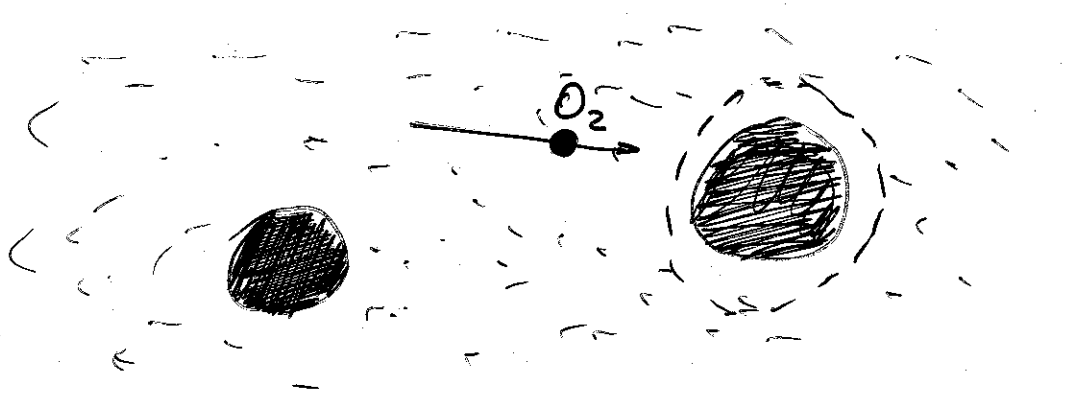
Fluorescence quenching

Molecular oxygen is a ground state triplet. Collision of oxygen with an excited state singlet leads to an enhancement of singlet to triplet conversion with subsequent quenching of the fluorescence.

When a molecule is free in solution it is very accessible to collisions with molecular oxygen. When the fluorescent group is inside a protein it is partially shielded. This effect can be used to discriminate residues on the protein surface from internal residues.

A different quencher is I_2^-. This molecule is charged. It is sensitive to the charge near the emitting group.

Consider the intensity of emission in the presence and in the absence of quencher.



We have seen that the fluorescence intensity is given by

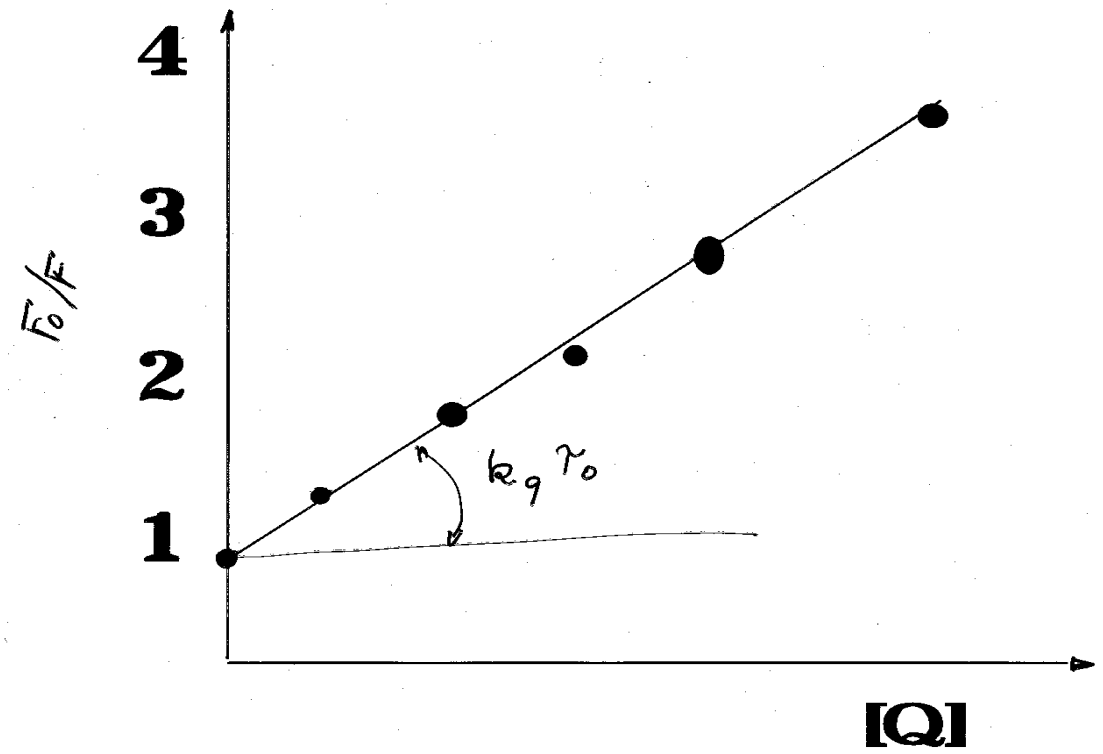
$$F \propto \Phi = \frac{k_F}{k_F + k_{ic} + k_{is} + k_q[Q]}$$

The ratio of fluorescence intensity with and without the quencher is

$$\frac{F_0}{F} = \frac{\Phi_0}{\Phi} = \frac{k_F + k_{ic} + k_{is} + k_q[Q]}{k_F + k_{ic} + k_{is}} = 1 + \frac{k_q[Q]}{k_F + k_{ic} + k_{is}} = 1 + k_q \tau_0 [Q]$$

This is called the Stern-Volmer equation.

Because τ_0, can be measured, the collision quenching rate k_q can be obtained from the plot of F_0/F versus [Q].



Typical values of k_q are on the order of 10^{+10} s^{-1} in aqueous solutions. This value is characteristic of a diffusion-controlled reaction

When the fluorescent residue is inside the protein, k_q is smaller

Table 8-4

Quenching of tryptophan fluorescence by collision with small molecules

Protein	Native protein (0.1 M phosphate, pH 7) $k_q \times 10^{-9}$ M^{-1} sec^{-1}		Denatured protein (6 M guanidine-HCl added) $k_q \times 10^{-9}$ M^{-1} sec^{-1}	
	Oxygen	Iodide	Oxygen	Iodide
Tryptophan	12.0	3.9	5.9	1.9
n-Ac-Try-NH_2	11.6	3.8	7.3	2.1
Pepsin	5.7	1.7	4.3	1.8
Trypsinogen	4.3	0.3	6.1	1.2
Carboxypeptidase A	3.8	0.3	3.8	1.1
Carbonic anhydrase	2.6	0.2	4.2	1.0

SOURCE: Adapted from J. R. Lakowicz and G. Weber, *Biochemistry* 12:4171 (1973).

However, the fact that oxygen can diffuse inside a protein with a rate which is 1/3 of the diffusion-controlled rate is a very intriguing result!

Static Quenching

In some cases, the fluorophore can form a stable complex with another molecule. If this *ground-state* is non-fluorescent then we say that the fluorophore has been statically quenched.

