The truth, madam is ~~never pure~~ hardly pure
and never simple.
The Importance (O.W.)

Electromagnetic Radiation. Characterization.

Electromagnetic radiation, like any other periodic phenomenon can be characterized by its amplitude, or intensity, by its frequency ν and its phase ϕ.

The frequency is measured in waves per second. The number of waves per centimeter is the wavenumber $\bar{\nu}$, related to the frequency ν by the equation:

$$\bar{\nu} = \nu/c \qquad (1)$$

where c is the speed of light in the medium in which the waves propagate. The wavelength λ is the reciprocal of the wave number

$$\lambda = 1/\bar{\nu} = c/\nu \qquad (2)$$

While the frequency is independent of the medium of the propagation the wave number and the wavelength are not. The dependence of the velocity of propagation upon the medium gives rise to the phenomenon of dispersion, which is of fundamental importance in spectroscopy. The simplest harmonic motion may be described by the equation:

$$A = A_o \sin(2\pi\nu t + \phi) = A_o \sin(\omega_o t + \phi) \qquad (3)$$

or its equivalent forms

$$A = A_o \sin(2\pi ct/\lambda + \phi)$$

$$A = A_o \sin(2\pi c\bar{\nu} t + \phi)$$

The phase ϕ is important in phenomena involving diffraction, interference or image formation but it is of little interest in the phenomena of absorption and emission of light by molecules. The reason is to be found in the differences between 'coherent' and 'incoherent' [illegible] of which we shall talk further on in these lectures.

Photons

According to the quantum theory radiant energy is absorbed or emitted only in discrete amounts or quanta, also called photons in the case of light. The energy ϵ of the photon is related to the wave frequency by Planck's relation

$$\epsilon = h\nu = hc/\lambda = hc\bar{\nu} \qquad (4)$$

where h is Planck's constant and equals 6.625×10^{-27} ergs.sec.

In photochemical and other applications it is often convenient to deal with the energy carried by one mole of photons, thus referring the changes of energy occurring in absorption and emission to the energy content per mole, of the substance responsible for the absorption or emission. The energy in one such mole is the Einstein

$$N\epsilon = Nhc/\lambda \qquad (5)$$

with N = Avogadro's number. If the Einstein is expressed in kg.calories and the wavelength of the light in microns,

$$E = N\epsilon = 28.6/\lambda \quad = 1.24/\lambda \text{ e.v.} \qquad (6)$$

Equation (6) gives the enrgy in electron-volts, as well, when λ is in microns. For the center of the visible spectrum $\lambda = 0.5\ \mu$ and E= 56.2 kg.calories or 2.48 electron volts.

Range of wavelengths involved.

We shall be concerned with the absorption of electromagnetic energy with wavelengths of 0.2 μ (200 nm) to 1 μ (1,000 nm). Quanta of energy in the upper limit of this range carry sufficient energy to produce the dissociation of the absorbing molecule into radicals, often followed by irreversible chemical reactions. This follows from the fact th[at] the chemical bond energies are under 100 kg.cal. which corr[espond] following equation(6) to $\lambda = 0.286\ \mu$.

When the shortest wavelengths of this range are used the instability of the substances under study presents special problems which are not encountered at the longer wavelengths, where photochemistry is not such a general phenomenon. The longer wavelength limit is set at 1 μ, by the rarity of electronic transitions at wavelengths longer than these and by the very strong infrarred absorption of water, which restricts observation, at least in the case of biologically important molecules.

Absorption Phenomenology. Bier's law.

Let us assume that the absorbing elements, which are molecules dissolved in a transparent solvent, or molecules in the gas phase, are placed in a cuvet B of x cm. depth and illuminated by a bomogeneous beam of light of 1 cm^2 section. If a similar cuvet A, containing everything as in B except the absorbing molecules is placed in the beam, the intensity reaching a detector placed behind the cuvet may be identified with I_o the intensity of the illuminating beam by supposing that similar losses due to reflection. imperfection of the solvent transparency and scattering occur in A in the same way as in B. Therefore any further change in the intensity of the radiation incident upon B must be due to the absorbing elements themselves.

A stream of particles, photons pass through the cuvet and interact with the absorbing molecules. We shall associate a given area, or effective cross section, with every molecule in B. This area σ_m, is such that a photon is absorbed if it passes through it, but not otherwise. Consider an area of beam section of 1 cm^2, over a very small depth x, so that there are very few ~~emitting~~ elements in this volume. According to the definition of effective cross section the

fraction $\Delta I/I$ of the incident light absorbed in this depth must equal the fraction of the beam section covered by the effective cross section of the molecules. Therefore,

$$-\Delta I/I = \sigma_m L \Delta x \qquad (7)$$

where L is the number of absorbing molecules in 1 cm^3 of solution in B. Expressing the concentration in moles/liter, [c], we have

$$L = [c]\ N\ 10^{-3} = [c]N' \qquad (8)$$

From (7) and (8)

$$-\Delta I/I = \sigma_m\ [c]N'\ dx \qquad (9)$$

which integrated between x=x and x=0 gives

$$\ln(I_o/I) = [c]x \cdot \sigma_m\ N' \qquad (10)$$

The product $\sigma_m N' = \sigma_{mM}$ is obviously the milimolar cross-section, or cross section of one milimole. If decimal rather than natural logs are used

$$\log (I_o/I) = 0.434\ \sigma_{mM}\ [c]\ x = k\ [c] \cdot x \qquad (11)$$

The quantity $\log I_o/I$ is referred to as the absorbance, or optical density of the solution, while $\ln I_o/I$ is the much less used "natural optical density", a quantity 2.3 times larger then the absorpbance.

The molar absorbance k appearing in the last equations is evidently the absorbance of a solution of unit concentration (1 mole/lt) observed in unit depth (x=1 cm.). The dimensions of k or of σ_{mM} are both $cm^2\ mM^{-1}$ since they are clearly milimolar cross sections.

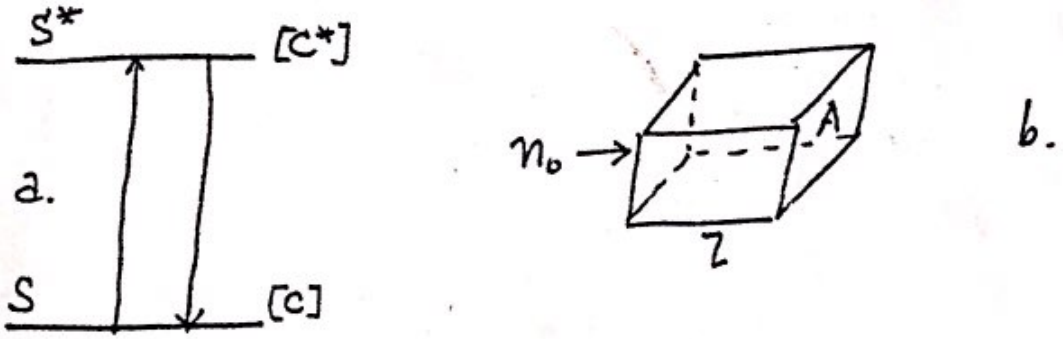
The molar absorbance of organic molecules in solution reach in the most extreme cases the magnitude of 10^5 cm^2/mM. This gives for the molecular cross section $\sigma_m = \sigma_{mM}/N'$ a value of app. 4.10^{-16} cm^2 or 4 $Å^2$. Since usually these molecules have physical cross sections of 15 $Å^2$ or more, it follows that in the usual case the effective cross

section is much smaller than the actual cross section only approaching it in exceptional cases. An equivalent statement is that "The probability of photon capture in a collision between photon and molecule is usually much less than unity ".

Equation (11) is the well known Biwr-Lambert law stating the linear dependence of the absorbance upon the concentration and the depth of the absorbing layer.

Independence of the absorbance from the intensity of the source.

It is an empirical fact that the intensity of the monochromatic radiation used to measure absorption xs may be varied over very wide range without appreciable changes in the observed absorbance. The possibility of such a change may be considered in relation to the system in the figure below



a. Energy levels. Ground level S and excited state S* ,and stationary concentrations under illumination : [c] and [c*]. b. Enclosure of homogeneously illuminated area A and length l containing the absorbing molecules

Upon absorption of light the molecules pass from the ground state S to the excited state S*. Since a steady value of the absorbance is obtained upon illumination the numbers of molecules passing from

S to S* by absorption must be compensated by the return of an equal number from S* to S . There must therefore exist stationary concentrations [c] and [c*], such that

$$k_{S-S^*} [c] = k_{S^*-S} [c^*] \qquad (12)$$

where k_{S-S^*} and k_{S^*-S} are respectively the rate constants giving the number of transitions per molecule per second in the two stated directions.

To calculate k_{S-S^*} consider an enclosure of A cm^2 cross section and length l, as shown in the previous figure in b, containing the illuminated molecules. A homogeneous stream of n_o photons per cm^2 per second enters the front face. The quanta emerging through the back face are

$$n = n_o A \exp - (\sigma_{mM} [c] l) \qquad (13)$$

The number n_a absorbed in the enclosure is therefore,

$$n_a = n_o A (1 - \exp-(\sigma_{mM} [c] l) \qquad (14)$$

the number of absorptions per second per molecule equals n_a/ molecules in the enclosure or

$$k_{S-S^*} = n_o A (1 - \exp-(\sigma_{mM} [c] l)/(Al [c] N') \qquad (15)$$

The number of absorptions per molecule, k_{S-S^*} is a maximum when the numerator has the highest value, and this will occur when

$$\exp-(\sigma_{mM} [c] l) \sim 1 - \sigma_{mM} [c] l$$

Introducing this into the last equation gives,

$$k_{S-S^*} = n_o \sigma_{mM} / N' = n_o \cdot \sigma_m \qquad (16)$$

The maximum number of absorption transitions depends therefore <u>only</u> upon the molecular cross section and the intensity of the illumination. As an example we may take a very powerful light source, like a 1,000 Watt

Xenon arc. No more than 20% of the power, or some 200 watts will be converted into light, the rest ~~dis~~appearing as heat. ~~The collecting~~ A beam of 5 cm diameter at 5 cm from the point source will capture 2% of this. system of lenses will not be able to capture more than ~~2-4~~ % of this reducing the imput to the enclosure to ~~4-8~~ watts. If we select the part of the spectrum for which σ_m has a large value, say 10^4 a few nanometers out of a continuous spectrum of some ~~4~~,000 nm. ~~We have reduced our~~ The effective input by a factor close to 10^3 leaving us with ~2 m ~~Less than 0.1~~ Harcourt watt. Assuming for the value of the Einsteim some 50 kg.cal our ~~0.~~1 watt will represent 5.10^{15} quanta. In the most favourable case $\sigma_m \sim 2.10^{-16}$ cm^2 which gives

$$k_{S-S^*} = \frac{1}{10^8} \text{ sec}^{-1}$$

As we shall see further on k_{S^*-S} is—for emission in the visible and ultraviolet regions—independent of the electromagnetic field and of the order of 10^8 sec^{-1} or greater. Therefore from equation (12)

$$[c]/[c^*] < 10^8$$

One molecule in one/100 million, often much less is in the excited state at any given time. There will be therefore no appreciable depopulation of the ground state and there cannot be any appreciable dependence of the absorbance upon the intensity of the excitation when light sources of ordinary intensity are used for absorption measurements. If very short, intense flashes are used it is possible to increase, for a short period lasting miliseconds the intensity of the illumination by a factor of 10^3 to 10^4. This would still be too small to depopulate appreciably the ground state were it not for the fact that a metastable state S^{**} may be reached from S^* and $k_{S^{**}-S}$ is of the order of 10^3–10^6 sec^{-1} rather than 10^8 sec^{-1} as is the case with k_{S^*-S}.

Master laser sources

* the linear dependence ~~between~~ concentration predicted by Bier's law.

Departures from Bier's law.

In practice it is often found that* Bier's law does not hold accurately. Some of the causes of failure are:

1. Broad band excitation.
2. Scattering
3. Fluorescence.
4. Molecular aggregation.

1. Broad band illumination. This is obvious and of relatively little importance but worth while remembering for the cases in which a solution ~~is illuminated~~ source with a continuous spectrum is used to illuminate a solution through a broad band filter, as is often done in photochemical experiments.

2. Scattering. To a certain extent scattering must always occur in B cuvet in excess of A due to the local fluctuations in the concentration of added molecules, but this it adds to an important contribution only in colloidal solutions, solutions of large particles, emulsions and other turbid media. Unfortunately the case presents itself often to the biochemist who works with partcles like mitochondria, microsomes or even large macromolecules. The scattering errors can be completely overcome by ensuring that the scattered, non-absorbed light reaches the detector. This may be best done by using an "integrating sphere". This is simply a box of any shape-not necessarily spherical- the inside of which is coated with a substance of very high reflectancce. and low absorptivity. In practice a coating with magnessiun oxide is used.

3. Fluorescence. In this case part of the absorbed light is reemitted at a different wavelength, and if it reaches the detector it decreases the apparent measured absorbance. Inpractice the errors

caused by fluorescence may be overcome by placing the light detector as far away as possible from the cuvet containing the solution. As the fluorescence has a almost spherical spatial distribution the amount of fluorescence reaching the detector decreases with the square of the distance from cuvet to detector. and is limited to the solid angle subtended by the effective aperture of the detector. It must be acknwledged that when very high optical densities, of the order of 3 or higher, which as sometimes the case with experiments in Biochemistry, are measured, small changes may be due to changes in fluorescence emission rather than absorbance, even when the precaution mentioned above has been taken. In these cases a more reliable way of elliminating fluorescence errors consists in the introduction of a second monochromator between cuvet and detector.

