer from two interacting donors (D1, D2) to an acceptor (A). See Figure 8 and Table I for the results of the calculations based on these models.

are equal, and the R_0 chosen is twice this value. At $\mu_A = 1$ the measured transfer would drop from nearly 27% when the spheres are in contact, to near 5% when the distance between closest surfaces is equal to 0.75 of R_0. The calculations do show, however, that such experiments are still possible if the donor–acceptor pair is wisely chosen. An experimental test of these calculations on two-sphere systems is presented in the accompanying manuscript. The results are highly encouraging (Gennis *et al.*, 1972).

Donor–Donor Interaction. Up to this point we have ignored interactions between donors. In many cases this will not be a problem merely because there will be no substantial overlap between the absorption and fluorescence of the donor. This is certainly the case with the frequently used label dansyl which has a remarkably large Stokes shift.

TWO DONORS—ONE ACCEPTOR. The donor–acceptor transfer efficiency for a system of two donors and one acceptor (all at fixed positions) is derived in the Appendix.

$$\bar{E} = \frac{1}{2}\frac{P_{1A} + P_{2A} + P_{12}P_{2A} + P_{21}P_{1A}}{1 - P_{12}P_{21}} \qquad (10)$$

P_{1A} is the probability of transfer from donor 1 to acceptor, P_{12} the probability of transfer from donor 1 to 2, etc. The effect of allowing the two donors to interact can be illustrated by model I seen in Figure 7a. Both donors and the single acceptor are fixed on a straight line. The average transfer has been calculated both with and without donor–donor interaction. The results are shown in Figure 8. We express R_0's in terms of the donor–donor distance which is defined as 1.0. If the R_0 for the donor–donor interaction, R_0 (DD), and for the donor–acceptor interaction, R_0 (DA), are 1.0 and 1.5, respectively, there is no significant effect on the transfer efficiency. If both R_0's are larger, 3.0, there is a substantial effect for large donor–acceptor distances.

In model II (see Figure 7b) the two donors are moved to nine different positions on a circle of diameter twice the distance of closest donor acceptor approach. R_0's are expressed as fractions of this diameter. The energy-transfer results are seen in Table I. The calculations for both models I and II illustrate several points. The effect of donor–donor interaction can be quite substantial depending upon the R_0's and the geometry involved. However, for realistic R_0's (*i.e.*, R_0 of a magnitude between the protein radius and diameter) the effect of including donor–donor interaction will be negligible. Although there will be geometries where the effects will be substantial (as in positions 1 and 6 for model II), the average over many positions will reduce their importance.

The effect of donor–donor interaction is to increase the observed transfer. This has been the case in all our calculations of this type. More energy is passed to the acceptor from the most distant donor, D1, *via* D2, than is passed from D2 to D1 and eventually emitted (or otherwise lost).

Calculations were also made for two donors and an acceptor on the surface of a sphere. One donor was fixed and the other donor and the acceptor allowed to take 108 positions representative of a random distribution of each. The transfer was calculated for each position with R_0 values chosen to correspond to a realistic experimental case. These were then averaged. The effect of donor–donor interaction on the transfer efficiency was an increase (as expected) of less than 3%.

THREE DONORS—ONE ACCEPTOR. Calculations were also performed for several systems of three donors and one acceptor using the Markov techniques previously described. Results are shown for two different geometrical arrangements. The first configuration (a), illustrated in Figure 9, consists