In such a case, the dependence of the fluorescence as a function of the quencher concentration follows the relation:

$$F_0/F = 1 + K_a[Q]$$

where K_a is the association constant of the complex. Such cases of quenching via complex formation were first described by Gregorio Weber.

THE QUENCHING OF FLUORESCENCE IN LIQUIDS BY COMPLEX FORMATION. DETERMINATION OF THE MEAN LIFE OF THE COMPLEX.

By G. Weber.

Received 8th July, 1946, as revised 25th April, 1947.

If a quencher is added to a solution of a fluorescent dye the ratio of the fluorescent intensities before and after quenching is [1, 2]

$$I_0/I = N_0\tau_0/N\tau \quad . \quad . \quad . \quad . \quad (1)$$

where N_0 and N are the numbers of excited molecules that may be de-activated with emission in the absence and presence of quencher respectively, and τ_0 and τ the corresponding values of the mean life of the excited state. Only in an ideally collisional quenching is $N/N_0 = 1$. In the quenching by complex formation we may assume that the molecules forming part of the complex are unable to emit. Then $N/N_0 = \alpha$, the degree of dissociation of the complex. The new mean life of the excited state of the fluorescence after addition of quencher is

$$\tau = \frac{1}{\left(\frac{1}{\sigma} + \frac{1}{\tau_0}\right)} \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

where σ is the mean free life of the fluorescent molecules. In general, the degree of dissociation of the complex cannot be directly determined, but α can be eliminated from eqn. (1). Calling the mean life of the complex Σ, and the total concentration of dye [F], at a definite concentration of quencher an equilibrium exists of the form

$$1/\sigma \,.\, \alpha[F] = 1/\Sigma \,.\, (1 - \alpha)[F] \quad . \quad . \quad . \quad (3)$$

where the left-hand side represents the number of complexes formed in unit time and the right-hand side the number broken down in the same period. Replacing $1/\sigma$ by its value given in eqn. (2), solving for $1/\alpha$ and introducing this in (1), we have

$$I_0/I = \tau_0/\tau[1 + \Sigma/\tau_0(\tau_0/\tau - 1)] \quad . \quad . \quad . \quad (4)$$

It is evident that if $\Sigma \ll \tau_0$

$$I_0/I = \tau_0/\tau \quad . \quad . \quad . \quad . \quad . \quad (5)$$

a rule proposed by Perrin [1] for the quenching by collisions. Eqn. (4) shows that Σ/τ_0 can be determined if the ratios I_0/I and τ_0/τ are known. The first can be easily obtained by photometric measurements while the second can be determined from the polarisation of the fluorescent light. According to Perrin's theory of the polarisation of the fluorescence in liquids [3, 4]

$$\frac{\tau_0}{\tau} = \frac{\frac{1}{p_i} - \frac{1}{p_0}}{\frac{1}{p} - \frac{1}{p_0}} \quad . \quad . \quad . \quad . \quad . \quad (6)$$

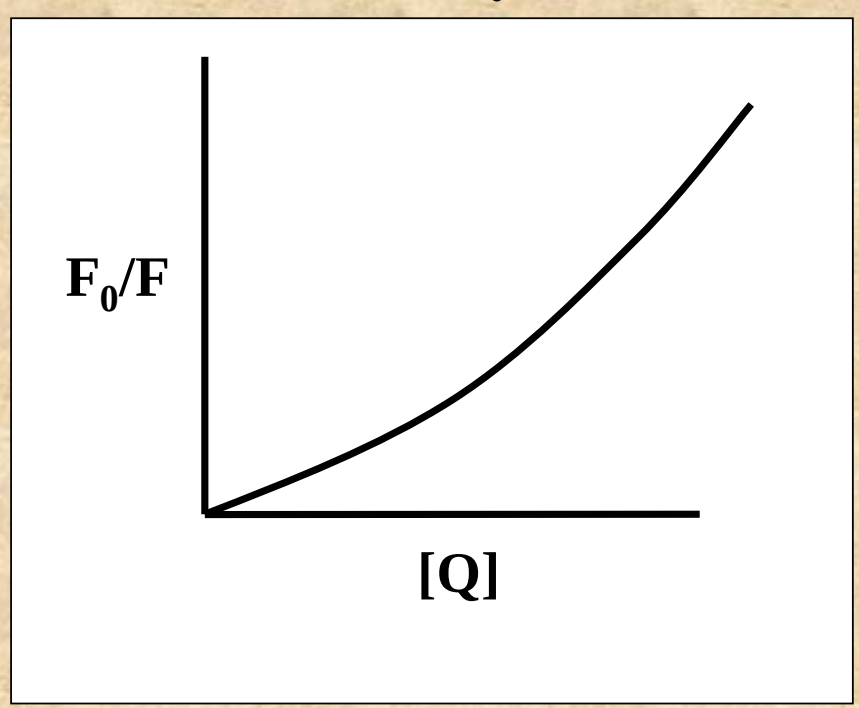
[1] Perrin, *Ann. Physique*, 1929, **12**, 169.
[2] Wawilov and Franck, *Z. Physik.*, 1931, **69**, 100.
[3] Perrin, *J. Physique*, 1926, **7**, 390.
[4] Sveshnikoff, *Acta Physicochim.*, 1936, **4**, 453.

In the case of static quenching the lifetime of the sample will not be reduced since those fluorophores which are not complexed – and hence are able to emit after excitation – will have normal excited state properties. The fluorescence of the sample is reduced since the quencher is essentially reducing the number of fluorophores which can emit.

If both static and dynamic quenching are occurring in the sample then the following relation holds:

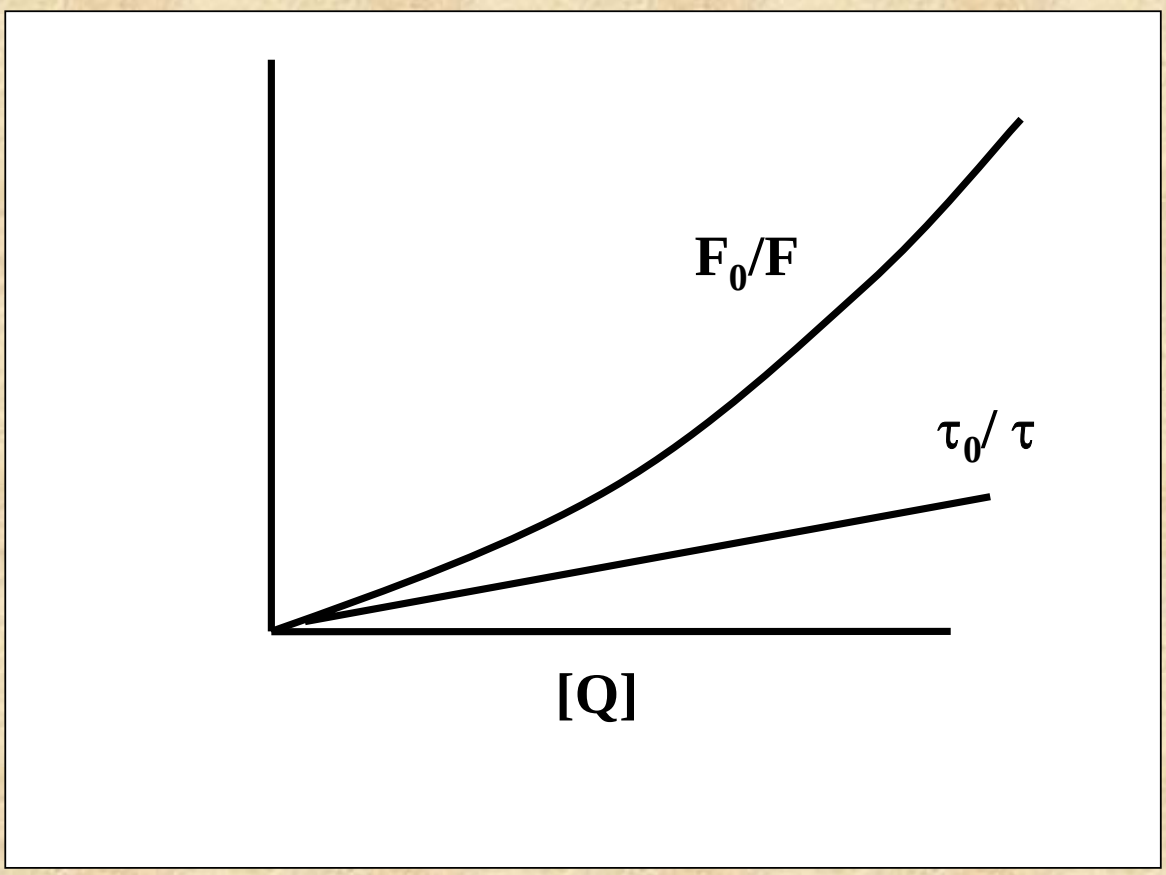
$$F_0/F = (1 + k_q \tau[Q]) (1 + K_a[Q])$$

In such a case then a plot of F_0/F versus [Q] will give an upward curving plot



The upward curvature occurs because of the $[Q]^2$ term in the equation

However, since the lifetime is unaffected by the presence of quencher in cases of pure static quenching, a plot of τ_0/τ versus [Q] would give a straight line



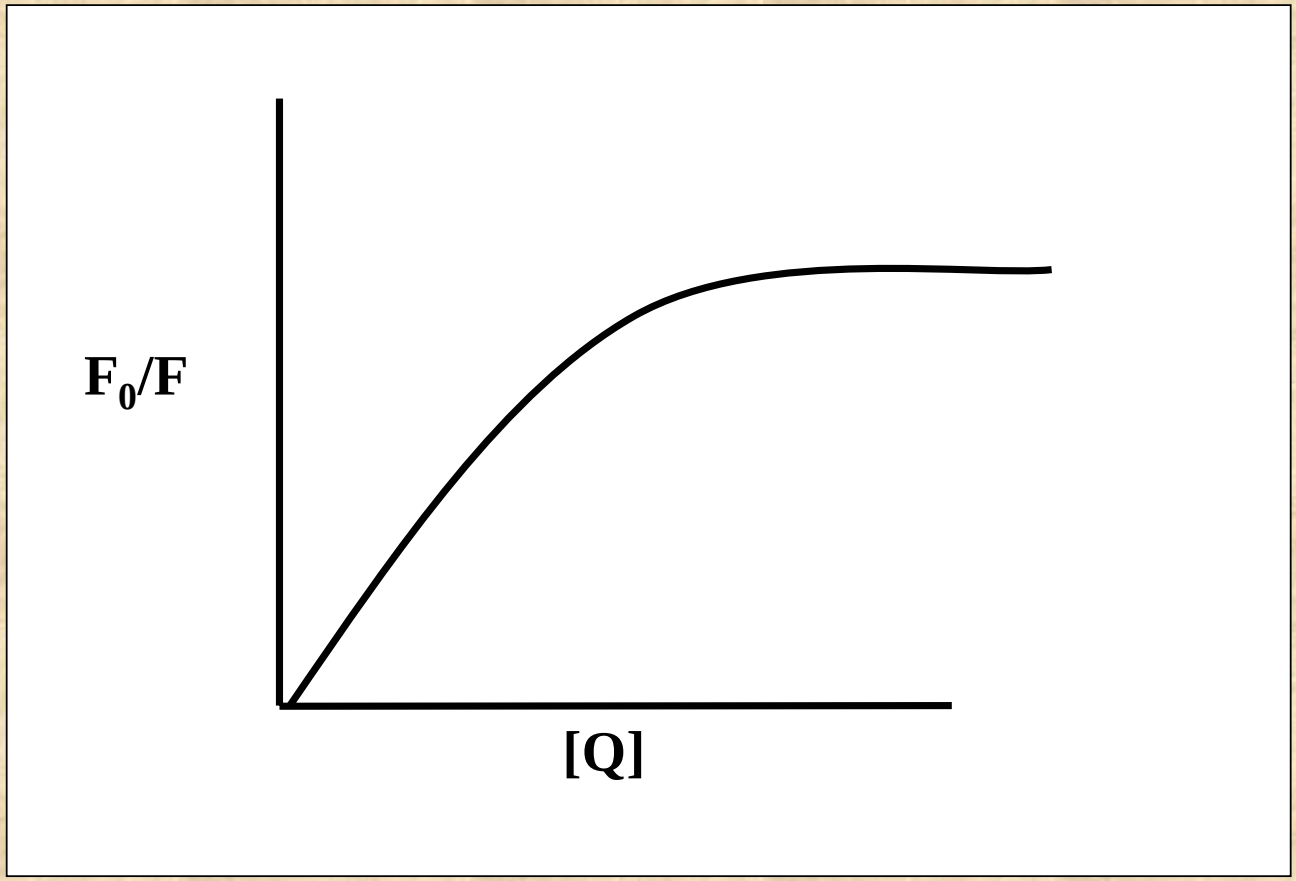
Sometimes you will see the equation for simultaneous static and dynamic quenching given as:

$$F_0/F = (1 + K_{SV}[Q])\mathbf{e}^{\mathbf{V[Q]}}$$

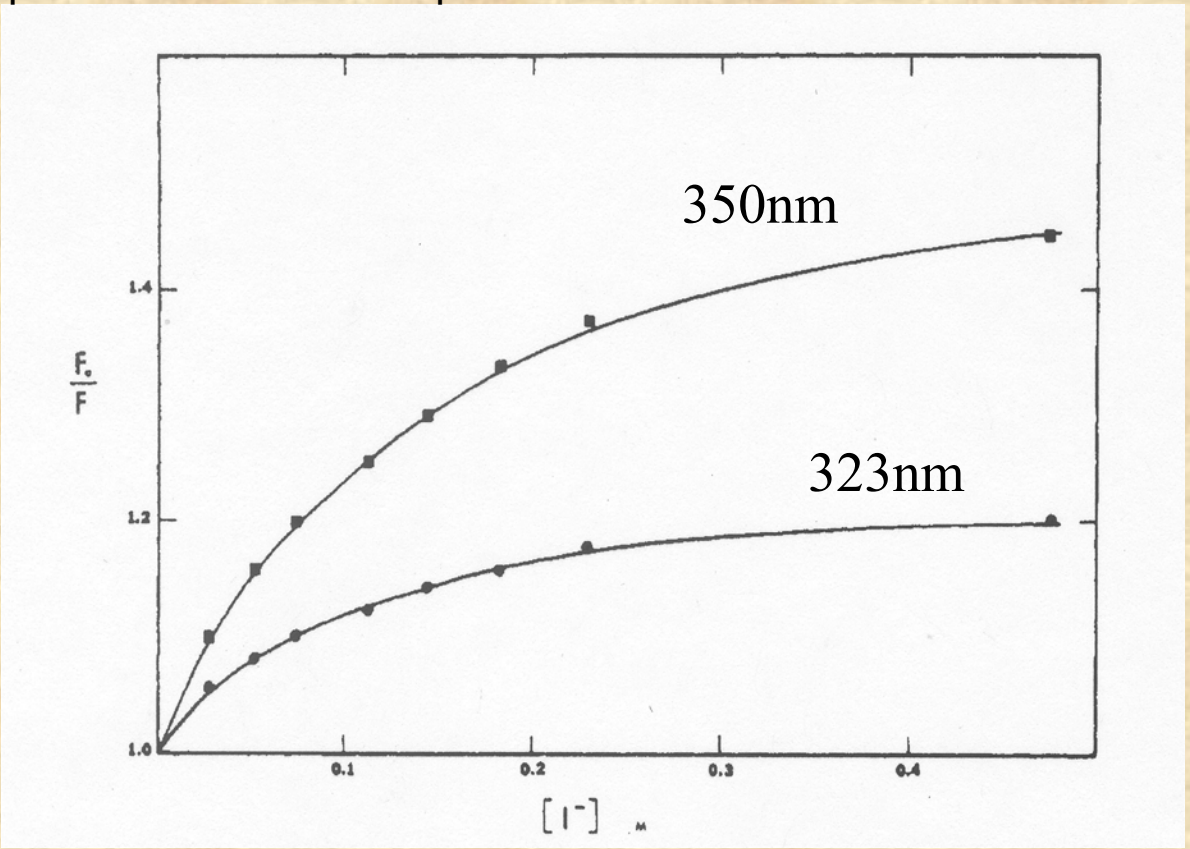
where the term $\mathbf{e}^{\mathbf{V[Q]}}$ is used as a phenomological descriptor of the quenching process. The term V in this equation represents an *active volume* element around the fluorophore such that any quencher within this volume at the time of fluorophore excitation is able to quench the excited fluorophore.

Non-linear Stern-Volmer plots can also occur in the case of purely collisional quenching if some of the fluorophores are less accessible than others. Consider the case of multiple tryptophan residues in a protein – one can easily imagine that some of these residues would be more accessible to quenchers in the solvent than other.

In the extreme case, a Stern-Volmer plot for a system having accessible and inaccessible fluorophores could look like this:



The quenching of LADH intrinsic protein fluorescence by iodide gives, in fact, just such a plot. LADH is a dimer with 2 tryptophan residues per identical monomer. One residue is buried in the protein interior and is relatively inaccessible to iodide while the other tryptophan residue is on the protein's surface and is more accessible.



In this case (from Eftink and Selvidge, Biochemistry 1982, 21:117) the different emission wavelengths preferentially weigh the buried (323nm) or solvent exposed (350nm) trytptophan.

Föster Resonance Energy Transfer (FRET)

An excited singlet can transfer energy to another absorbing group even if this group is far away. This process has very important consequences,

Description of the process

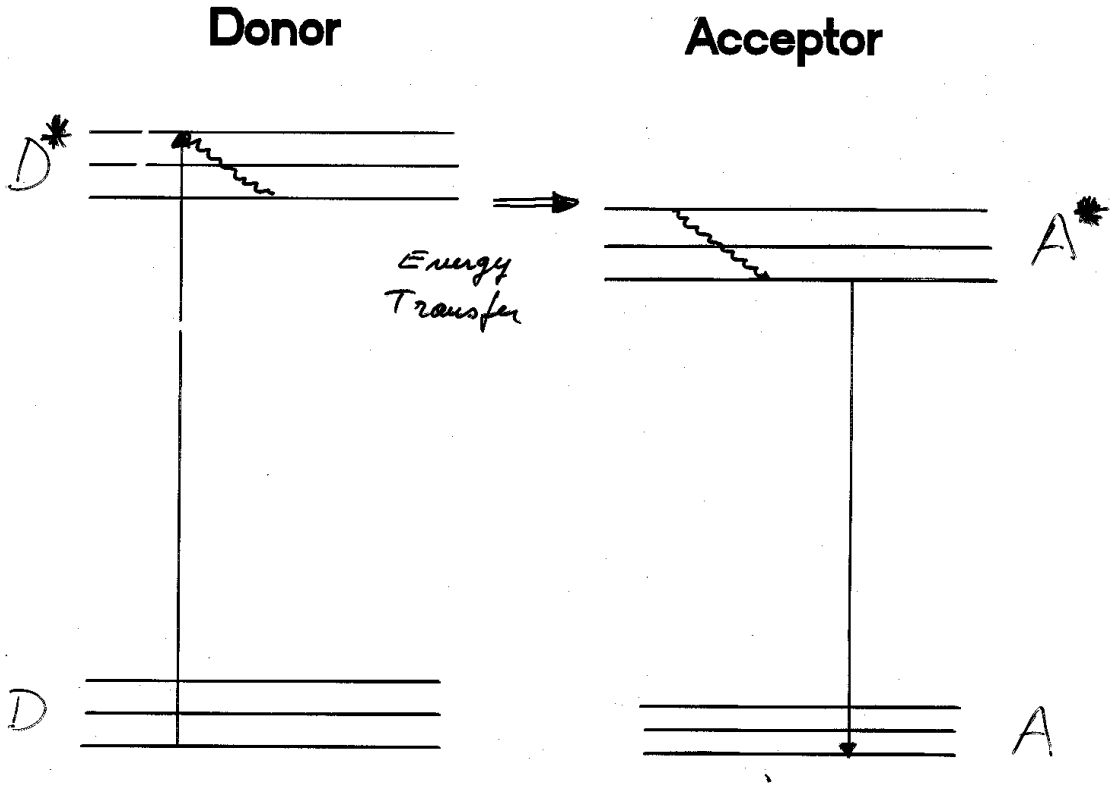
Consider a group called donor D which absorbs light. After absorption, because of the fast internal conversion, the molecule will be at the bottom (lower vibrational state) of the excited state.