4. Molecular aggregation.

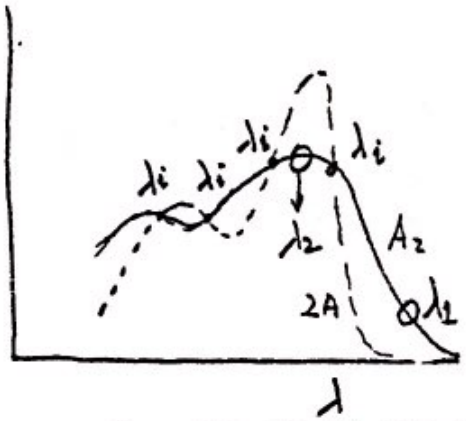
In contradistinction to the previous cases, which may be considered artifactual we have here an intrinsic cause of failure of Bier's law. Suppose a chromophore A capable of associating into dimers, so that we have in the solution an equilibrium,

$$A + A = A_2$$

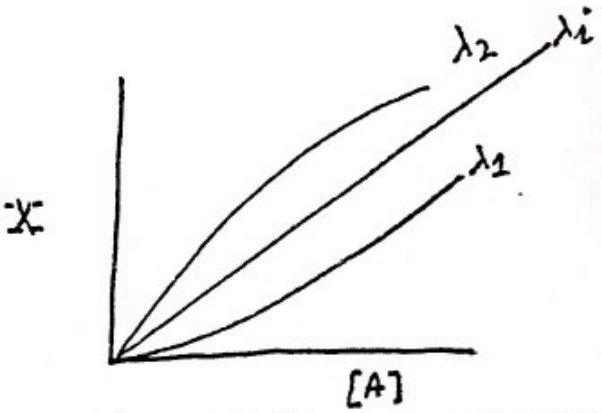
Suppose that A and A_2 have different absorption spectra. At wavelengths, those at which the molar absorbance of A_2 exceeds twice the molar absorbance of A an increase in molar absorbance with concentration will be observed over the range of concentrations within an order of magnitude of the dissociation constant of the dimer. The opposite will occur in those spectral regions in which the molar absorbance of A_2 is less than twice the molar absorbance of A.

molar of 2A and A_2

At the points of equal absorbance (isosbestic points) Bier's law will be obeyed. The effects are depicted in the figure below.



a. Spectra of 2A and of A_2



b. Plots of Absorbance X against concentration at different wavelengths.

The changes in sign of the observed effects with wavelength are very characteristic of molecular aggregation and may be used to test its presence in opposition to other causes of failure of Bier's law mentioned before.

Concentrations at which dimerization takes place

Oscillators.

In the process of light absorption the electromagnetic energy of the photon is converted into the potential energy of the absorbing atom or molecule.by a change in the relative possitions of nuclei and electrons with respect to those that characterize the minimum potential energy or "ground state" of the system. The atom or molecule may be represented by a devise the potential energy of which changes as a result of the periodic variation of the electromagnetic field of the light wave. In its simplest form such a devise-called an oscillator consists of a mass m which,if displaced from equilibrium becomes subjected to a restoring force proportional to its displacement x. Its equation of motion is therefore,

$$m\,(d^2x/dt^2) = -kx \qquad (1)$$

where k is a force constant and x the displacement. The solution of the differential equation (1) has the form,

$$x = A \sin (\sqrt{k/m})t \qquad (2)$$

which obviously satisfies eq. 1.

The motion of m is periodic,an oscillation of period $T = 2\pi/\sqrt{k/m}$ since for integral values of n, $t=nT$, x recurs to an initial value. The circular frequency of the oscillation is

$$\omega_o = 2\pi/T = \sqrt{k/m} \qquad (3)$$

In terms of this characteristic frequency ω_o equation (2) becomes

$$m(d^2x/dt^2) + m\omega_o^2 x = 0 \qquad (4)$$

The solution of (4) ,or (2) is the infinite sine wave,which as already discussed (see under Fourier spectrum) cannot represent a real physical phenomenon. The finite character of the oscillation is determined by introducing a damping term or viscous resistence,which is proportional

to the velocity of the diplacement. This damping term equals,

$$m\mu\, dx/dt$$

where μ is the damping constant per unit mass. Equation (4) becomes,

$$m(d^2x/dt^2) + m\mu(dx/dt) + m\omega_o^2 = 0 \qquad (5)$$

Free decay.

If the oscillator is subjected to a periodic force of frequency ω_o, but at t= 0 this periodic force is discontinued, at all subsequent times equation (5) will be obeyed. The behaviour of the oscillator becomes then independent of the previously applied conditions, and constitutes the so-called free decay, The solution of (5) is the typical damped oscillator equation,

$$x(t) = C_1 \exp(a_1 t) + C_2 \exp(a_2 t) \qquad (6)$$

where

$$a_{1,2} = -(\mu/2) \pm ((\mu^2/4 - \omega_o^2)^{\frac{1}{2}} \qquad (7)$$

If $\mu^2/4 \gg \omega_o^2$, a_1 and a_2 are real non-zero and the amplitude decreases exponentially and dies away. This behaviour is of no interest, since for oscillators with properties ressembling those of atoms and molecules $\mu \ll \omega_o$. In this case the exponentials of (6) become imaginary and

$$x(t) = \exp-(\mu t/2)(C_1 \exp jSt + C_2 \exp -jSt \qquad (8)$$

where

$$S = (\omega_o^2 - \mu^2/4)^{\frac{1}{2}}$$

or since $\mu \ll \omega_o$.

Therefore

$$x(t) = \exp(-\mu t/2) \times (A \cos(\omega_o t + \phi)) \qquad (9)$$

The term between the brackets is a periodic function of the time and exp $(-\mu t/2)$ is a continuously decreasing factor so that the motion is damped oscillatory with many periods of oscillation within the damping

time $2\mu^{-1}$. The intensity returned by the oscillator to the field is proportional to the square of the amplitude or

$$I(t) = A^2(t) = \exp(-\mu t)\, A^2 \cos^2(\omega_o t + \phi) \qquad (10)$$

Equation 10 describes therefore the emission of energy from an oscillator that previously absorbed it foom the field. It is seen that emission occurs at the proper frequency of the oscillator, ω_o with a characteristic rate of decay μ.

Forced behaviour of the oscillator.

If the zero on the right hand side of equation (5) is replaced by $E_o \sin \omega t$ where ω is an arbitrary circular frequency, the equation corresponds to the case of an oscillator of proper frequency ω_o submitted to an arbitrary field frequency. The oscillation of the right hand side is that of the electric field of the wave. According to H.A.Lorentz, it is considered that atoms and molecules are essentially electronic oscillators set in forced motion by the periodic electric field of the light waves, which acts to separate the lighter electrons from the heavier nuclei, while the restoring force and viscous damping result from the electric and magnetic actions among the charges. The steady state solution of the equation

$$m(d^2x/dt^2) + m\mu\, dx/dt + m\omega_o^2 = E_o \sin\omega t \qquad (11)$$

is also a periodic motion of the applied frequency ω but of different amplitude and phase ϕ,

$$x(t,\omega) = A(\omega) \sin(\omega t + \phi(\omega)) \qquad (12)$$

By introduction of the solution (12) into the original equation (11) we find:

$$\tan\phi = -\mu\omega/(\omega_o^2 - \omega^2) \qquad (13)$$

and $$A(\omega) = E_0/m \cdot ((\omega^2 - \omega_0^2)^2 + \mu^2 \omega^2)^{-\frac{1}{2}} \qquad (14)$$

Maximum amplitude is observed when $\omega = \omega_0$, that is when the applied frequency coincides with the proper frequency of the oscillators. When this is the case equation (13) shows that field and oscillator are 90^o out of phase, the oscillatot lagging behind the field. The ratio of the amplitude $A(\omega)$ obtained at an arbitrary frequency and $A(\omega_0)$ the amplitude of the ocillations at the proper field frequency is,

$$A(\omega)/ A(\omega_0) = \mu\omega/ ((\omega_0^2 - \omega^2)^2 + \mu^2\omega^2)^{\frac{1}{2}} \qquad (15)$$

The transfer of energy from the field to the oscillator is proportional to the square of the amplitude. Maximum energy absorption will be found at ω_0, and half maximum at a frequency $\omega_{\frac{1}{2}}$, such that

$$(A(\omega_{\frac{1}{2}})/ A(\omega_0))^2 = 1/2 \qquad (16)$$

or according to the latter equation, for the case $\omega_0 \gg \mu$

$$\omega_{\frac{1}{2}} = \omega_0 \pm \mu/2 \qquad (17)$$

A plot of $A^2(\omega)$ against ω is shown in figure . This typical response curve is called a Lorentzian. It shows the Fourier spectrum of the energy absorbed by the oscillator. The width of the spectrum equals μ and therefore we expect the duration of the transfer process to be of the order μ^{-1} in accordance with the equation $\Delta\gamma \cdot \Delta t \sim 1$.

For an oscillator emitting visible light ω_o is of the order of 10^{15} 5^{-1} while the damping time μ^{-1} is of the order of 10^8 s^{-1}, so that half-amplitude is reached where ω differ from ω_o by one part in 10^7. Such radiation, which could rightly be termed 'monochromatio' is never seen in the ordinary emission by atoms or molecules. Doppler effect and pressure broadening due to collisions determine the emission of atoms in the gas phase (Ditch burn). In molecules in ~~solution there is a~~ broadening of the elementary Lorentzian (eqs. 10 - 14), of the same origin ~~than~~ as the pressure broadening in the gas emission, ~~which~~ raises the half width to some 70 - 700 cm^{-1}. Moreover each electronic transition includes also some concomitant changes in vibrational and/or rotational energy. These form a broad band of energy levels separated by intervals which are small in comparison with those of the pressure-broadened Lorentzian and the result is that each single electronic transition is enormously broadened. The extent of this broadening is revealed by examination of a vibrationally resolved spectrum that shows a minimum of interference from interaction with the solvent e.g. the absorption spectrum of benzene or anthracene in hexane, or better still (Lipsky) in a profluoro-hydrocarbon. The resolved vibrational levels show half-widths of cm^{-1} corresponding to Lorentzians in which ω is already an appreciable fraction of ω_o. The apparent half-width of the band does not bear any relation to the decay time of the emission any longer, it is determined in fortuitous manner by the broadening and superposition of nearby electronic transitions.

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Oscillator strength and integrated molar absorption.

An ideal electronic oscillator consists of an electron of mass m and charge e which is bound to an 'infinitely heavy' positively charged nucleus by the elctrostatic force alone. If this oscillator is placed in abeam of light where the energy density is $\rho(\nu)$ ergs/cm^3 at frequency ν ,the rate of energy absorption by the oscillator from the beam equals

$$(dE/dt)_{ideal} = B\,\rho(\nu) \qquad (1)$$

B is a proportionality constant dependent only upon the mass and charge of the electron and the refractive index n of the medium in which the oscillator is placed and equals,

$$B = \pi^2 e^2/3\,mn^2 = 8.3031\times10^8/n^2 \qquad (2)$$

A real oscillator differs from an ideal one by the multiplier f (oscillator strength) which is introduced to balance the equation (1) in the form,

$$(dE/dt)_{real} = B\,f(\nu)\,\rho(\nu) \qquad (3)$$

In a light-absorption experiment the absorption is due to oscillators numbering [c]N' per cm^3. Therefore,

$$dE/dt = B\,\rho(\nu)\,f(\nu)\,[c]\,N' \qquad (4)$$

An expression for dE/dt can also be obtained from Beer's law. 1 cm^3 of sample is illuminated through the front face by a homogeneous beam of light of frequency ν . If in the absence of the oscillators the density of the radiation is $\rho(\nu)$,the energy lost by the beam in traversing the solution equals,

$$\Delta E(\nu) = \rho(\nu)(1 - \exp(-\sigma_\nu[c])) \qquad (5)$$

where σ_ν is the milimolar absorption cross section. To satisfy equation (1) $\rho(\nu)$ must be constant within the sample, and this requires that a negligible fraction of energy be removed from the beam. $(\Delta E(\nu)/\rho(\nu) \ll 1)$ This condition implies that

$$\exp-(\sigma_\nu[c]) = 1 - \sigma_\nu[c] \qquad (6)$$

or

$$\Delta E(\nu) = \rho(\nu)\,\sigma_\nu\,[c] \qquad (7)$$

The radiation traverses the sample with the speed c/n where c is the speed of radiation in vacuum and n the refractive index of the medium in which the oscillators are placed. Therefore in unit time the energy absorbed is $\Delta E(\nu)c/n$, giving

$$dE/dt = \rho(\nu)\,\sigma_\nu\,[c]c/n \qquad (8)$$

Equating the values of dE/dt given by (8) and (4),

$$f(\nu) = c\,\sigma_\nu/nBN' = 2.302\,c\,k_\nu/nBN' \qquad (9)$$

where k_ν is the molar absorbance.

Equation (9) gives the desired relation between the oscillator strength and the molar absorbance at any wavelengths, or frequency. The absorption takes place over a discrete band of frequencies $\Delta\nu$ and consequently (9) must be integrated over this interval to have the total f value. Molar absorption coefficients are given in terms of $\bar{\nu}$, wavenumbers rather than frequencies. It is therefore convenient to integrate over the wavenumber interval $\Delta\bar{\nu}$ after replacing $d\nu$ by $c\,d\bar{\nu}$, giving

$$f = (2.302\,c^2/BnN')\int_{\Delta\bar{\nu}} k_{\bar{\nu}}\,d\bar{\nu} = 4.125\cdot10^9 n \int k_{\bar{\nu}}\,d\bar{\nu} \qquad (10)$$

If the band over $\Delta\nu$ is Gaussian with standard deviation ξ

$$k_\nu = k_{max}\exp-(\bar{\nu} - \bar{\nu}_{max})^2/\xi^2$$

$$\int_{\Delta\nu} k_\nu \, d\nu = k_{max}\sqrt{\pi} \cdot \varepsilon \qquad (12)$$

Equations (10) and (12) permit a rapid estimation of the oscillator strength using the molar absorption of the maximum and the half-width of the absorption band.

Notice that the derivation shows that the oscillator strength is a classical concept, and as such no supposition about the quantum nature of the absorption process is necessary for its ~~calculation~~ derivation.

In the emission by atoms in the gas phase the elctronic absorption bands are sharp lines well separated from each other. (17b) In polyatomic molecules, particularly in solution, the absorption bands are broad and often overlap each other. For this reason it is often difficult to estimate the oscillator strengths of the different electronic absorption bands in these cases. The separation of the overlapping transitions is often done arbitrarily.

Origin of the variable oscillator strength. Selection rules.