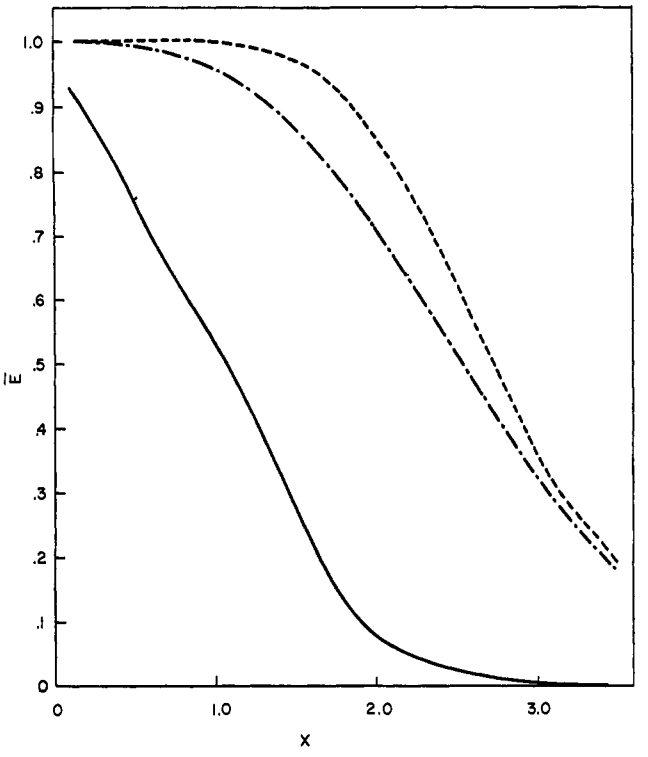

FIGURE 8: Results of energy-transfer calculations using model I. Average energy transfer as a function of X (see Figure 7). The distance between the two donors is defined as 1.0. (a) R_0(DA) = 1.5, R_0(DD) = 1.0; same result for R_0(DD) = 0.0, (——). (b) R_0(DA) = 3.0, R_0(DD) = 3.0, (- - - - - - - -). (c) R_0(DA) = 3.0, R_0(DD) = 0.0, (-·-·-·-·-·-).

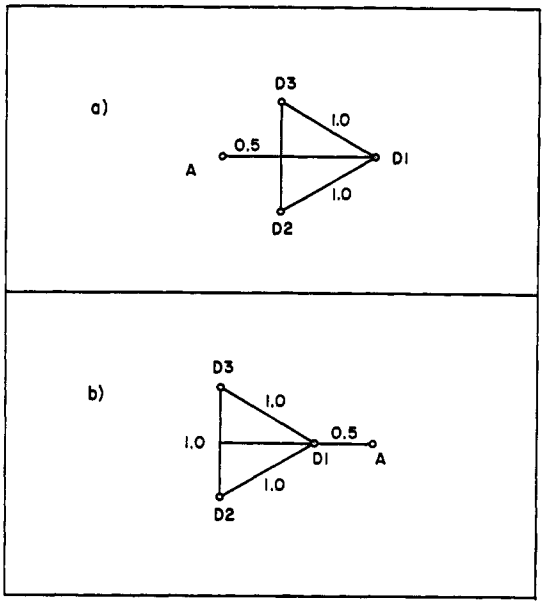

FIGURE 9: Configurations a and b used for energy-transfer calculations with three donors and one acceptor. See Table II for the results of these calculations.

of the donors at the corners of an equilateral triangle with the single acceptor (A) equidistant from two donors (D2, D3) but closer to the third (D1). The other configuration (b) is identical but the acceptor is now closer to D2 and D3. R_0's are expressed as fractions of the donor–donor distance. The results, as expected, are sensitive to the choice of the donor–donor and donor–acceptor R_0 values. Again, it is emphasized that we are at all times considering only very weak dipole–dipole coupling and are not including the possibility of exciton coupling of a set of equivalent chromophores in close proximity.

The calculations are performed in a manner which allows one to follow the probability that a photon absorbed by any of the donors has been transferred to the acceptor as a function of the number of steps or jumps. This is done for each of the donors and the final answers (transfer efficiencies for photons absorbed at D1, D2, and D3) are averaged. The calculations were taken to 99 Markov steps, though for the specific examples illustrated here this was many more than necessary. The donor–donor coupling is the primary determinant of this parameter. In the limit of no donor interaction, then, of course, there is only one possible step, after which there is complete deexcitation. That is, all the initial excitation has been either transferred to the acceptor, or otherwise lost (fluorescence or quenching). If the donor coupling is very strong, the probability is that the energy will be transferred back and forth among the donors before finally being dissipated. Many steps will then be necessary to follow this system to a state where the excitation is removed completely from the donor network.

Two different values for R_0 (DD) were used in these representative calculations, with R_0 (DA) fixed at 1.0: (1) R_0 (DD) = 0.5; (2) R_0 (DD) = 1.0.