If the donor emission energy coincides with the absorption energy of a different molecule called acceptor A, the following resonance process can take place.

$$D^* + A \underset{k_{-T}}{\overset{k_T}{\rightleftharpoons}} D + A^*$$

The energy transfer occurs at a rate k_T.

The acceptor then rapidly decays to the bottom of the excited state. From this level the acceptor molecule can decay by fluorescence emission or by non-radiative processes



Because of rapid internal conversion the process A* to D* is very unlikely to occur unless the donor is of the same kind of the acceptor.

This process is called **sensitized emission** of the acceptor A.

This process strongly depends on the distance between the two groups.

The complete theory was developed by Föster. Sometimes this process is called Föster cycle. Foster calculated the rate of transfer to be

$$k_T = \frac{1}{\tau_D}\left(\frac{R_0}{R}\right)^6$$

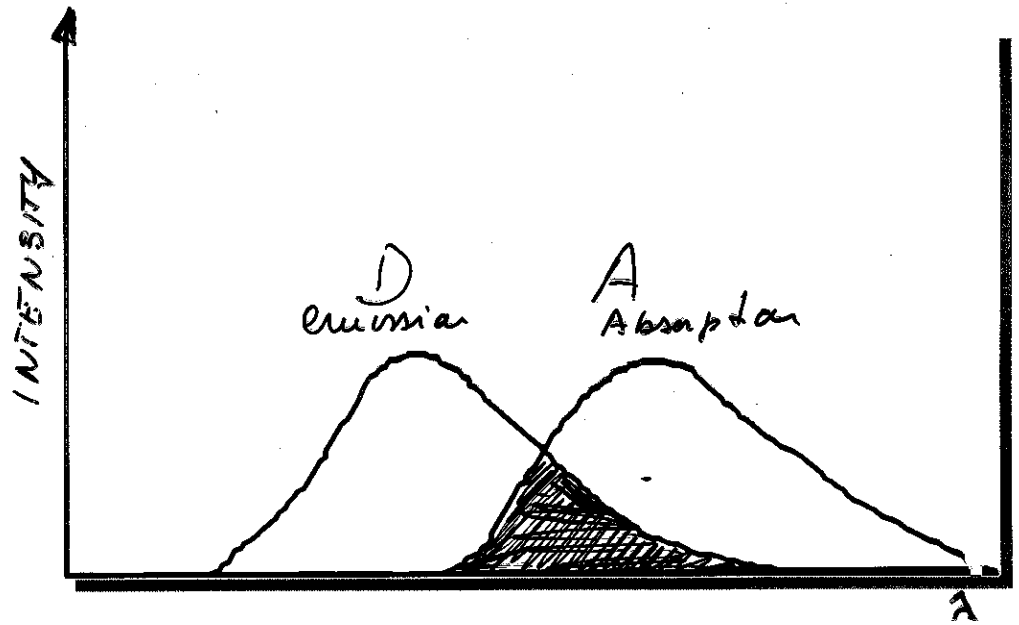
τ_D is the lifetime of the donor in the absence of the acceptor.

R is the distance between the two groups

R_o is called the Föster characteristic distance

$$R_0 = 9.7x10^3\left(J\kappa^2 n^{-4}\Phi_D\right)^{1/6} \quad \text{(in cm)}$$

J is a measure of the spectral overlap



n is the refractive index of the medium between acceptor and donor

Φ_D is the quantum yield of the donor

κ^2 is a complex geometrical factor which depends on the relative orientation of donor and acceptor.

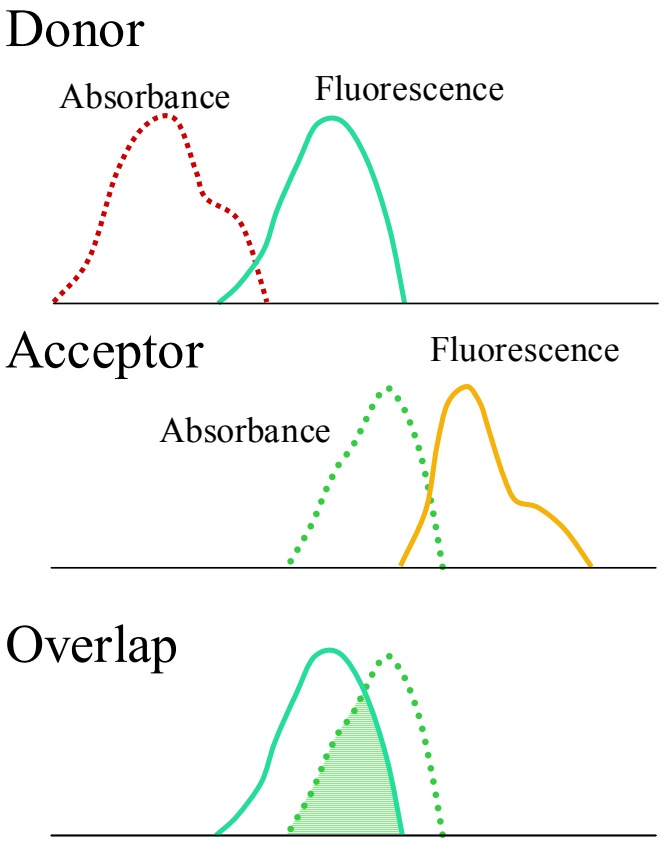
The overlap integral *J* is defined by:

$$J = \int_0^\infty f_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$$

Where λ is the wavelength of the light, $\varepsilon_A(\lambda)$ is the molar extinction coefficient at that wavelength and $f_D(\lambda)$ is the fluorescence spectrum of the donor normalized on the wavelength scale:

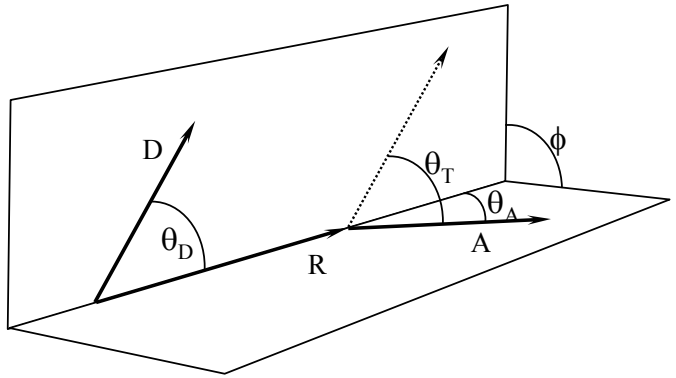
$$f_D(\lambda) = \frac{F_{D\lambda}(\lambda)}{\int_0^\infty F_{D\lambda}(\lambda)d\lambda}$$

Where $F_{D\lambda}(\lambda)$ is the donor fluorescence per unit wavelength interval



The orientation factor κ^2

$$\kappa^2 = (\cos\theta_T - 3\cos\theta_D \cos\theta_A)^2$$



Where θ_T is the angle between the D and A moments, given by

$$\cos\theta_T = \sin\theta_D \sin\theta_A \cos\phi + \cos\theta_D \cos\theta_A$$

In which θ_D, θ_A are the angles between the separation vector R, and the D and A moment, respectively, and ϕ is the azimuth between the planes (D,R) and (A,R)

The limits for κ^2 are 0 to 4, The value of 4 is only obtained when both transitions moments are in line with the vector R. The value of 0 can be achieved in many different ways.