In polyatomic molecules the oscillator strengths observed vary greatly. The longest wavelength transition in benzene has an oscillator strength of 2.10^{-3}, while shorter wavelength transitions in the same molecule have f greater than 1. ~~For example~~ The last absorption band in NADH has f=0.23 and the Soret band in Haemoglobin has f=1.05. The variable oscillator strength depends upon the existence of selection rules, which are founded in the quantum mechanical description of atoms and molecules. These rules are well understood for atomic absorptions (see G.Herzberg, Atomic Spectra and Atomic Structure), but much less well understood in the case of molecules. The main consideration refers to the distribution of charge in the ground and excited states

In polyatomic molecules in solution there are virtually no gaps in the absorption spectrum starting from a well defined long-wave limit and proceeding bluewards. This continuous absorption spectrum results from variable overlap of neighbouring electronic transitions ~~which~~ and makes it often impossible to separate, or even enumerate them in an unequivocal manner. Polarization characteristics (see under 'Polarization spectrum') may be used to separate neighbouring bands in fluorescent chromophores, but in many cases enumeration and separation of the transitions can only be done in an arbitrary manner, like providing a Gaussian or Lorentzian tail to each well defined maximum. In consequence the oscillator strengths calculated cannot often be given a very precise value, but can serve well as a basis for comparison. 'Strong absorption' is usually employed to indicate an oscillator strength greater than 0.5 and weak absorption implies an oscillator strength below 0.1.

of the molecule. The probability of absorption of a photon depends upon the existence of a difference in charge distribution in the ground and excited states of the molecule. The interaction between photon and molecule cannot lead to absorption, or at least to absorption with high probability unless there is an appreciable difference in the charge distribution of the two states. The necessity for such a rule may be understood by a classical analogy: The energy in the field may be likened to an alternating current and the molecule to a rectifier into which the current flows. As a result of the rectification process + and - charges appearing in a ~~condenser~~ capacitor attached to the rectifier. If the charge distribution of the ~~condenser~~ capacitor is not allowed to change no energy may be drawn from the field. The absorption is then forbbiden. If the charge distribution in the excited state is very different from the ground state the energy in the field may be easily rectified and the transition is strongly allowed. It must be stressed that the structures of both ground and excited states determines the probability of absorption so that for one kind of excited state reached from the ground state the absorption probability may be very small while for another excited state it may be considerable.*

Absorption of light by molecules. Franck-Condon Principle.

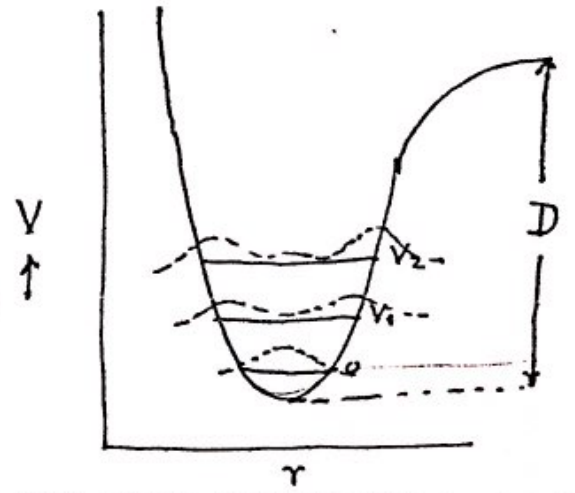
In atoms in the gas phase the electronic absorption bands are very sharp The line width is determined solely by the pressure and temperature, and is usually 10^{-2} - 10^{-1} Å. The energy levels are sharply separated and the absorbing states may be completely characterized. In molecules the situation is considerably more complicated. Instead of an absorption line a broad band is observed. The broadness originates in the simultaneous electronic ~~and~~ and vibrational changes upon absorption.

This analogy of the absorbing molecule to the rectifier can be pursued further. The rectifier in question is a freuency-sensitive or synchronous rectifier having a proper frequency ω_0. If the resistence and capacity of the output circuit of the rectifier are R and C respectively, an alternating voltage of frequency ω generates an output voltage

$$V_o = \frac{V_i}{\sqrt{(\omega - \omega_0)^2 + \frac{1}{R^2C^2}}}$$?

RC is the time constant of the system. A long time constantbespeaks a sharp spectrum of rectified frequencies and a short time constants a broad spectrum. It will be noriced that the frequency dependence of the efficiency of the rectifier is virtually the same as that of the ~~dependence of the~~ damped oscillator (equation).

The effects expected for a molecule are best presented by considering the case of a simple diatomic molecule. The potential energy V as function of theinternuclear distance r is given by the familiar Morse curve,



The minimum in the curve corresponds to to the equilibrium internuclear distance r_o. V_o is the zero point energy; $V_1, V_2 \ldots$ corresponds to the potential energies of a molecule with increasing numbers of quanta of vibrational energy. In the dark the thermal equilibrium produces a distribution of the molecules among the levels following Boltzamn statistics. The molecules in the nth level and above are given by

$$\exp-(E_n/RT)$$

where E_n is the energy content per mole for level n. Vibrational energy levels are typically separated by some 3 kg.cal/mole so that at room temperature only some exp(-6) or 10^{-3} are found in the levels above zero. For practical purposes (with exceptions) we can consider that the absorption of light is always from molecules in the zero level of the ground state. Note that the distribution along r is chracteristicall different for molecules in the zero level and in the other levels. The most probable distance for the zero level is r_o the equilibrium internuclear distance. For $n > 1$, the distribution becomes bimodal or plurimodal, with the extreme values being the most prominent.

For a certain value r_d of r the interaction becomes negligible and the atoms may pull apart without further change in V. The difference in energy between this state and n=0 is the dissociation energy D of the molecule which is determined by the bond energy between the atoms. Because of the Boltzman distribution we can state that virtually all absorptions take place from the zero level of the ground state. To see what changes are possible in absorption we construct the potential energy curve that we expect from a molecule in the electronic excited state. For this purpose we take into account the proposition that the electrons involved in the changes brought about by absorption of light are the outer or valency electrons. The inner electrons are held much more tightly and the energies necessary to modify the relations of these with the atomic nuclei are correspondingly greater (x-ray region) Since the valency electrons are involved in the rearrangement due to the absorption of light the binding of the nuclei to each other will be less in any excited state than in the ground or most stable state, giving rise to three effects:

a. Increase in the equilibrium internuclear distance r_o^*

b. Decrease in the energy of dissociation D*.

c. Decrease in the change in potential energy with r, dV/dr* in the neighbourhood of r_o^*

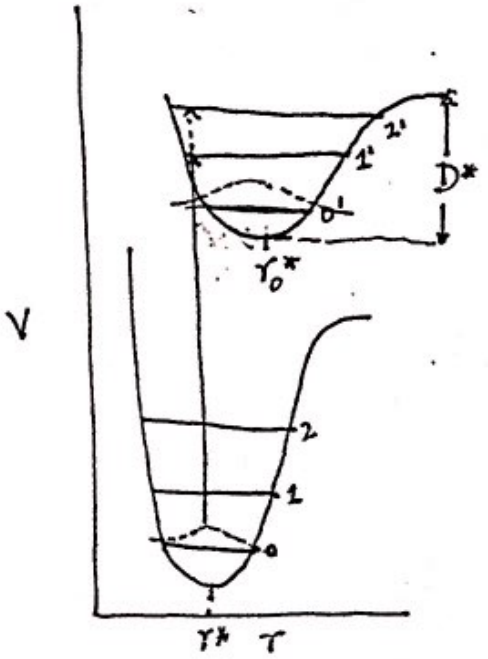
Thus, to construct the potential energy curve in a typical excited state we use the rules:

$$r_o^* > r_o$$

$$D^* < D$$

$$\left(\frac{dV}{dr^*}\right)_{r \to r_o^*} < \left(\frac{dV}{dr}\right)_{r \to r_o}$$

The result is indicated in the following figure.

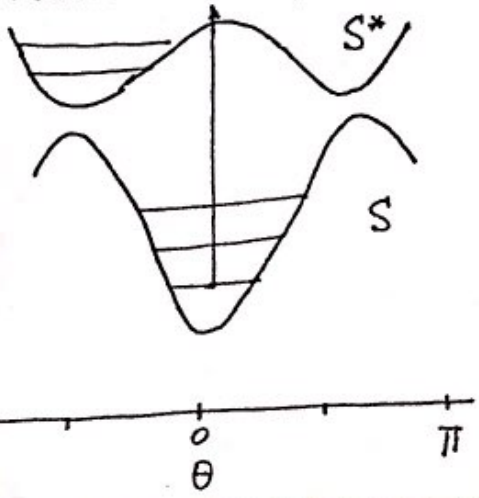


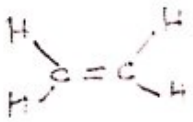
The principle governing the absorption ,or emission ,in polyatomic molecules is the basic principle of molecular spectroscopy,the principle of Franck & Condon. In its simplest form the Franck-Condon principle may be formulated as follows: Changes in electronic ~~energy take place~~ distribution occur very fast in comparison with changes in bond angles and bond distances Therefore the nuclear configuration of a molecule,that is the set of bond distances and bond angles, ~~does not~~ cannot change appreciably during the absorption,or emission,of light by the molecule. In the simple case of a diatomic molecule this means that an electronic transition is represented by a vertical line starting from r_o and ending in an excited state with this very same value. Since $r_o^* > r_o$ it follows that almost virtually always an excited vibrational level is reached in absorption. Evidently, levels above 2 can be reached provided that the field contains the necessary energy. Energies below the lowest that can be reached by a vertical transition from r_o are possible by two mechanisms:

1. Some molecules in the electronic ground state are thermally excited so that values conspicuously larger than r_o exist from which the molecule may reach the levels 0',or 1'.
2. Some molecules in the ground state,but not thermally excited have

values of r much larger or much smaller than r_o, since there is a distribution of r values in the molecules in the zero level of the ground state, corresponding to the existence of a finite zero-point energy. The combined effects of these two causes is to provide absorption bands with long-wave tails rather than abrupt cut-off terminations. The absorption at the long-wave tails of bands is often found to be thermally sensitive. In the case of broad absorption bands lowering of the temperature results almost invariably in a displacement of the band edge to shorter wavelengths, owing to the reduction of the thermally excited population.

2a → In polyatomic molecules the Franck-Condon diagram would be much more complicated since it would involve coordinates for every bond distance and angle in the molecule. However in conjugated molecules one would expect all bond distances in the conjugated chain to be similarly if not equally altered, so that r could be replaced by an appropriate mean value $\bar{r}$. Sometimes it is only one particular bond distance, or more often a bond angle that alters dramatically on going from ground to excited state. In ethylene, for example, the ground state is planar while in the lowest excited state the planes of the the two CH_2 halves are at right angles to each other. The Franck-Condon diagram for this case is shown below. The potential energy of twisting about the double bond is plotted against the angle between the two planes, θ. One can see that in this case also, increasing the temperature will result in absorption at longer wavelengths.





The scheme of fig , corresponding to a single diatomic molecule, would essentially apply to a polyatomic one like ethane, if the internuclear distance r is taken to be the C-C distance in the molecule and the electrons involved in the absorption transition are exclusively those of the σ bonds between the carbon atoms. In a molecule like ethylene ($CH_2 = CH_2$) the electrons involved in the absorption transition would be the π electrons forming the double bond between the carbons, since these are bound to nuclei by considerably smaller energies. The excited states would represent promotion of an electron to an excited molecular orbital generally designated as a π^* orbital. The transition itself would be of the type $\pi \rightarrow \pi^*$ while in ethane it would be a $\sigma \rightarrow \sigma^*$ transition. The quantity D would correspond in ethylene to the formation of the biradical $\dot{C}H_2 - \dot{C}H_2$ ~~matter~~ rather dissociation of the molecule, but has otherwise the same significance.

In molecules in the gas phase there are well defined quantized rotational levels, which fill the gaps betwen the vibrational levels. Rotational structure has been observed and analyzed, in the last years in molecules as large as phenol, indole and anthracene. In solution the rotational structure is completely obliterated since free rotation, with a quantized energy is no longer possible. The vibrational structure may be still recognized within an absorption band, by the presence of peaks separated by about 3 kg.cal. in energy. The last absorption band of aromatic hydrocarbons in non-polar solvents, exhibits often this structure.* 23b

Prohibition of intercombinations.

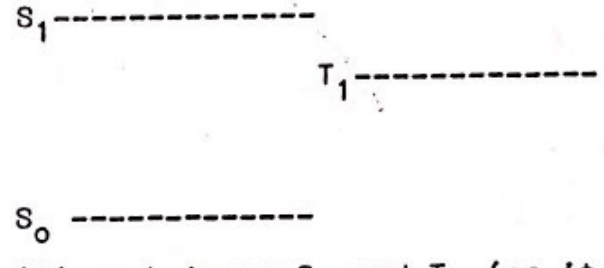
One very important selection rule refers to the prohibition of radiation with change in multiplicity. We shall now define this term: The spinss of the electrons in an atom are paired in accordance with Pauli's principle. The unpaired spins are responsible for the multiplicity of the state. Any unpaired spin is assigned a multiplicity of $\frac{1}{2}$ and the total spin S is obtained by simple addition of the unpaired spin values. The multiplicity M equals 2S+1. The origin of the concept is to be found in the fine structure of atomic emission lines. A line emitted by a state with multiplicity M has that number of hyperfine components because the S unpaired spins can combine with the orbital motion giving 2S+1 distinct values of the energy. In the alkali atoms, for example there is one unpaired electron spin, and consequently the state is a doublet, like the familiar sodium lines at 5890-5896 Å.

Most organic molecules have no unpaired electrons. For them S=0, or M=1, They are singlet states. If one of the paired electrons inverts its spin we have S=1, or M=3, thus becoming a triplet.

A further influence, of particular interest to the chemist or biologist arises because of possible molecular interactions between the solvent and chromophore molecules. An aspect often forgotten in practical consideration of the spectra of molecules in solution is that it cannot be [~~considered to be~~] the result of identical, or even very similar interactions for all molecules. It clearly must be considered as resulting from a population of interactions in which ~~the~~ individual cases contribute with unknown weights. This heterogeneity must be kept firmly in mind as it puts definite limits to our ability to single out excited states which can be strictly defined and subjected to analysis using the quantum-mechanical rules that have been successfully applied to the spectra of atoms in the gas phase.

It could be conceived that absorption of radiation takes place with simultaneous inversion of a spin. Such absorption would constitute a singlet-triplet transition (S-T). An important selection rule forbids any change in multiplicity during absorption or emission. Transitions like T-S or S-T are therefore forbidden transitions, or more explicitely they cannot occur with simultaneous absorption of radiation. Non-radiative changes in multiplicity can and do occur without difficulty. The rule refers to radiative changes, or more accurately to radiation by electric dipoles.

Following a rule due to Hund (see Herzberg: Atomic Structure and atomic Spectra) the triplet excited states of molecules are of lesser energy than the corresponding singlet states. Thus the energy scheme for an organic molecule with a ground state singlet would be:



Although the transitions between S_o and T_1 (or its reciprocal T_1-S_o) cannot occur with high probability, they can still take place by a mechanism of very low probability in comparison with those that produce transitions like S_o-S_1 or T_1-T_2. The former are weaker by a factor that varies from 10^{-6} to 10^{-9}.

The vast majority of organic molecules have all their electrons paired in the ground state, and following the prohibition of intercombinations, the same holds for all the excited states that are easily reached by light absorption. The set of these states constitutes a singlet manifold. A corresponding manifold of triplet states may be generated by uncoupling a pair of electrons spins, and according to a rule due to Hund these corresponding triplets are of lower energy than the generating singlets. It follows that the first triplet state, T_1 is energetically placed between the lowest excited singlet, S_1 and the ground state S_o. Each triplet state is, in molecules as in atoms, triply degenerate. The energy splitting of the states is very small and is completely erased in the optical absorption spectrum by the broadness of the electronic transitions. However, the characteristic degeneracy of the state may be revealed in the optical detection of magnetic resonance.