The results are given in Table II. Note that, as before, the overall effect of donor–donor interaction is to increase the observed average transfer efficiency. The results are obviously sensitive to the choice of R_0 (DD). It is interesting to note that when R_0 (DD) = 0.5 there is only a minor effect on the average transfer due to donor–donor interaction. The relative distances and interaction strengths are not unrealistic. However, there are dramatic changes seen when the R_0 (DD) is doubled to become more competitive with the donor–acceptor interaction. This is especially true for configuration a where so much energy is essentially drained from the two distant donors to the closest and from here to the acceptor. Such a strong coupling may apply to donors such as fluorescein where the extinction coefficient is large and the Stokes shift is small.

TABLE I: Energy Transfer from Two Donors to One Acceptor. Model II.[a]

Position No.	θ_1	θ_2	$\bar{E}$ R_0 (DD) = 0; R_0 (DA) = 1.5	$\bar{E}$ R_0 (DD) = 1.0; R_0 (DA) = 1.5
1	120	60	0.388	0.465
2	120	180	0.070	0.075
3	120	300	0.388	0.391
4	240	60	0.388	0.391
5	240	180	0.070	0.071
6	240	300	0.388	0.465
7	360	60	0.839	0.878
8	360	180	0.522	0.529
9	360	300	0.839	0.878
			Average = 0.433	Average = 0.460

[a] See Figure 7b.

TABLE II: Three Donors—One Acceptors. Transfer Efficiencies.

Configuration[a]	R_0 (DA)	R_0 (DD)	D1	D2	D3	Average Transfer Efficiency
a	1.0	0.0	0.984	0.095	0.095	0.392
a	1.0	0.5	0.984	0.108	0.108	0.400
a	1.0	1.0	0.970	0.511	0.511	0.664
b	1.0	0.0	0.136	0.892	0.892	0.640
b	1.0	0.5	0.156	0.891	0.891	0.646
b	1.0	1.0	0.597	0.864	0.864	0.775

[a] See Figure 9.

It is impossible to generalize from a system so critically dependent on several parameters. It is better to calculate the effect for each specific case of interest as the need arises. These calculations do, however, give a feeling for the magnitudes of the effects that can be expected and the situations where they become important. Many cases can be ruled as insensitive to the effects of donor interaction by examining the measured magnitude of that interaction in relation to the rest of the system. Experimentally fluorescence depolarization experiments can be helpful in verifying such an interaction. In those cases where donor interaction is substantial it is clear that possibly large effects can result.

Discussion

The calculations reported here show that random labeling of proteins with fluorescent dyes can yield important information, particularly about protein size and the distance between proteins in a complex. Using reasonable values for R_0 and the protein radii we have shown that the expected energy transfer is of sufficient magnitude to make such experiments feasible in complex systems of biological importance. Furthermore, if all the assumptions on which we have premised our calculations hold reasonably well, then such experiments can be used for more than an "all or nothing" result; semiquantitative answers should be possible. The calculations show the importance of using relatively large number of acceptors. They also show that by varying the ratio of acceptors to donors, one can gain additional confidence in the experimental results. In the case of single proteins the molecular size (or volume) is the crucial parameter to be determined. By varying the degree of labeling of the acceptor and observing the transfer efficiency the result should give (as in Figure 1) the radius of the protein (or its equivalent sphere). If the data deviate in a substantial manner from one of the predicted curves it would indicate nonrandom labeling. One might then learn something about the distribution of reactive groups on the protein surface. See for example the results on horseradish apoperoxidase in the accompanying paper (Gennis *et al.*, 1972).

For systems with more than one protein, singlet energy transfer could be used to make a low-resolution map of the structure. In a three-protein system, measuring three distances would establish the geometrical arrangement of the subunits.

It should also be possible to detect conformational changes using these techniques. The denaturation of double-labeled proteins should be easily observable. Using a spherical model, changes in the energy transfer would be related to the changes of the protein radius.

These methods will not only apply to single proteins but also to multiple protein complexes and even larger particles such as the ribosomal 30S particles. Double labeling of this particle with a dye pair of sufficiently large R_0 would be useful in helping to analyze any conformational changes associated with its functions.

These calculations have provided a framework on which experiments can be designed. They have indicated what can and cannot be expected from such experiments and have clearly shown ways of optimizing the chances of success.