If the molecules undergo fast isotropic motions (dynamic averaging) then $\kappa^2 = 2/3$

Energy transfer studies give information

- Distance between groups
- Orientation of two groups
- The refractive index between two groups

Notice that D and A can be the same kind of molecule provide emission and absorption overlap.

How to perform an energy transfer experiment?

1. Define

E = efficiency of transfer

$$E = \frac{k_T}{k_T + k_F^D + k_{IC}^D + k_{IS}^D} \leq 1$$

where D refers to donor

Energy transfer efficiency (E) $$E = \frac{k_T}{k_T + \sum_{i \neq T} k_i}$$

Where k_T is the rate of transfer and k_i are all other deactivation processes.

Experimentally, E can be calculated from the fluorescence lifetimes or intensities of the donor determined in absence and presence of the acceptor.

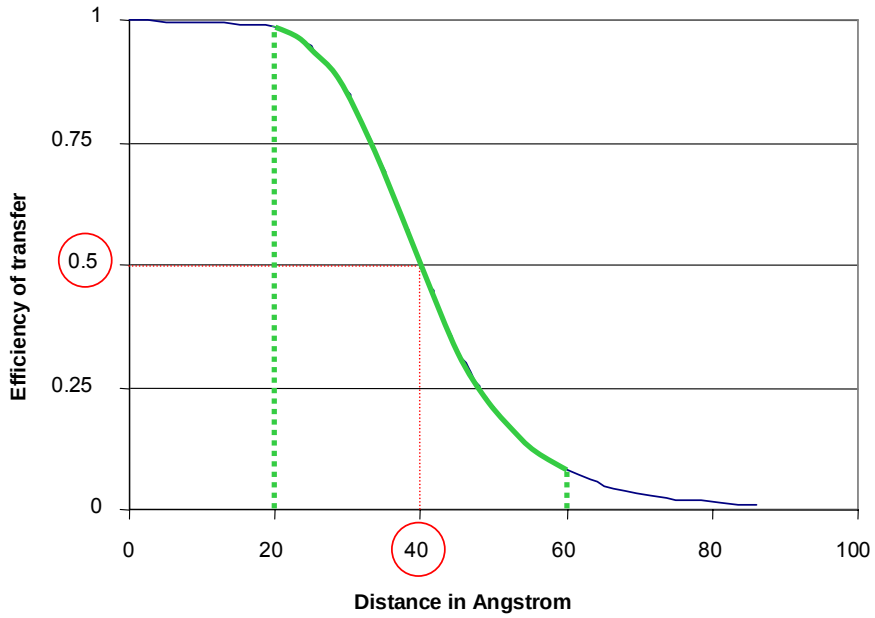
$$E = 1 - \frac{\tau_{da}}{\tau_d} \quad \text{or} \quad E = 1 - \frac{F_{da}}{F_d}$$

Distance dependence of the energy transfer efficiency (E)

$$R = \left(\frac{1}{E} - 1 \right)^{1/6} R_0$$

Where R is the distance separating the centers of the donor and acceptor fluorophores, R_0 is the Förster distance.

The efficiency of transfer varies with the inverse sixth power of the distance.



R_0 in this example was set to 40 Å.
When the E is 50%, $R=R_0$

Distances can generally be measured between ~0.5 R_0 and ~1.5R_0

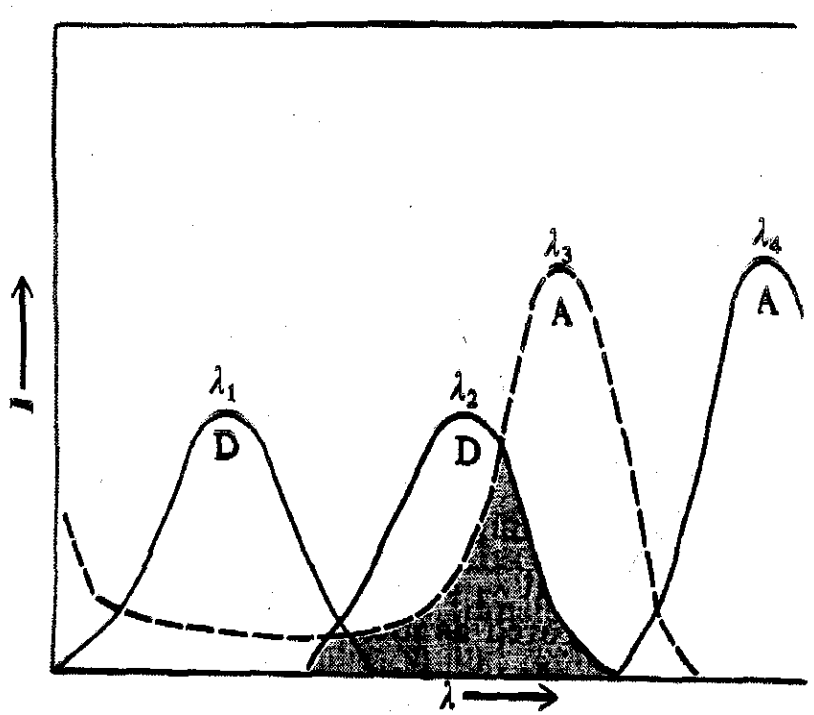
2. Prepare two samples: One containing only the donor, the other containing donor and acceptor. If the concentration of the donor is the same, the intensity of the fluorescence of the donor can be measured and compared in both samples.

$$\frac{\Phi_{D+A}}{\Phi_D} = \frac{\dfrac{k_F^{D^*}}{k_F^{D^*} + k_{IC}^{D^*} + k_{IS}^{D^*} + k_T}}{\dfrac{k_F^D}{k_F^D + k_{IC}^D + k_{IS}^D}}$$