Molecular structure and light absorption in organic compounds.

We shall be concerned with the optical absorption properties of compounds containing C,H,O,N ans S. A saturated hydrocarbon containing only C and H shows no absorption in the region of the ultraviolet to which air is transparent ($\lambda > 220$ nm). The outer of valence electrons of these compounds are involved in C-H or C-C sigma bonds (Bond energy = 80 kg.calories). An absorption band would require the promotion of an electron from a sigma to a sigma * orbital, ($\sigma \rightarrow \sigma^*$ transition) the necessary energy of which lies in the far UV, starting nearly at 150 nm. Compounds in which absorption will take place at wavelengths of 200 nm or longer require electron less tightly bound to the nuclei that those of the sigma bonds. Such are the electrons that participate in the π bonds of unsaturated hydrocarbons. The simplest case is possibly that of ethylene which has an shows electronic transition at 165 nm. and a much weaker absorption band ~~is also observed~~ at 200 nm. The ~~$\pi \rightarrow \pi^*$~~ transition at 165 nm is an example in which one of the bonding π electrons is promoted to an excited orbital (π^*). In butadiene the longest wavelength transition is at 220 nm, and with further extension of the conjugated chain the longest wavelength absorption moves regularly towards the red end of the spectrum:

Longest wavelength absorption

$H(CH=CH)_nH$

n	nm	K_{max} (mM)
2	217	21
3	268	35
5	334	121
6	364	--
8	410	--
10	447	--

The displacement of the absorption maximum to the red is accompanied by an increase in the oscillator strength. A simple qualitative, and almost quantitative explanation of these facts is given by the FEMO (Free-electro molecular orbital) theory. Although such theory is obviously insufficient it has the merit of explaining the approximate region of spectral absorption from ~~simple~~ molecular structure properties in very simple fashion and ~~it contains no~~ without appeal to adjustable parameters. The set of conjugated double bonds is supposed to constitute a "box" inside which the π electrons (two for each double bond) can move freely. The possible energy levels that the electrons can occupy inside the box is given by a simple application of quantum mechanics (see e.g. Pauling & Wilson): The energy E_n associated with the nth level is

$$E_n = n^2h^2 / 8m\ell$$

where m is the elctron mass, h is Planck's constant and l the length of the box. n=1, the lowest energy level corresponds to a standing wave without nodes inside the box, so that ~~the~~ its half-length ~~is~~ equals the length of the box, ℓ. In the second level one whole wavelength is contained exactly in the box, in the third level 3/2 lengths and so on. Two electrons, with opposite spins occupy each level of the box, in accordance with Pauli's principle. In butadiene, with four π electrons, these occupy the lowest two levels while the third is unoccupied. The absorption transition of least energy (i.e. longest wavelength) results from the promotion of an electron in the uppermost filled level to the ~~the~~ lowest unoccupied level. The energy difference between these, the nth and the (n+1)th levels is given by,

$$E_{n+1} - E_n = \frac{h^2}{8ml^2}\left((n+1)^2 - n^2\right) = \frac{h^2}{8ml^2}(2n+1)$$

According to Bohr's frequency condition

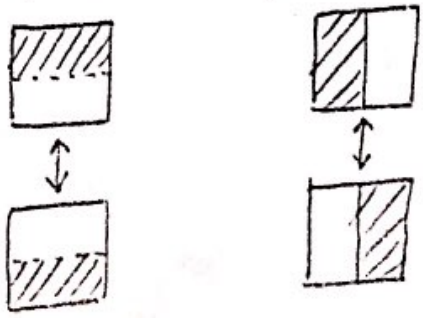
$$E_{n+1} - E_n = hc/\lambda \qquad \text{and therefore,}$$

$$\lambda = hc/(E_{n+1}-E_n) = 8ml^2c/(2n+1) = 33\ l^2/(2n+1)\ \text{nm}$$

if l is given in Å units.

The agreement is not very good: Thus for n=4 the formula predicts absorption at 471 (maximum) against an observed value of 304 nm. Nevertheless it should be acknowledged that the equation predicts the approximate place of absorption, by an extremely simple equation containing only universal constants h, mc and the length of the conjugated chain. The qualitative agreement is important in ensuring that the physical picture given by this simple theory is in the main correct.

An more complex situation presents itself in molecules in which the conjugated path is closed, as is the case of aromatic hydrocarbons. In these cases the electronic oscillations resemble those of a membrane in which the perimeter is fixed and the standing waves are those of the surface. It is known that in this case (see Lord Rayleigh, The theory of Sound) the fundamental mode of vibration is one without nodes, all the points of the membrane moving in the same direction above or below the fixed perimeter. The first and all other overtones are degenerate. A rectangular membrane with sides of comparable dimensions has two first overtones with nodal lines at right angles to each other.



All energy levels above the first are also doubly degenrate, with nodal lines which are at right angles to each other. This makes a considerably difference between a linear conjugates molecule, like hexatriene and the correspodding closed structure (benzene). In hexatriene the six π electrons occupy 3 energy levels. In Benzene the same six electrons occupy only two levels: The first, without any nodes is filled with two electrons, and the second, doubly degenerate is occupied by four electrons.

Benzene Naphthalene

Electron levels in Benzene and Naphthalene

In naphthalene the remaining four electrons completely fill the next doubly degenrate level, and in general polyciclic aromatic hydrocarbons fill completely n levels since they have exactly 4m+2 π electrons, ~~It will be observed that~~ where m is the number of aromatic fused rings. Hückel attributed the stability and large resonance energy of the polyciclic aromatic hydrocarbons to the complete filling of the energy levels in the same way ~~in which~~ as the saability of the noble gases ~~which~~ is due to the existence of completely filled electron shells. [On this basis Huckel predicted that cyclo-octatetraene would turn out to be non-aromatic. It is in fact non-planar and behaves like a non conjugated molecule.]

in the aromatic hydrocarbons.

( ) The double degeneracy of the filled levels has a simple qualitative consequence that is experimentally easy to observe: The absorption of the aromatic hydrocarbons occurrs at shorter wavelengths than the absorption of the linear conjugated compounds with the same number of π electrons. For example decaene absorbes at 447 nm, while naphthalene absorbes at 305 nm. and the difference is even more marked in the members with larger numbers of π electrons. Promotion of an electron to the next unfilled orbital of an aromatic hydrocarbon results in a state with 2 nodes (B state) or 2n+4 nodes (L state). From the number of nodes it follows that L states are multipolar states while B states are dipolar. Since the distribution of charge in the ground state is highly symmetric in the unsubstituted hydrocarbons, the transitions to L states have small oscillator strength

( ) while the transition to the B states have much larger f values. Both B and L states are degenerate since the nodal lines can cut bonds (L_a and B_a states) or atoms (L_b and B_b states). Thus we have L_a, L_b, B_a and B_b states. All these transitions are singlet-singlet transitions.

Hydrocarbon		L_a	L_b	B_a	B_b
Benzene		208	263		183
	f	0.1	2.10^{-3}		0.7
Naphthalene		289	302	170	220
	f	0.18	2.10^{-3}	0.5	1.7
Anthracene		379	?		256
		0.1			2.4
Naphtacene		474			272?
		0.1			1.8

As pointed out by Platt, who among other investigators developed the FEMO model, promotion of an electron from the uppermost filled orbital to an unoccupied higher level results in a composite 'hole and electron' state, which may have only two nodal lines (B state) if hole and electron belong to the same nodal system or $2n+4$ (L state) if they belong to systems with orthogonal nodes. From the number of nodes it follows that L states are multiple states exhibiting a smoothed-out distribution of charge, while B states are dipolar. In unsubstituted aromatics the ground states have uniform charge distribution, and transition to an L state is consequently difficult (quasi forbidden) with f values often smaller than 0.1. On the other hand transitions to the strongly dipolar B states are fully allowed and have oscillator strength that can reach unity or higher (see Jaffee and Orchin).

Both B and L states are doubly degenerate corresponding to the original geometric degeneracy of the nodal lines and the two states thus produced are termed L_a and L_b, or B_a and B_b. In benzene and its substituted derivatives the longest wavelength transition is an L_b clearly separated from the L_a transition by 10,000 cm^{-1}. This splitting due to the geometric degeneracy is a minimum when the two planar dimensions of the aromatic molecule are equal, a situation approached in aromatics made up of two fused rings, like naphthalene, quinoline and indole. In naphthalene the transitions maxima of L_a and L_b are separated by only 1400 cm^{-1}, and since this is smaller than the corresponding bandwidths the transitions are overlapping and appear unresolved in the absorption spectrum. The overlap of two nearby transitions in the first excited level lends considerable complication to the absorption and fluorescence properties of the molecules because chemical substituents as well as changes in the solvent environment can produce a variety of effects by acting separately upon the twin levels. Such complexity, which often hinders a straightforward interpretation of the spectroscopic observations, is a further potential source of information of particular interest in the case

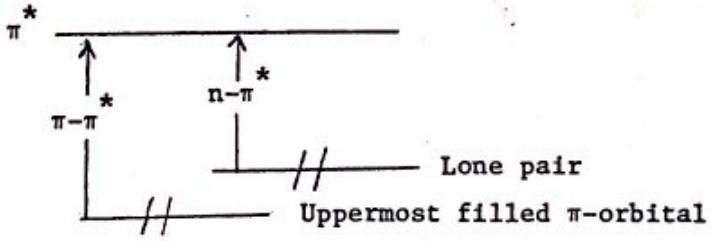
of the tryptophan residue of proteins, and the naphthalene fluorescent probes.

$n-\pi^*$ transitions.

While $\pi-\pi^*$ transitions are universally responsible for the brilliant colours of plant and animals, there is an important category of transitions that involve the promotion of non-bonding electrons to a π^* orbital. These non-bonding electrons belong to the lone pairs of S, N and O and the corresponding transitions are called $n-\pi^*$ transitions. They are identified because of the following characteristics:

1. They often lie on the long-wave side of $\pi-\pi^*$ transitions, so that they appear as the longest wavelength transition of the ultraviolet or visible spectrum.
2. They are often weak, 'forbidden' transitions.
3. They undergo a characteristic 'blue shift' in hydroxylic solvents.
4. They disappear (shift to very short wavelengths) on protonation of the corresponding lone pair.

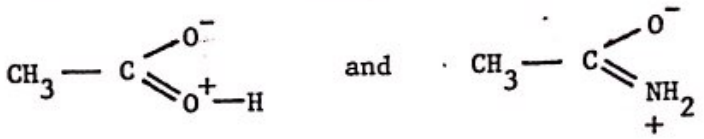
In an energy level diagram the relation of $n-\pi^*$ to $\pi-\pi^*$ transitions is often as follows:



When the lone pair level is well separated from the uppermost filled orbital the $n-\pi^*$ transition is distinct and well separated from the nearby $\pi-\pi^*$ transition. It is in these cases that the $n-\pi^*$ transition is characteristically weak (forbidden). As an example of the weak, isolated $n-\pi^*$ transitions we have

	λ	$k_{max.}$ $(cm^2 \mid mMole^{-1})$
Acetone	280	15
Acetaldehyde	293	8
Acetic acid	204	60
Acetamide	214	30

The much shorter wavelength at which the transition occurs in Acetic acid or acetamide as compared to the other derivatives may be understood by an increased contribution of electron distributions like



and

to the corresponding ground states. Typical n-π^* transitions are observed in quinone and benzophenone. In the heterocyclic aromatics pyridine, quinoline, etc. the n-π^* transitions are apparently overlaid by broad π-π^* transitions and are difficult to characterize. When the uppermost filled π-level and the lone pair level are close in energy, a situation that presents itself where the lone pair atom is attached to an aromatic ring, the lone pair is to a certain extent conjugated with the π-electrons of the ring. In these cases promotion of a lone pair electron to the ring is much more probable and the corresponding transition are much stronger than the typical isolated n-π^*, but may still be recognized by the blue shift in hydroxylic solvents and the disappearance upon protonation. This is the general case of the aromatic amines, where the longest wavelength absorption band disappears on protonation, the spectrum taking the general character of a methyl substituted aromatic.

Blue shift of n-π^* transitions in hydroxylic solvents.

This effect may be seen in acetobe where the maxima of absorption are

hexane	279 nm.
ethanol	272
water	265

The energy difference between the values in hexane and water equals approximately 5 kg. calories /mole. In N-nitroso dimethylamine the value between water and hexane equals 7 kg.cal. Similarly the aromatic amines show blue shifts of some 6 kg.cal. If the N atoms of these molecules is replaced by C the corresponding shifts are less than 1/10th of the N values. The shift to shorter wavelength is easily explained : because of the promotion of the lone pair electron to a π^* orbital the heteroatom comes to bear a partial positive charge and the hydrogen bond ~~in which the lone pair takes part~~ disappears in the excited state. Consequently the energy necessary to break it has to be added to the energy of promotion of the lone pair electron.

Protonation effects.

In a medium of suficient acidity lone pairs can be protonated. On addition of the proton the transition "disappears",or at least moves to shorter wavelengths. Disappearence of the bands in strong acid are observed in acetone,in aromatic amines,etc.

The ffects of substitution in the aromatic ring.

When the hydrogen atoms in aromatic rings are substituted by other chemical entities two main effects are observed: 1. There is a decrease in the symmetry of the molecule that facilitates the interaction with the field,that is the production of a dipole. In general the effect is to increase ~~the f value~~ appreciably the oscillator strength of the ~~longest wavelengths~~ L,

transitions. Thus the maximum molar absorption coefficient of L_b in benzene is 220 cm^2/mM. Replacement of a hydrogen by a methyl(toluene) causes only a small increase in absorption, but replacement by OH (phenol) results in an increase to 1,380 cm^2/mM, that is by a factor of six over benzene itself. It is also observed that although the position of the band is not much altered in toluene, in phenol the maximum displaces to 275 nm from the benzene value of 250 nm. The difference between toluene and phenol represents the difference between a group which merely perturbs the benzene absorption and a group, OH that extends the conjugated path of the π electrons and provides for possible localization of charge in the excited state. The extension of the conjugated chain is responsible for the bathochronic shift of the maximum, while the facilitated localization of charge is responsible for the increased oscillator strength. The effect of groups like OH, SH and NH_2 in shifting the absorption to longer wavelengths and increasing the absorption intensity have been well known to the dye chemists interested in the synthesis of these compounds, and they were designated because of their effects as 'auxochrome' groups.