Appendix

Energy Transfer between Two Interacting Donors (D1 and D2) and One Acceptor (A). Consider the four possible paths the absorbed energy may follow: (I) absorbed by D1 and eventually transferred to A from D1; (II) absorbed by D1 and eventually transferred to A from D2; (III) absorbed by D2 and evenutally transferred to A from D2; (IV) absorbed by D2 and eventually transferred to A from D1.

There may be many steps between the initial absorption and final transfer to the acceptor. We know the probability of each event, however, for any one step. Let us define the following rate constant: k_{1A} and k_{2A}, the rates of transfer to A from D1 and D2, respectively; $k_{12} = k_{21}$, the rate of transfer between the two donors; k_{QF}, the sum of the rates of fluorescence and the rates of all the other ways of deexcitation of the donor. k_{QF} is assumed to be the same for both donors. We then define the probabilities for each alternative using these rate constants. For example, $P_{12} = k_{12}/(k_{12} + k_{1A} + k_{QF})$. Note that P_{12} need not equal P_{21} since k_{1A} and k_{2A} are independent. Consider the case where the energy is absorbed by D1 and also transferred to A *via* D1. One way in which this may happen is by direct transfer, the probability being given by P_{1A}. The next shortest path leading to this same result would be for the energy to jump to D2, then back to D1 and from D1 be transferred to A. The total probability of this event is given by the simple product $(P_{12}P_{21})P_{1A}$. Clearly, the next shortest path will have a total probability of $(P_{12}P_{21})(P_{12}P_{21})P_{1A}$. Taking all the possbilities into account gave us the following probability for case I

$$E_I = P_{1A} + (P_{12}P_{21})P_{1A} + (P_{12}P_{21})^2P_{1A} + \cdots = P_{1A}\sum_{N=0}^{\infty}(P_{12}P_{21})^N \quad \text{(A-1)}$$

A similar treatment of the other three cases yields the following results

$$E_{II} = P_{12}P_{2A}\sum_{N=0}^{\infty}(P_{12}P_{21})^N \quad \text{(A-2)}$$

$$E_{III} = P_{2A}\sum_{N=0}^{\infty}(P_{12}P_{21})^N \quad \text{(A-3)}$$

$$E_{IV} = P_{21}P_{1A}\sum_{N=0}^{\infty}(P_{12}P_{21})^N \quad \text{(A-4)}$$

The probability of transferring energy from either of the donors to the acceptor will be the sum of these probabilities

divided by two to normalize the fact that two donors are absorbing the energy. Using the relation $\sum_{N=0}^{\infty} X^N = 1/(1 - X)$, we finally get eq 10.

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Singlet Energy-Transfer Studies on Associating Protein Systems. Distance Measurements on Trypsin, α-Chymotrypsin, and Their Protein Inhibitors†

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ABSTRACT: Singlet–singlet energy transfer has been measured between fluorescent dyes covalently attached to single proteins and to trypsin–trypsin inhibitor complexes. To maximize the probability of surface labeling, Celite-bound reactive dyes were used. These included fluorescein and Rhodamine B isothiocyanates and dansyl chloride. The Celite technique permits the production of heavily labeled proteins which retain nearly full activity. The experimental energy-transfer results are in excellent agreement with calculations which assume random surface labeling of spherical proteins with known anhydrous radii and a Poisson distribution of the degree of labeling. The experiments on complexes of trypsin with lima and soya bean trypsin inhibitors show that singlet energy transfer is a practical and simple method for determining the distance between specific proteins in a large protein complex. Extrinsic fluorescence labels are capable of providing semiquantitative distance information. Preliminary energy transfer results indicate that trypsin and chymotrypsin form a stable complex at low concentration. The experimental protocol and interpretive framework should be easily generalizable to a variety of complex systems. This rapid and sensitive technique will be of general utility in studies of the size, spatial arrangement, stoichiometry, and kinetics of associating macromolecules.

The use of singlet energy transfer to investigate qualitatively the distances between known sites on a macromolecule (Beardsley and Cantor, 1970) or changes in conformation upon complex formation by proteins (Edelhoch and Steiner, 1965; Millar *et al.*, 1962) is well established. Quantitative measurements of distances by energy transfer have been made only indirectly and only on small molecules (Stryer and Haugland, 1967).

The method of singlet energy transfer seems elegantly suited to the study of the stoichiometry, conformation, and

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