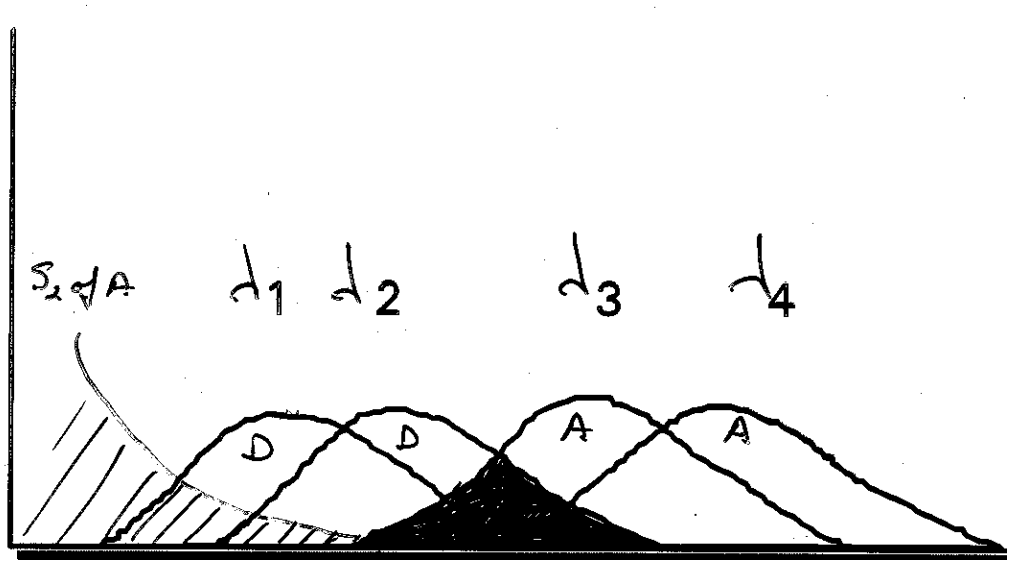
If we assume that the only influence of the acceptor is to introduce the k_T term, and that there is no direct interaction between donor and acceptor then D*= D

$$\frac{\Phi_{D+A}}{\Phi_D} = \frac{k_F^D + k_{IC}^D + k_{IS}^D + k_T - k_T}{k_F^D + k_{IC}^D + k_{IS}^D + k_T} = 1 - E$$

3. If the acceptor is fluorescent, then it is possible to measure the intensity of the acceptor fluorescence in the absence and presence of the donor.



Consider the following configuration



The donor absorbs at λ_1 and emits at λ_2. The acceptor absorbs at λ_3 and emits at λ_4

The acceptor absorption band may superimpose to the absorption of D.

The sample is excited at λ_1. The fluorescence at λ_4 of the acceptor can be observed in the absence of the donor to check if there is any contribution of direct acceptor excitation

$$F_{A_{\lambda_1,\lambda_4}} \cong \varepsilon_{A_{\lambda_1}} C_A \Phi_{A_{\lambda_4}}$$

In presence of the donor the total fluorescence intensity at λ_4 is

$$F_{A+D_{\lambda_1,\lambda_4}} \cong \varepsilon_{A_{\lambda_1}} C_A \Phi_{A_{\lambda_4}} + \varepsilon_{D_{\lambda_1}} C_D E \Phi_{A_{\lambda_4}}$$

measuring F_A and F_{A+D}, the energy transfer efficiency E can be obtained

$$\frac{F_{D+A}}{F_A} = 1 + \frac{\varepsilon_D}{\varepsilon_A} \frac{c_D}{c_A} E$$

4. An independent measurement of E can be obtained by comparing the decay time of the donor in the presence and absence of the acceptor.

$$\frac{\tau_{D+A}}{\tau_D} = 1 - E$$

The efficiency of energy transfer can be written as

$$E = \frac{k_T}{k_T + \frac{1}{\tau_D}}$$

using equation

$$k_T = \frac{1}{\tau_D}\left(\frac{R_0}{R}\right)^6$$

we obtain

$$E = \frac{R_0^6}{R_0^6 + R^6}$$

For a given value of R_o, a measurement of E can give R. Notice the dependence on R^6.

The value of R_o depends on many factors, but it generally ranges from 5 to 50A.

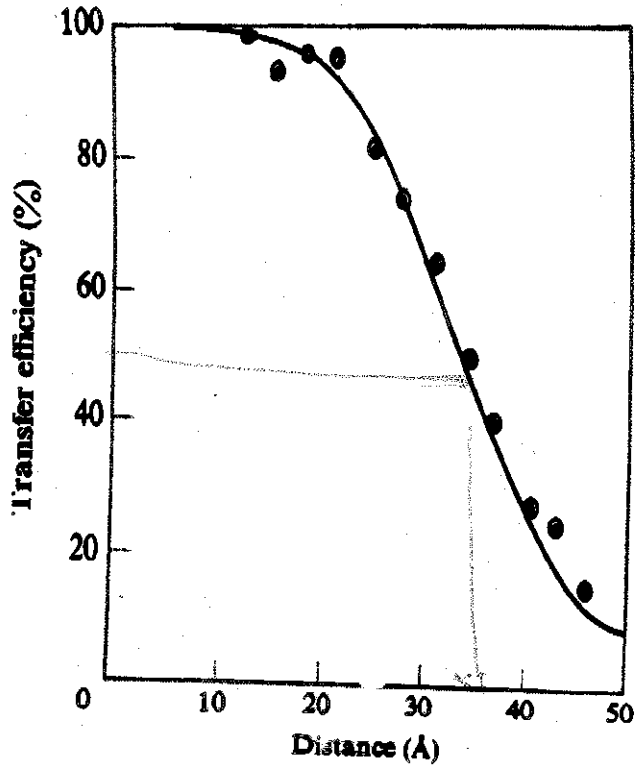


Figure 8-20

Efficiency of energy transfe
distance in dansyl-(L-prolyl
semicarbazide oligomers w
curve was fit to the data wi
[From L. Stryer and R. P.
Natl. Acad. Sci. USA 98:71

Dansyl - $(Pro)_n$ - NH - NH - CO - NH - Naphthyl

Spacer group.

Except in very rare case, κ^2 can not be uniquely determined in solution. What value of κ^2 should be used ?

1. We can **assume** isotropic motions of the probes and a value of $\kappa^2 = 2/3$, and verify experimentally that it is indeed the case.

***By swapping probes*:** The environment of the probe will be different and if κ^2 is not equal to 2/3, because orientations of the probes are not dynamically average (during the lifetime of the probe) due to restricted motions of the fluorophores, then the distance measured by FRET will be different.

By using different probes: If the distance measured using different probe pairs are similar (taking into account the size of the probes) then the assumption that κ^2 is equal to 2/3 is probably valid.

2. We can **calculate** the lower and upper limit of κ^2 using polarization data (Dale, Eisinger and Blumberg: 1979 Biophys. J. 26:161-93).

Elementary energy transfer theory

Different processes

a) **Trivial process**: Emission of a photon by D* and absorption by A

The probability of this process depends on R^{-2} and orientation. If A≠ D the apparent lifetime of D is not influenced by the presence of A.

b) **Radiationless energy transfer** D*→ A.