The lone pair of O in benzoquinone and of the N in the heterocyclics like pyridine and quinoline are not conjugated to any great extent with the ring, and their presence serves to introduce an additional $n-\pi^*$ transition in the system. In aniline and phenol the lone pair s of the O and N are partially conjugated with the ring. This can be seen in the ground state behaviour of these compounds: The pK of the aromatic amines is about 4.5 while the aliphatic amines is 9. Similarly the pK of phenol is 10 compared with 13 in an ordinary alcohol. These partially conjugated lone pairs give rise to transitions that are broad, displaced to longer wavelengths as compared to the L bands observed in their absence, and of moderate f values. They are blue-shifted in hydroxylic solvents and erased by protonation. They are of the same kind as $n-\pi^*$ transition but have become partially allowed by conjugation.

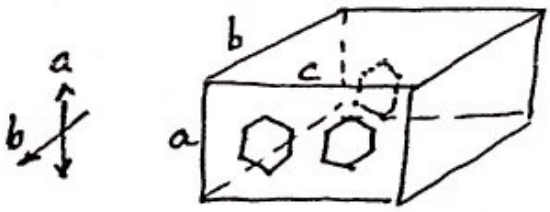
* and provides for possible localization of charge in the excited state. The extension of the conjugated chain is responsible for the bathochromic shift of the maximum, while the facilitated localization of charge is responsible for the increased oscillator strength

In general the effect of substituents of the aromatic nuleus is to increase the oscillator strength and displace the absorption towards the red. the largest differences in oscillator strength among the different transitions of the same molecule are observed in the unsubstituted hydrocarbons where oscillator strength ratios can be of the order of 10^2 or 10^3, as in benzene or pyrene. On the other hand a heavily substituted aromatic molecule like dimethyl-iso alloxazine, which carries the visible chromophore of the flavin coenzymes presents three transitions, at 450 nm, 375 nm and 260 nm. The oscillator strengths of which are 0.3 - 0.6 and differ among themselves by a factor of no more than two.

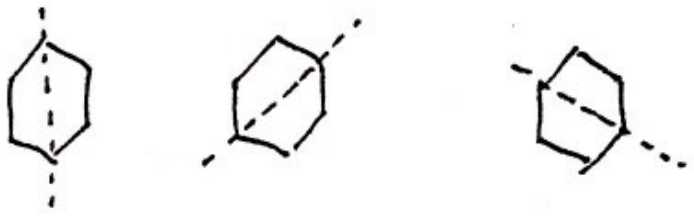
Polarization of Transitions. Linear dichroism.

A classical dipole oscillator interacts with the electric field so that the separation of charges occurs in the direction of the electric vector of the exciting light. Experiments carried out in the last century by Otto Wiener and repeated more recently by others show that the interaction is primarily with the electric field and only to a negligible extent with the magnetic field of the light. The classical oscillator is entirely isotropic and will absorb light with the same intensity regardless of its orientation with respect to the electric vector of the light wave. This is not often the case with real oscillators, and in most molecules the polarizability of the molecule is different in the different directions. In many cases molecules behave like, completely anisotropic linear oscillators. In these cases there is a priviledged direction in the molecule (the transition moment in absorption) for which the absorbance is a maximum value, and a direction normal to it for which it is zero. For any angle θ between the electric vector of the exciting light and the direction of the transition moment the probability of absorption is proportional to $\cos^2\theta$. Such a relation can be expected on classical grounds and also in quantum theory. Quantum theory does not predict a unique transition direction in the molecule. This depends in fact upon the molecular structure but in molecules with reduced symmetry experiment shows that in many cases, this direction is a single one in the molecule. To carry out a decisive experiment showing the preferential absorption along certain molecular directions requires a molecular population in which all members are similarly oriented. In fluid solutions it is not possible to generate such population, as application of an electric field or the laminar flow of the solutions can produce only a partial orientation of a small fraction of the molecules. The experiment is possible with

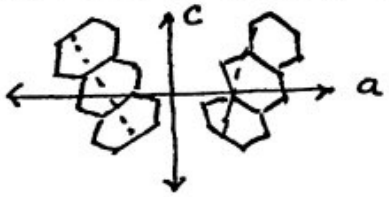
requires a crystal in which the molecules are related to each other by translations alone or with little rotation superimposed. Otherwise it would not be possible to orient the crystal with respect to the electric E vector of the light so that one molecular direction, alone, is parallel, or nearly so to it. In the crystals of hexamethyl benzene the molecules are placed in parallel sheets with benzene rings parallel to each other.



When the E vector of the polarized light is parallel to the a direction absorption is maximal. When the E vector is normal to the benzene rings plane (b direction) the absorption is very small. The difference from zero could be is probably due to scattering or and to imperfections in the crystal. or to a residual out-of-plane absorption.
Examination of this and other crystals shown of aromatic compounds show that invaribly the strong absorption transitions show a maximum when illumination is with light polarized in the plane of the aromatic rings and a minimum when it is normal to it. In the case of the crystals of hexamethy benzeme crystals illumination through the ac face of the crystal does not reveal changes in absorption with the plane of polarization: By symmetry all the three directions in the ring shown below are equivalent.



Therefore rotation of the plane of polarization of the light transmited in the b direction cannot appreciably change the absorbance. The effects obtained when the crystal is of lower symmetry is exemplified by the case of the anthracene crystals. The molecules are stacked in the crystal in nearly parallel planes so that illumination of the ab face gives results similar to those obtained with the hexamethylbenzene crystal. Both the L transitions (centered at 370nm) and the B transitions (centered at 250 nm) have maximum absorption for the E vector in the a direction. The two molecules of anthracenne in the unit cell are at a small angle to each other, as shown below



It should be possible therefore to distinguish whether the transitions are a or c polarized. The maximum at 370nm is clearly c polarized (also called longitudinally polarized). There is evidence however that the polarization of the 250 nm band is not the same in the free molecule and in the crystal. This is not surprising since there is evidence of strong interactions among the molecules in the crystal which are absent in the gas phase or in dilute solutions of anthracene. The main conclusion to be derived from all these observations is that the transition moments in aromatic molecules (π-π^* transitions) are all polarized in the plane of the rings.

Lyons L.E. & Morris G.C. J.Chem.Soc. 1551, 1959
H.M.Wright. Chem Rev. 67, 581(1967) (Arrangement of anthracene in crystal)
Clark L.B. J.Chem. Phys. 51, 5719 (1969)
Chen & Clark L.B. J.Chem.Phys 51, 1862 (1969)

GW

Environmental effects upon absorption.

The study of this aspect is of the greatest interest to the Biochemist or Biologist. Changes in the absorption properties of chromophore groups cab in principle provide information about their interaction with the surrounding elements in the natural biological systems like macromolecules, particles or membranes. Frequently the interpretation of the observations cannot be as certain as one would wish because of the present incomplete knowledge of the relation between spectroscopic properties and molecular structure in the isolated chromophore.

It is possible to recognize that on the one hand some spectroscopic properties changes result from the macroscopic properties of the surroundings, like the refractive index or the dielectric constant, while others depend upon the detailed structure and the functional properties of the molecules. A knowledge of the former influences is necessary in giving due weight to the latter," specific " factors. I shall describe first the general mode of interaction between excited chromophore and environment and then treat, in a phenomerological fashion, the most important types of spectroscopic changes that follow the establishment of well defined molecular complexes.

Characterization of the medium by its macroscopic properties:

If an external alternating electric field is applied to a medium the charged particles in it will undergo motions that depend upon the field frequency. If the frequency is so high that overall molecular motions or even changes in molecular configuration cannot occurr within one period the only effects will result from electron polarization that is from the displacement of the electrons alone with respect to the heavy, practically motionless nuclei. Such will be the case when the

electric field has the frequency of the E field of the light (10^{15} sec^{-1}
The polarizability at such high frequency depends upon the refractive index, n alone, and equals

$$(n^2-1)/(2n^2+1) \quad (1)$$

– including overall orientation of the molecules.

At very low frequencies, when nuclear motions as well as electronic displacements are possible the polarizability is given by

$$(D-1)/(2D+1) \quad (2)$$

where D is the static dielectric constant of the medium.
In a liquid made up of molecules with no apprecible dipole moment the electronic polarizability is the only contributor to the effects and the quantity

$$\Delta f = (D-1)/(2D+1) - (n^2-1)/(2n^2+1)=0 \quad (3)$$

the Maxwell relation $D=n^2$, obtains.

since in these cases $D=n^2$. Δf is therefore the orientation polarizability of the medium. For a typical solvent of non-polar molecul like hexane $\Delta f = 0.001$ and for a highly polar one, like water $\Delta f = 0.34$

Effects upon chromophores.

To calculate the effects of the environment upon the absoprtion, or the emission of light it is necessary to include [the energies of interaction ~~between~~ of the chromophore in the ~~ground and excited~~ two states with the solvent molecules ~~in the energy difference between these~~ and * electronic states, ~~and therefore, according to~~ this difference is incorporated in the Bohr frequency condition in the calculation of the wavelengths absorbed.

* difference in

If the energy in the ground state is U and the energy in the excited state is U* in the absence of all molecular interactions,

$$U^* - U = hc\bar{\nu}_o \quad (4)$$

where $\bar{\nu}_o$ is the wavenumber of the electronic transition (0-0') transition, and U and U* are molecular energies. If the energies of interaction of the solvent molecules with the chromophore in the ground and excited states are respectively u and u* we must have

$$(U^* + u^*) - (U + u) = hc(\bar{\nu}_o + \Delta\bar{\nu}) \qquad (5)$$

The displacemenf $\Delta\bar{\nu}$ of the electronic transition, due to the interaction with the solvent is therefore

$$\Delta\bar{\nu} \equiv (1/hc)(u^*-u) = (1/hc)\Delta u \qquad (6)$$

The problem is therefore the calculation of the difference u in the interaction of the chromophore with the solvent in the ground and excited states. Such interactions are electrostatic in nature and depend upon the detailed distribution of charge in the chromophore and solvent molecules. This molecular charge distribution may be expanded in terms of monopole, dipole, quadrupole...contributions. We shall assume our molecules to be neutral so that the dominant term in the distribution will come in most cases from dipole effects. It must be realized from the start that such treatment leaves out of consideration a number of possible causes of interaction like charge transfer effects or hydrogen bonding and that these may produce the dominant effects in some cases. However, the restricted dipole treatment is useful in providing a framework into which many practical cases may be fitted besides permitting to see the character and implications of the cases that do not conform to its predictions.

Chromophore dipole in a dielectric.

When a dipole molecule is placed inside a dielectric the latter becomes polarized. To make use of the macroscopic properties of the solvent it is supposed that a small spherical cavity of radius a is scooped in the medium and a "point" dipole of strngth μ is placed in its centre. The surrounding medium is characterized by its dielectric constant and refractive index. This model originated with N.Martin and R.P.Bell and was developped by Onsager. When the dipole is placed inside the cavity,the surrounding medium is polarized,that is charges appear on the surface of the cavity,that act to compensate those of the dipole. The field R that would produce,when externally applied the same effects as the dipole is called the reaction field.

Böttcher (Theory of electric polarization,Elsevier 1952) demonstrates that

$$R = \mu f / a^3 \qquad (7)$$

where f is the polarizability of the medium. This can be partitioned into the two contributions,one from the orientational polarizability R_{or} and another from the electronic polarizability R_{el}.Moreover,

$$R_{or} = (\mu/a^3)\,\Delta f \; ; \qquad R_{el} = (\mu/a^3)(n^2-1)/(2n^2+1) \qquad (8)$$

giving therefore,

$$R = (\mu/a^3)\,(\,\Delta f + (n^2-1)/(2n^2+1)\,) \qquad (8)$$

If field and dipole are antiparallel,the interaction energy is

$$u = -R\mu = -(\mu^2/a^3)(\Delta f + (n^2+1)/(2n^2+1)) \qquad (9)$$

Consider now a molecule at the time of the excitation. The equilibrium set up previous to the excitation corresponds to the dipole moment μ

of the chromophore in the ground state. At the time of the absorption μ changes into μ^* the dipole moment characteristic of the excited state that results from the absorption of the exciting radiation. The change in interaction energy Δu may be split into two parts corresponding to changes in the orientational and electoonic parts of equation (8).

$$\Delta u_{or} = R_{or}(\mu - \mu^*) \qquad (9)$$

where R_{or} is the reaction field of the ground state since, according to the Franck-Condon principle, nuclear configuration changes, required to modify R_{or} cannot take place during the excitation itself. Therefore in equation (9) giving R_{or} /and the last equation gives, the dipole moment equals μ

$$R_{or} = \mu \Delta f / a^3$$
$$\Delta u_{or} = \mu(\mu-\mu^*)\Delta f/a^3 \qquad (10)$$

On the other hand the reaction field due to the electronic polarizability R_{el} changes in absorption following the change from μ to μ^*:

$$\Delta u_{el} = \Delta u_{el} - u^*_{el} = (\mu^2-\mu^{*2}) \cdot (n^2-1)/(2n^2+1)\ a^3 \qquad (11)$$

the total energy/in absorption equals, change

$$\Delta u = \Delta u_{el} + \Delta u_{or}$$
$$\Delta u = \mu(\mu-\mu^*)\Delta f/a^3 + (\mu^2-\mu^{*2})(n^2-1)/(2n^2+1)\ a^3 \qquad (12)$$

Δu adds to the energy of the absorption transition resulting in a change $\Delta \bar{\nu}$ in the wavenumber of absorption given by

$$\Delta \bar{\nu} = \Delta u/hc$$

In vacuum D=n=1 u=0. The changes in absorption frequency are therefore changes with respect to the absorption in the gas phase in which the molecules are entirely isolated.

... is the time of the excitation the dipole moment associated with the molecule changes instantaneously from the ground state value μ to the excited state value μ^*.

... each dipole is surrounded by its own reaction field, respectively R and R^*. Each of these two is made up of an electronic and an orientational contribution. The electronic contributions to R and R^* are different, since the electronic reaction field can follow the change in dipole strength from μ to μ^*. On the other hand the orientational reaction field cannot follow sufficiently rapidly the change in dipole moment from ground to excited state. Therefore (because of the Franck-Condon principle) R_{or} is the same for both reaction fields. We can then write,

$$R = R_{or} + R_{el} \qquad R^* = R_{or} + R^*_{el}$$

$$R_{el} = 2\mu/a^3 \,(n^2-1)/(2n^2+1)$$

$$R^*_{el} = 2\mu^*/a^3 \,(n^2-1)/(2n^2+1)$$

From the above equations we obtain,

$$\Delta = \mu^*(R_{or} + R^*_{el}) - \mu(R_{or} + R_{el})$$

On substitution,

$$\Delta = (\mu^* - \mu)\, R_{or} + (\mu^{*2} - \mu^2)((n^2-1)/(2n^2+1))/a^3$$

of the chromophore in the ground state. At the time of the absorption μ changes into μ^* the dipole moment characteristic of the excited state that results from the absorption of the exciting radiation. The change in interaction energy Δu may be split into two parts corresponding to changes in the orientational and electoonic parts of equation (8).

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$$\Delta \bar{\nu} = \Delta u/hc$$

In vacuum D=n=1 u=0. The changes in absorption frequency are therefore changes with respect to the absorption in the gas phase in which the molecules are entirely isolated.

Absorption shifts. In a 'non-polar' medium $\Delta f=0$, and therefore $\Delta u=\Delta u_{el}$ or $R_{or}=0$.

$$\Delta u = (\mu^{*2}-\mu^2)/a^3 \cdot (n^2-1)/(2n^2+1)$$

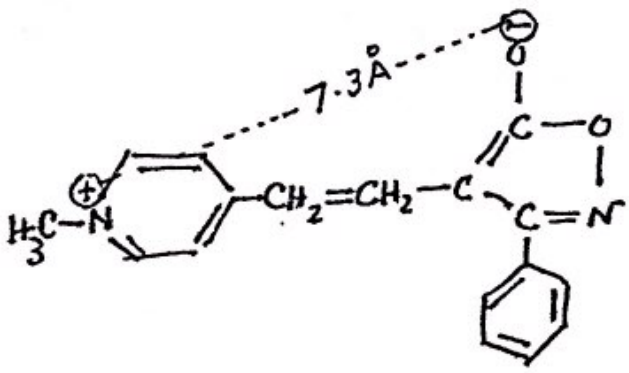
Therefore if $\mu > \mu^*$. Blue shift from the gas phase

$\mu < \mu^*$ Red shift from gas phase.

In a polar medium the same rules apply. The differences in refractive index between polar media like water or formamide and non polar media like hexane are sufficiently small for the differences in the term $n^2-1/2n^2+1$ to be neglected in comparison with the larger differences in Δf. Thus for hexane and water the differences in the terms due to the refractive index is 0.01 while the difference in $\Delta f = 0.33$. With this approximation we can write equation ~~(11). in the form~~ from eq (12)

$$\Delta u_{polar} - \Delta u_{non\ polar} \simeq \mu\,(\mu-\mu^*)\,\Delta f/a^3 \qquad (13)$$

We can take an example due to Lippert (1955) . The compound shown on the left has a dipole moment of 33 D in the ground state. The absorption maximum is at 16,000cm^{-1} in methylene chloride (non polar) and at 21,000 cm^{-1} in water (polar). The bluer absorption in the polar medium shows that the dipole moment in the excited state must be <u>smaller</u> than that in the ground state. Using equation (13)



$$\mu\,(\mu-\mu^*)\,\Delta f/a^3 = 1.10^{-12} \text{ ergs/molecule.}$$

For water $\Delta f = 0.34$ using $a \sim 5.10^{-8}$ cm.

$$\mu-\mu^* = 11\times10^{-18} \text{ c.g.s units}$$

$$\mu^* = 33-11 = 22 \text{ D.}$$

~~To achieve~~ A decrease in the dipole moment of 11 D in a molecule the linear dimensions of which are a few Å units, implies ~~that the absorption transition must result~~ in a shift of electric charge corresponding to a considerable fraction of an electron. In these cases one is entitled to speak of a charge-transfer transition. Various causes of uncertainty in the above treatment may be mentioned, among them the disregard of any specific relations between the chromophore and the solvent molecules, the assumption of a spherical cavity, the negligible dimensions of the dipole and the assumption of a continuous medium surrounding the molecule. Finally an added uncertainty in the final value is introduced by the dependence upon the third power of the molecular radius. Of all these sources of uncertainty the most important is the neglect of differences in hydrogen bonding or partial charge transfer in both ground and excited state. For example the blue shift of the $S_o \rightarrow S_1$ transition in aromatic amines in hydroxylic solvents would lead one to the conclusion that $\mu^* < \mu$, while the changes in fluorescence spectrum with solvent polarity, and much incidental evidence, shows that μ^* is considerably greater than μ. In general the spectral displacements in absorption are considerably smaller than in emission and the qualitative predictions obtained from eq. are considerably less trustworthy than those from observation of the fluorescence. The theory offers however a framework to separate the cases due to pure changes in dipole moment from others - like the case of the amines ~~discussed~~ in which other molecular interactions enters into play.

The effects of molecular interactions upon absorption.

We consider the interactions possible between two molecules A and B dissolved in a 'transparent' solvent. In general a well defined molecular complex must be formed for the absorption to be conspicuously modified . The absorption spectrum observed will be the result of the juxtaposition of contributions from the free partners A and B and the molecular complexes A_iB_j where i and j ~~are small integers, usually less than 2, which~~ define the stoichiometry of the complex. It is implicit that spectroscopic observations will be extremely useful, often indispensable, in the determination of the stoichiometry and the thermodynamic properties of molecular complexes.

From a purely descriptive point of view it is convenient to consider the effects belonging to three separate spectral regions,

1. The region where neither A or B have conspicuous absorption.
2. The region where one of the partners alone shows strong absorption.
3. The region where both absorbe intensely.

Changes in the region of small or null absorption.

In some cases interaction of the partners results in the appearence of a strong absorption band situated ~~at longer wavelengths to~~ in a spectral region in which absorption by both ~~the spectra of~~ either A ~~and~~ or B ~~alone in the same solvent~~ in the same solvent is weak or absent. A case of this type is illustrated by the finding of Benesi & Hildebrandt (1948) of a strong absorption band (λ_{mx}= 298 nm; k_{max}= 9,000) when I_2 and Benzene are mixed. I_2 in an indifferent solvent like hexane shows a principal absorption band at 500 nm with k_{max}=1,000, while benzene does not appreciably absorbe beyond 260 nm. with an oscillator strength of order 10^{-3}. Mulliken suggested as a possible origin of this band that,

while the ground state of the complex could be described by the apolar structure AB, where orientation polarizability and dispersion forces provide the binding energy, the state characteristic of the new absorption was a strongly polar one in which one molecule, the donor gave an electron to the other, the acceptor. Thus the process of light absorption would result from a change that may be crudely characterized by

$$AB + h\nu = A^- B^+$$

The new excited state belonging to the complex is a 'charge transfer state' and the new absorption band may be called a charge-transfer band. (CT).

Certain consequences that may be tested by experiment follow from this idea.

1. It is possible to estimate the spectral region in which CT bands should occur: If I_D is the ionization potential of the donor, B and E_A the electron affinity of the acceptor, A

$$I_D - E_A$$

represents the energy necessary to create the isolated species A^+ and B^- from A and B, where A^+ and B^- are at such large distance from each other that no electrostatic interactions take place. If they are now allowed to interact, coming to an equilibrium distance r, the attractive enrgy released is e^2/r and the total energy involved in creating the polar structure A^+B^- from A B equals,

$$\Delta u = I_D - E_A - e^2/r \qquad (14)$$

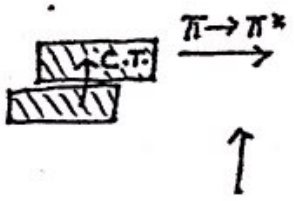
The wavelength of the charge -transfer absorption should be at

$$\Delta_{CT} = hc/\lambda \quad (I_D - E_A - e^2/r) \qquad (15)$$

The ionization potentials of many organic molecules are known with certainty, they are in the neighbourhood of 8 e.v. or 180 kg.cal. The electron affinities are less well known but they are experimentally estimated to be 1-2 e.v. or 20-45 kg.cal. If the charge transferred is that of a whole electron and r is assumed to be the sum of the van der Waals radii of the atoms or molecules, say 5 Å, the term e^2/r amounts to some 65 kg.cal. Therefore the energy Δu is typically some 3.5 e.v. or 75 kg.calories, corresponding to a transition in the U.V. or perhaps in some cases the visible range of the spectrum. As it is obvious from the above calculation, the large energy difference between the electron affinity and the ionization potential of organic molecules ensures that the ground state has only a very small contribution from charge transfer processes, which will require the energy supplied by the radiation to take place to an appreciable extent. This was not fully realized when first the idea of charge transfer bands was proposed by Mulliken, and the complexes which showed such bands were called charge transfer complexes. There are no reasons to believe that the ground state of complexes showing a charge transfer band differ in any manner from the many other molecular complexes which do not show them. The detailed measurements of the dipole moments of complexes with CT bands by Czekalla and his co-workers show that they these are essentially normal, of the magnitude expected from the vector sum of the moments of the isolated partners.

2. Always according to equation (15) we expect a large dipole moment only in the excited state. Such moment cannot be measured in as direct a fashion as in the ground state, but it may be indirectly estimated in other ways, for example by the dependence of the fluorescence spetrum upon the polar characteristics of the solvent, or from the dependence of the fluorescence polarization upon an external electric field. The values of u^* measured in this way are in good agreement with the idea of an almost total transfer of the electronic charge in the excited state.

3. In molecular complexes between formally uncharged aromatic rings the main forces maintaining the complex are dispersion forces between the rings. The maximum interaction brings the rings into v der Waals contact over their flat faces so that the geometry of the complexes is expected to be as shown below

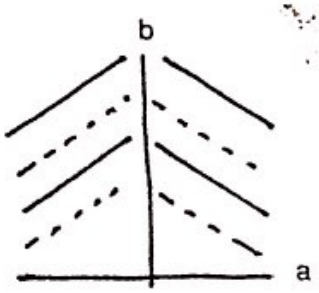


Direction of $\pi - \pi^*$ aromatic transitions

Expected direction of CT transition

The π-π^* transitions have moments contained in the plane of the aromatic rings. On the other hand the transition due to the transfer of charge may be expected to lie in a direction corresponding to the charge separation and therefore normal to the plane of the rings. Nakamoto has investigated the crystal dichroism of some molecular complexes showing CT bands. The crystals of quinhydrome are made up of alternating layers of benzoquinone and hydroquinone. The unit cell contains two quinone-hydroquinone complexes which are, unfortunately, at a considerable angle to each other, as shown in the accompanying

figure.

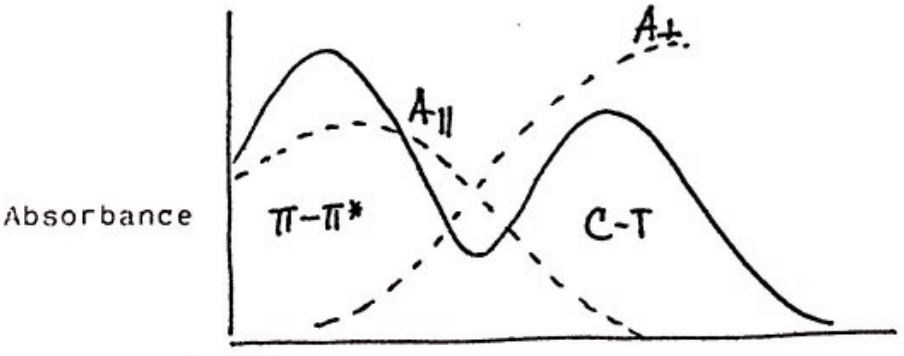


Quinone

Hydroquinone.

Nevertheless it is possible to observe that while the $\pi-\pi^*$ absorption transitions is most intense when illuminated with light polarized along the a direction (E vector parallel to a), the crystal shows maximal absorption in the CT band when the electric vector is along the b direction.

Hexamethylbenzene and tetrachlorophthalic anhydride form a molecular complex that crystallizes in a favourable form for optical observations of this type. The orientation of the molecules in the unit cell is such that it is possible to set the plane of polarization of the light either parallel or perpendicular to the plane of the rings. The variation in the absorption is that predicted for this case by the charge transfer hypothesis.



When the new absorption band observed upon molecular interaction is

strong and lies clearly to the long-wave side of the spectrum of the partners there is little difficulty in assigning it to a charge transfer process. When the new band is buried within the region of π-π* absorption, or it is very weak it may be difficult or impossible to reach a decision. The criteria under 1,2 or 3 above may be used, but the answer may not be completely clear since not all cases are equally favorable for their use. It is being increasingly recognized that many previous assignements of CT bands in molecular complexes of biological interest are very doubtful. Processes of partial charge transfer in the excited state may be interesting and important to the Biologist but for the present we are lacking in criteria that would permit us to determine them in quantitative fashion.

Absorption changes in the region where one partner alone absorbs.

The 'non-absorbing' species takes the part of the transparent solvent, the effect of which as already beeen studied. The effects are usually less marked than those seen upon change of solvent since the partner will correspond to a fraction of the cavity in which the chromophore is placed. In most cases the complexes involve primarily dispersion forces between aromatic rings and because of the increased polarizability of the molecule in the excited state the main effect is one of shift to the red upon complex formation. An added complication in these cases is introduced by the fact that the ~~complex~~ solvent may not be indifferent in the interactions of A with B. Many of these complexes could be really ternary complexes in which the solvent plays an importnt role both in the thermodynamic and spectroscopic properties of the complex. Thus, nuclear magnetic resonance observations show that chlorophyll molecules undergo associations in which water molecules enter in stoichiometric amounts. (Ballschmitter & Katz, J.A.C.S.)

X-ray diffraction observations have shown that complexes occur in which

occur in which molecules of water are ibdispensable in maintaining the structure of the complex. (E.Sefter,Science)

Effects observed in the region in which both partners absorbe strongly.

In these cases the effects are dominated by the geometrical relation of the transition moments of the molecules making up tb complex.

Consider the case of oscillators belonging to identical molecules oriented parallel and in v. der Waals contact with one another. The light waves generates identical distribution of charges in both molecules and the repulsive effects between the charges has the effect of decreasing the absorption intensity

a. b.

If the transition moments are sequential ,as in b the effect is to promote absorption since the separation of charges in the two molecules tend to stabilize each other. The effect is to hinder absorption in one case and to facilitate in the other. The first effect is one of hypochromism, the second is one of hyperchromism.

Hypochromism is observed in stacked compexes of aromatic molecules. Some degree of hypochromism must be expected in these cases since the π-π^* transition moments are all contained in the plane of the ring. If the transition momentswere perpendicular to the ring planes the general case would be one of hyperchromism. The fact that the usual observation is one of hypochromism shows that the transition moments in absorption must be contained in the ring plane. Apart from hypocchromism it is often seen,particularly in the case of dimers of dyes like fluorescein or fuchsin,that the longest wavelength absorption band

is split into two, the weaker of the transitons appearing at longer wavelengths and the stronger at shorter wavelengths as compared to the single band in the [illegible] molecule.