Give a system with two states ψ_1 and ψ_2 the transition probability between ψ_1 and ψ_2 is dependent on the square of the matrix element

$$\beta = \langle \psi_1 | V | \psi_2 \rangle$$

Resonance Integral

V is the interaction that causes transition

Assume D*A and DA* representing the two states. Several mechanisms can operate

1. **Coherent transfer**: the phases of the excitation of a number of molecules are correlated. This happens in molecular crystals with A=D

2. **Incoherent transfer**: the phase relation between excited molecules D* is lost rapidly. In solution dephasing occurs in few picosecond due to random interactions with the solvent

a. **Very weak- coupling** $|\beta| < \Delta E$

where ΔE is the amplitude of the energy fluctuations in the surrounding matrix (solvent), for example from thermally excited phonons. In this cage D* undergoes many internal vibrations before exchanging its electronic energy with A

b. **Weak coupling** $|\beta| \approx \Delta E$

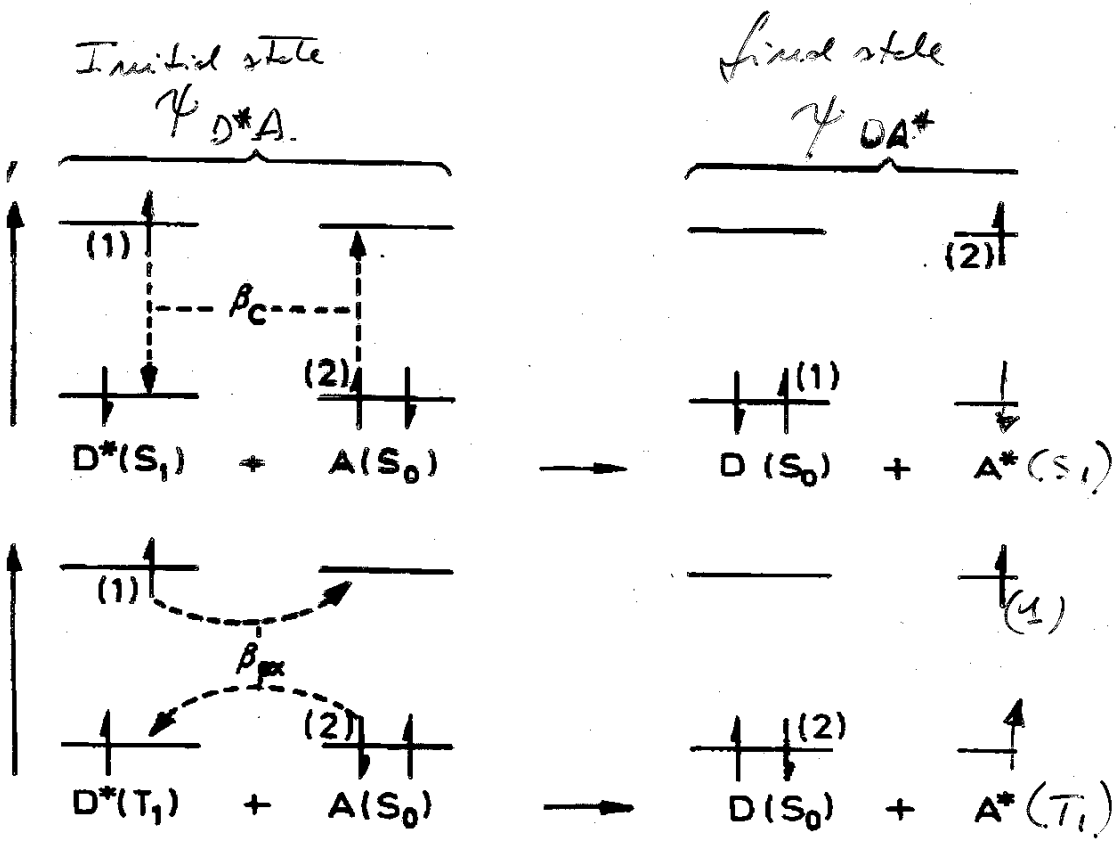
Only a few vibrations occur

c. **Strong Coupling**. The excitation energy cannot be thought as localized either in A* nor D*. Also the absorption spectrum is not the sum of A and D.

In biology only weak coupling occurs.

Föster Theory (1946).

Assume energy levels of D and A are the same. Relevant states as shown below



Assume only two electrons are involved in the process.

The relevant anti-symmetric normalized wavefuntions are

$$\psi_{D^*A} = \frac{1}{\sqrt{2}}(\psi_{D^*}{}^{(1)}\psi_A{}^{(2)} - \psi_{D^*}{}^{(2)}\psi_A{}^{(1)})$$

$$\psi_{DA^*} = \frac{1}{\sqrt{2}}(\psi_D{}^{(1)}\psi_{A^*}{}^{(2)} - \psi_D{}^{(2)}\psi_{A^*}{}^{(1)})$$

V is the coulombic interaction between electrons 1 and 2

$$V = \frac{1}{r_{12}}$$

$$\beta = \beta_c + \beta_{Ex}$$

$$\beta_c = Coulomb \quad term = \langle \psi_{D^*}(1)\psi_A(2) | V | \psi_D(1)\psi_{A^*}(2) \rangle$$

$$\beta_{Ex} = Exchange \quad term = \langle \psi_{D^*}(1)\psi_A(2) | V | \psi_D(2)\psi_{A^*}(1) \rangle$$

This is valid in the weak coupling case. Otherwise wavefunctions ψ_D and ψ_A are no longer appropriate

Spin selection rules require conservation of the spin of the system (D ... A)* and forbid transition $T_1 \leftrightarrow S_0$ in a molecule alone. Hence only the exchange mechanism can contribute to triplet D to triplet A transfer. For singlet-singlet transfer both exchange and coulombic processes can occur.

The exchange process is effective only if there is spatial overlap of wavefunctions of D* and A. This case can occur only during contact of D* with A* (typically below 5Å)

Consider only the coulomb term. Do multipole expansion of 1/r. The dipole term is dominant.

$\beta_c = \beta_{\text{dipole-dipole}} \approx M_D M_A R^{-3} n^{-2}$

Where M_D and M_A are the transition moments of donor and acceptor, R is the distance and n the index of refraction at optical frequencies. (Only if M_D or M_A are zero, quadrupole or other higher order terms can be important).

Apply Fermi's "golden rule" to calculate transition rate constant.

$$k_{D^* \to A} \cong \frac{2\pi}{\hbar} |\beta|^2 f$$

f is a measure of the density of interaction between the initial and final state of the real system.

If there is only one acceptor at a distance R from the donor:

a) for exchange $$k_{D^* \to A} \cong \frac{1}{\tau_D} e^{-\gamma(R-R_0)}$$

b) for Coulomb $$k_{D^* \to A} \cong \frac{1}{\tau_D} \left(\frac{R_0}{R}\right)^6$$

$$R_0 = \frac{C\kappa^2 \Phi_D I}{n^4}$$

What happens in a matrix of donor and acceptors?

$$\phi(t) = e^{-AC_A\sqrt{t}}$$

The decay of the fluorescence intensity depends on the square root of time!