In other cases like the dinucleotides and polynucleotides this band splitting is hardly seen and only a decrease in absorption with little or no change in [illegible] maximum is present.

Multimolecular interactions.

The strength of the interactions between neighbouring dipoles dexreases with the cube of the distance ($1/r^3$) thus in a stack of flat molecules the interaction between two oscillators separated by an intermediate molecule may be expected to be 1/8 th or less of the interaction between nearest neighbours. In an infinite stacked array virtually the whole of the effect is therefore due to the three or four nearest neighbours. It is then understandable that in simple dinucleotides the hypochromism may reach [illegible] while in helical polynucleotides and in the natural nucleic acids it rarely reaches 20% . The appearance of hypochromism does not require the molecules to be equal or similar in nature. It is only necessary that absorption by the two chromophores take place at the same wavelength. Thus the proximity of [illegible] gives rise to a deffect absorption of 17% at 260 nm. In NAD+ a similar situation arises as a result of common absorption in the region of 260 nm by reduced nicotinamide and adenine. Although most of the hypochromic affects have been described in aromatic molecules, because of the common association of such molecules along the planes containing the transition moments, they may also be observed in non-aromatics. Thus Imahori and Tamiya[19] observed that the helical form of polyglutamic acid has a much weaker absorption at 195 nm. (amide absorption) that the random coil of the same molecule. The former has ε_{max}= 4,500 cm^2/mM

per residue, the second 7,000 cm^2/mM. Rosenheck and Doty (196)) have made similar observations in polylysine. The phenomenon is due to the parallel orientation of the transition moments of the amide group absorption imposed by the formation of the helix.

Fluorescence

General Remarks

The emission of light by molecules in solution can provide considerable information commonly not available from absorption studies. If the emission processes were the strict reciprocal of the absorption the study of both processes would provide the same information. Two factors intervene in making the emission process different from that of absorption. In the first place the excited molecules emit after an interval of 10^{-8} sec. after the absorption. This time is long enough to permit extensive molecular rearrangements of various kinds involving the molecular geometry, tautomerizations or proton dissociation, by which reasons the emitting molecules may differ essentially from those existing at the time of the excitation. In the second place a number of competitive processes take place after excitation and reduce the number of molecules capable of radiation. All these processes can only affect the emission if they occur with appreciable probability in their fluorescence lifetime. Therefore, fluorescence emission permits the recognition and measurement of a variety of molecular processes that take place at rates of 10^{7}-10^{10} sec^{-1}. This time range is particularly interesting to the chemist and biologist. With the exception of the phenomenon of electronic energy transfer, which can occur when the excited and unexcited molecules are separated by several molecular diameters, all other modifications of the emission by molecular interaction require direct contact of the partners. If the modifying molecules - or chemical groups have a diffusion coefficient D, they will be able to diffuse, in the time of the fluorescence lifetime to an average distance Δx, determined by the Einstein relation

$$\Delta x^2 = 2D\Delta t \qquad (1)$$

For small molecules in solution D is typically 4.10^{-6} cm^2/sec at room temperature. Since the time allowed for diffusion and encounter of the molecules is 10^{-8} sec.,

* With the exception of the phenomenon of electronic energy transfer, which can occur when the ~~molecule~~ excited and unexcited molecules are separated by several molecular diameters, all other modifications of the emission by molecular interaction require direct contact of the partners. If the modifying molecule - or chemical groups have a diffusion coefficient D, they will be able to diffuse, in the time of the fluorescence lifetime τ an average distance Δx, determined by the Einstein relation:

$$\sqrt{\Delta x^2} = 3.10^{-7} \text{ cm.} \qquad (2)$$

and only those elements in the solution that were within some 30 Å of the excited molecule will be able to affect the emission. If the interval between excitation and emission is reduced to 10^{-10} seconds

$$\sqrt{\Delta x^2} = 3.10^{-8} \text{ cm.} \qquad (3)$$

and only those elements in the immediate vicinity of the excited molecule can affect the fluorescence emission. In times of the order of 10^{-12} sec. molecular motions become completely negligible. It follows that in explaining the changing molecular interactions so important in Biology the simplest elementary steps that can be conceived will take place during times commensurate with the fluorescence lifetime. This is the most important reason for the interest of the Biologist or Biochemist in fluorescence. In some instances the molecular elements capable of modifying the fluorescence emission may be found well within the critical volume determined by eq. (1) but contact with the fluorophore requires overcoming of a potential energy barrier of height E. If transfer of energy between nearby elements occurs with a frequency ν modification of the fluorescene is possible if

$$\nu \exp - (E/RT) \geqslant 7/\tau \qquad (4)$$

the last equation gives

$$E \leqslant 2.3 \text{ RT log } (\tau\nu) \qquad (5)$$

At room temperature, employing the most likely value of ν, 10^{12} and $\tau = 10^{-8}$ sec.

$$E \leqslant 5.4 \text{ kcal/mole}$$

We can therefore associate with the processes that modify the fluorescence emission a characteristic true τ, and characteristic radius of action and maximum energy of activation given respectively by eqs. (1) and (5).

The fluorescence emission from solutions may be characterized much more completely than the absorption. In other words it is possible to obtain more complete information about the molecular processes responsible for the former than for the latter. To characterize completely the emission from a solution

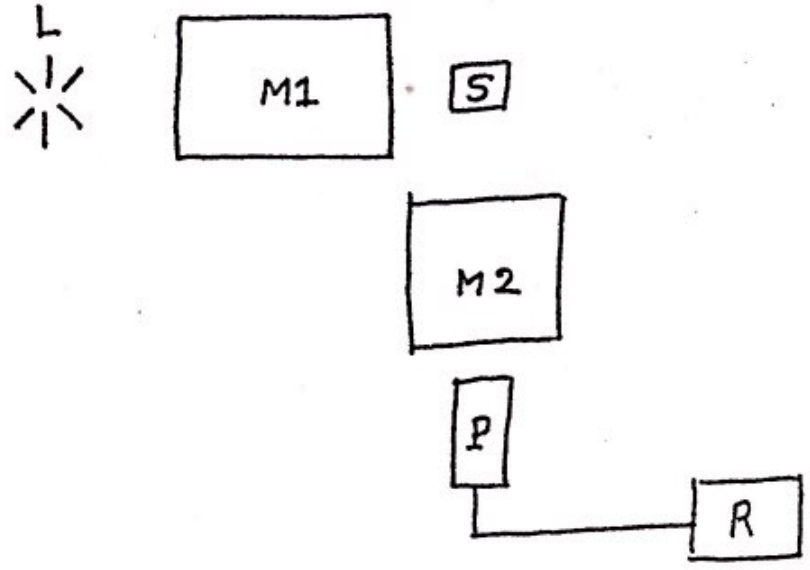
we shall examine:

1. The fluorescence emission spectrum.
2. The excitation spectrum of the fluorescence.
3. The quantum yield.
4. The fluorescence lifetime.
5. The polarization of the emission.

Points 2 and 3 have counterparts in absorption, namely the absorption spectrum and the intensity of the absorption. The fluorescence spectrum, fluorescence lifetime and polarization of the emission of solutions have no counterpart in absorption and provide the additional information.

Fluorescence emission spectrum.

To observe and record the fluorescence emission we require an exciting source, as nearly monochromatic as possible, a monochromator to disperse the fluorescence emission and a detector, usually a photomultiplier to measure it. The monochromatic excitation is best provided by a continuous light source like the Xenon arc, and an additional monochromator, M1. The plan of a spectrophotofluorimeter is shown below:



L. Exciting light source.
M1, M2. Monochromators
S. Fluorescent sample.
P. Photodetector
R. Readout devise: DVM, recorder, in line computer.

Using the spectrofluorimeter it is possible to study the dependence of the fluorescence emission upon the the exciting wavelength, and therefore upon the absorption spectrum of the solution. Examination of a large number of cases has resulted in the formulation of certain general rules:

1. In a pure substance existing in solution in a unique form the fluorescent spectrum is invariant, remaining the same whatever the wavelength of excitation.
2. The fluorescence spectrum lies at longer wavelengths than the absorption. If the absorption maximum of the band of least frequency is at wavelength λ_{abs} and the maximum of the fluorescence spectrum is at λ_{fl} it is always found that,

$$\lambda_{abs} < \lambda_{fl}. \quad \text{or} \quad \bar{\nu}_{fl} < \bar{\nu}_{abs}$$

3. The fluorescence emission spectrum is, to a good approximation, a mirror image of the absorption band of least frequency. The wavelength of reflection is found midway between $\bar{\nu}_{abs}$ and $\bar{\nu}_{fl}$ and corresponds to the energy of the pure electronic transition (0-0')

[I shall take up the explanation of the origin of these regularities, and then discuss some exceptions, real and apparent.]

If the fluorescence spectrum is invariant it is clear that the emission must take place as a transition between fixed energy levels, independently of the energy absorbed at the excitation. Since $\bar{\nu}_{fl} < \bar{\nu}_{abs}$ but still close to it, the origin of the emission, or upper level of the transition must correspond to the lowest electronic band reached in absorption. If this is the first singlet excited state, S_1 then the longest wavelength absorption transition,

~~taking place as it does from the ground state singlet~~ is $S_0 \rightarrow S_1$, and the fluorescenceemission must correspond closely to the reverse process $S_1 \rightarrow S_0$, <u>irrespective of the state initially reached in absorption</u>. Observation of the absorption spectrum of organic compounds in solution reveals no gaps between the electronic transitions. [Each observable absorption maximum corresponds either to a vibrational level of an electronic transition or is itself the absorption maximum of an independent electronic absorption band.] ~~Very seldom~~ Excentionally does the absorption fall at the minima to 1/2oth. ~~or even~~ and very seldom to 1/10th of the value at the nearby maxima. Most often the ~~ratio of~~ molar absorption of maxima ~~and minima is five times or less~~ is only in two to five times the absorption at the minima. It is possible then to speak of the absorption spectrum of organic molecules in solution as of a virtual continuum extending from a long-wave limit into the vacuum ultraviolet without gaps or even very ~~sudden~~ abrupt changes in intensity of absorption. The different electronic energy levels within this continuum must therefore have considerably overlap with the states of neighbouring energy. It follows that the energy gained in absorption can be easily redistributed within the molecule, or exchanged with be solvent, as the emeergy content drops from from the original reached in absorption to the S_1 level. Here the continuum of states becomes interrupted and it is this interruption that is responsible for the energy, as well as the invariant character of the fluorescence emission. It is possible to imagine that the process following excitation are distinctly two:

1. Redistribution of excess vibrational energy within the excited molecule.
2. Loss of the excess vibrational energy to the surrounding medium.

was originally

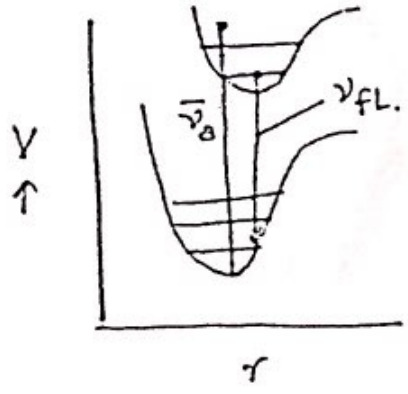
The existence of these two processes has been ~~well~~ documented by the studies of B.S. Neporent in Russia, using naphthylamine vapour at various pressures. In the most dilute vapours the molecules of naphthylamine are virtually isolated from each other in the time from excitation to emission. Since they undergo no collisions with other molecules in this interval of time, the only way in which the energy of the emission may be modified is by redistribution among their own degrees of freedom. Naphthylamine has 20 atoms or some 54 (3n-6) vibrational degrees of freedom. If ΔE, the excess vibrational energy above the lowest vibrational level of S_1 is equipartitioned among the exisitng degrees of freedom, the result is a hot molecule its 'temperature' being determined by the relation

$$\Delta E = C_v \cdot T_{vib}$$

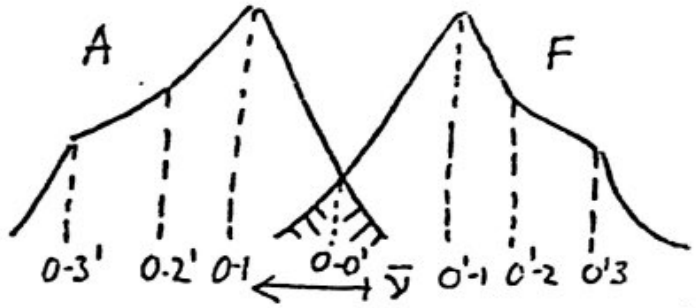
where C_v is the specific heat at constant volume and T_{vib} the tempeature that would correspond to the equipartitional vibrational energy. The emission will take place from molecules distributed in Boltzman fashion above the 0' level of S_1. At 0^o K the emission would consist exclusively of transitions from the 0' level of S_1 to the variuos vibrational levels of S_o. At higher temperatures the emission from levels above 0' would become appreciable, and show itself in a displacement of the maximum towards shorter wavelengths. From the blue shift of the maximum of emission, T_{vib} may be calculated. It is possible therefore to compute C_v from the spectroscopic data of very dilute vapors excited at different wavelengths, and compare it with the value obtained by conventional calorimetric methods. Neporent obtained ~~in this way~~ excellent agreement demonstrating thus the reality of the redistribution

of energy within a complex polyatomic molecule. If a foreign gas like H_2 or He is admitted together with the dilute naphthylamine vapour, at a sufficient pressure of the foreign gas the excitation-dependent blue shift completely disappears. Clearly a process of thermal equilibration with the foreign gas takes place between excitation and emission and as a result of this 'thermalization' the fluorescence spectrum becomes independent of the exciting wavelength. The small pressures of foreign gas required to achieve this result indicates that a few collisions are sufficient to equalize the temperature of the excited molecules with the surroundings. Boudart & Dubois have further shown that a molecule with many degrees of freedom, like pentane is much more efficient in removing energy than He or H_2. It is therefore quite clear why in solution a single fluorescent spectrum, independent of the exciting wavelength is observed: Collisions with the solvent occur at the rate of 10^{12} sec^{-1} so that virtual thermalization must be reached in 10^{-11} sec or thereabouts, which represents a very small fraction (10^{-2} to 10^{-3}) of the total fluorescent lifetime. This conclusion, currently accepted for many years has received direct configuration by studies of Kaiser and others, who utilized the decay of the Autistokes Raman lives to determine the lifetime of states in liquids.

2. The displacement of the fluorescence maximum towards longer wavelengths as compared with the absorption maximum is a simple consequence of the Franck-Condon principle, and the condition that the internuclear equilibrium distances are different in the ground and excited state increase as a result of the changed electronic distribution.



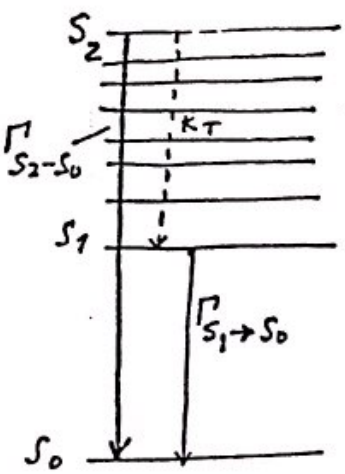
3. The mirror image rule may be grounded in the fact that reciprocal transition probabilities are equal. Thus the probability that the absorption takes place from the 0 level of S_o to the nth level of S_1, must be equal, except for a universal multiplier the ratio of the A and B coefficients of Einstein, to the probability of emission from level 0' of S_1 to the nth level of S_o. A simple construction shows how the mirror image arises:



The shaded region, common to both spectra corresponds to the absorption taking place from levels between 0 and 1, and the emission from level between 1' and 0'. The 0–0' or pure electronic transition may be located midway between the absorption and emission maxima with fair accuracy, while it would not be possible to deduce the position of it by either the absorption or emission spectra alone. Observe that irrespective of bandwidth there would be no overlap of the absorption and emission bands if all absorptions originated in the 0 level of S_o and all emissions in the 0' level of S_1. This would correspond to the Boltzman distribution at 0^o K. As the temperature is raised emission and absorption increasingly overlap due to contribution originating from levels above the zero level. The thermal overlap would be expected to be the more important the closer the absorption maximum is to the 0–0' transition, that is other things being equal the sharper the absorption and emission the more important the overlap. Fluorescein and chlorophyl furnish examples of this phenomenon. The energy difference $hc(\bar{\nu}_a - \bar{\nu}_e)$ is called the Stokes' shift. The Stokes shift observed in indifferent solvents, like hexane and cyclohexane, is roughly

equal to twice the bandwidth of the $S_o \rightarrow S_1$ (or $S_1 \rightarrow S_o$) transitions. This half width, which is less equivocally determined in the fluorescence than in the absorption spectrum, vary typically from 300 to 2500 cm^{-1}, (Stokes shifts range from 200 to 5000 cm^{-1}). In polar media the Stokes shifts can increase by as much as 5000 cm^{-1} in the extreme cases.

is in this case dependent upon the pH of the solution but not upon the exciting wavelength. This characteristic behaviour permits us to deduce that the additional, green fluorescent species is formed only in the excited state. The absence of any apparent contribution to the emission from the upper level (S_2 ... etc.) when these are originally reached by the excitation can be understood in terms of the relative rate of the competing processes of radiation transition from the upper levels ($S_2 \rightarrow S_o$, $S_3 \rightarrow S_o$ etc...) and the rate of thermalization. (Fig). Let the rate of emission from the S_2 level be



Γ_2, the rate of emission from S_1 be Γ_1 and the rate of thermalization be K_t. The new ratio of the fluorescence emission from S_2 to the fluorescence emission from S_1 will then equal $\frac{\Gamma_1}{\Gamma_2 + k_t}$. From the oscillator strengths of the transitions we can assume that Γ_1 and Γ_2 are both of the order of $10^8\ 5^{-1}$ while K_t, from the observations of Kaiser and others is the vibrational deactivation in liquids is at least of order $10^{12}\ 5^{-1}$ and may be even higher if the process of crossing from S_2 to S_1 is determined only by intramolecular redistribution of the excess vibrational energy. In anycase the ratio of emission from upper level transition from S_1 can be no greater than 10^{-4}, and would be exceedingly difficult to demonstrate in practice. In azulene and its derivatives a rather exceptional situation prevails. The lowest energy electronic transition ($S_o \rightarrow S_1$) is peculiar to substances with this nucleus, nothing similar being observed in other aromatic hydrocarbons: It covers a large potion of the visible spectrum and is devoid of vibrational structure. The shorter wavelength transitions are very much like those of other aromatic hydrocarbons, and the overall spectrum is not unlike that of naphthalene, though displaced to longer wavelength. The fluorescence is weak, with a yield of about 1%, and its spectrum is a mirror image of the $S_o \rightarrow S_2$ absorption. No detectable fluorescence is observed on

True and apparent exceptions to the rules regarding the fluorescence emission.

Because of the rapid thermalization the observation of strong dependence of the fluorescence spectrum upon the exciting wavelength may be taken as a proof of the existence of more than one emitting species in solution. Fluorescence impurities may be often detected by this method. Less trivial is the case due to the presence of forms differing by the dissociation of a proton. For example the naphthylamines associate a proton to the aromatic NH_2 at pH values below its 4.5 pK. The absorption by unprotonated naphthylamine extends to nearly 400 nm, with a maximum of the $S_o \rightarrow S_1$ transition at 340 nm, and the fluorescence occupies a broad band in the visible spectrum with maximum at 440 nm. The protonated form-naphthyl - has absorption and emission that resemble those of methyl-naphthalene. The $S_o \rightarrow S_1$ transition has a maximum at 320 nm and the emission is entirely in the ultraviolet. At pH values close to the pK, practically within two units of it a change in the fluorescence spectrum with exciting wavelength will be detected, as well as the presence of two fluorescence bands corresponding to the two species, if the excitation falls upon a wavelength absorbed by both.

In yet other cases there may be a single ground state species but two distinct fluorescence spectra may be emitted if proton association or dissociation occurs in the excited state. A simple example of this kind is that of naphthionic acid which has a blue fluorescence in neutral solution and a green fluorescence in alkaline solution (0.1 M NaOH) (Boas E. Rollef) By adjusting the concentration of alkali from 10^{-5} M to 10^{-1} M it is possible to observe the transition from the blue emission (neutral molecule) to the double emission (neutral molecule and NH^- ion both present in solution) to the green emission (molecular ion alone). In these cases there is no ground state difference, since the species NH^--Ar-SO_3^- does not exist at any pH value in the ground state. The fluorescence spectrum

excitation to the S_1 level. The very different nature of this level appears responsible for the sluggish crossing to S_1 from upper levels. This rate of crossing does not appear determined by, or to be of the same order as, the rate of thermalization but is slow enough to permit emission of fluorescence from S_2 with a lifetime of the order of 1 nsec. Evidently the second of our rules is violated since the longest wavelength absorption clearly exceeds the fluorescence in this particular case. The mirror image rule is valid, but the emission is the mirror image of the $S_o - S_2$ and not of the $S_o - S_1$ transition.

In many cases a very careful study of the dependence of the emission upon the exciting wavelength shows the existence of very small differences, on the order of a few percent. What do these reveal? The experimenter has here a difficult choice. They may be due to the presence of a very small amount of a second ground-state component, perhaps a chemically modified molecule, different, but not very different from the bulk, or they may be ground state conformers or tautomers that undergo interconversion in times that are long compared to the fluorescence lifetime, or complexes of the fluorophore with the solvent existing in very small concentration but absorbing preferentially at certain wavelengths. If all these possibilities can be discarded by experiment it becomes necessary to accept the existence of two fluorescence levels, and that the probability of populating one or the other depends upon the excitation wavelength. The compounds more amenable to this kind of investigation are undoubtedly the aromatic hydrocarbons: They can be obtained in high purity using zone refining methods, and because of the planar character of the molecule the existence of tautomers, or stable solvent complexes in a fluid solvent is not likely.

Additional points in 'fluorescence spectrum'

1. Experiments of Duccuing and Ricard on the vibrational deactivation
2. Experiments of Reutzepis on radiationless crossing in levels.
3. Emission from S_2 due to thermal excitation.

Fluorescence excitation spectrum.

The experimental setup used to study the fluorescence emission spectrum may be used to investigate the dependence of the fluorescece intensity upon the exciting wavelength. Evidently the constancy of the fluorescence spectrum does not imply by itself that all exciting wavelengths will result in fluorescence emission proportional to the number of excitations. To clarify experimentally this aspect we can select an interval of fluorescence wavelengths by means of M_2, which shall remain fixed and vary the region of spectral excitation by means of M1. Let the sample S consist of a solution of a pure substance in a transparent solvent. We shall define the quantum yield of fluorescence, or ~~more~~ simply the fluorescence yield as the ratio:

$$q = \frac{\text{quanta emitted as fluorescence}}{\text{quanta absorbed by the solution}} \qquad (16)$$

If the intensity $I(\lambda')$ of the exciting light entering S, and the fluorescence intensity $F(\lambda)$ leaving S, are both given in quanta we can write,

$$F(\lambda) = I(\lambda')A(\lambda')\, q(\lambda') \qquad (17)$$

where $A(\lambda')$ is the fraction of the exciting light absorbed in S and q the quantum yield corresponding to excitation with wavelength λ'. The quantity $F(\lambda)$ is not the quantity detected by the phototube PM. The number of quanta arriving at the photocathode depends upon,

a. The geometry of the exciting beam.

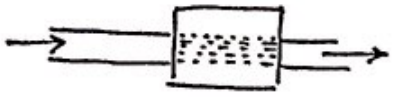
b. The effective aperture of the PM system

c. The transmission of M2 for the quanta of wavelength λ

d: The sensitivity of the detector to quanta of wavelength λ.

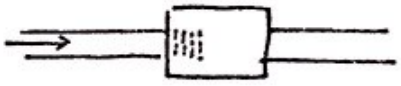
It is seen that a and b ~~are~~ can be influenced ~~determined~~ by the exciting wavelength, wwhile c and d are determined by the fluorescence wavelebgth selected.

The dependence of ~~a and b upon~~ the results upon a and may be ~~shown~~ demonstated. by considering a cuvet of square section as the container S of the solution. When λ' is set at a value at which very little absorption accurs, the fluorescence 'source' viewed by M2 has uniform intensity accross the cuvet length as shown in A below.



A

Fluorescence distribution across the cuvet at a poorly absorbed wavelength.



B

Fluorescence distribution across the cuvet at a wavelength that is strongly absorbed.

In case B the efluorescence emission is restricted to a zone confined to the front face of the cuvet, and it is far from uniform.

It is virtually impossible to adjust the geometry and aperture of the system so that all portions of the illuminated path are seen equally by the detector, but there are two approximate and very useful solutions to the problem. The first is to limit the observations to solutions of such low concentration in fluorophore that the condition in A is always fulfilled. In practice this requires that the maximum absorbance remains below 0.05 or $A(\lambda')$ below 0.15. Then a and b become constant and independent of the exciting wavelength.

Since the absorbance is very low at all wavelengths we can set,

$$A(\lambda') = 1 - \exp-(\sigma_{\lambda'}[c]x) \sim 2.3\, k_{\lambda'}[c]x \qquad (18)$$

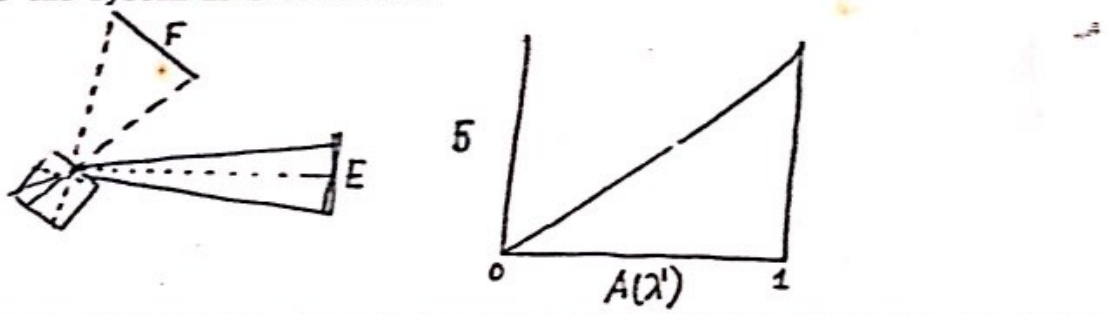
where c is the molar concentration, k the molar absorption at wavelength and x the path length. The signal registered by the detector is,

$$S(\lambda,\lambda') = I(\lambda').2,3\ k_{\lambda}'[c]\ x\ C(a,b).q(\lambda') \qquad (19)$$

where C is a constant dependent upon the conditions a and b. For a constant concentration and cuvet thickness, we have

$$S(\lambda,\lambda')/I(\lambda') = \text{constant}.\ k_{\lambda}'.\ q(\lambda') \qquad (20)$$

Suppose that the relative distribution of the intensity of the exciting light among the different wavelengths is known. Then a plot of $S/I(\lambda')$ against λ' must reproduce the absorption spectrum if $q(\lambda')$ is a true constant independent of the exciting wavelength. Front face collection of the fluorescent light provides an additional method of minimizing the difficulties caused by the variable penetration of the exciting light into the sample. The geometry of the system is shown below:



If the fluorescence lens is focused 1 to 3 mm below the front face of the cuvet containing the solution the light is collected with almost equal efficiency at all optical densities. Equation (19) takes the simple form

$$S(\lambda,\lambda') = A(\lambda')q(\lambda')f(A) \qquad (21)$$

where f(A) is a function of the absorbance for the exciting light which, by judicious adjustment of the collecting lens F may be made to differ by less than 5% between low and high optical densities. F(A) is determined by keeping the

excitation wavelength constant and varying the concentration of fluorophore to obtain absorbances between 0.05 to 2. If this method is used a plot of $S(\lambda,\lambda')/I(\lambda')f(A)$ will reproduce the fractional absorption spectrum $A(\lambda)$ the relation of this to the absorption spectrum is given by eq. (18). At a sufficiently high concentration of fluorophore virtually all the exciting light is absorbed at every wavelength, $A(\lambda')$ to 1 and $S(\lambda,\lambda')$ reproduces the profile of the exciting source, if $q(\lambda')$ is independent of the wavelength of excitation. Used under conditions of complete absorption, a fluorescent solution with a quantum yield independent of wavelength of excitation becomes a proportional quantum counter, since it is able to convert with uniform efficiency the excitation quanta, irrespective of color, into quanta with a unique spectral distribution. Direct measurement of the light source by a photomultiplier, after dispersion by a prism or grating, demands correction for the inevitable spectral differences in grating transmission and photomultiplier response. The proportional quantum counter does away with these two operations. An ideal quantum counter of this kind should emit fluorescence at the longest visible wavelengths and the ratio of maxima to minima of absorption should be as low as possible to prevent large differences in the penetration of light into the solution at the different wavelengths. Additionally it must be sufficiently soluble in a transparent solvent to reach a concentration at which all wavelengths are absorbed in a very thin layer without, however forming molecular aggregates with different absorption spectra and fluorescence yield. A concentrated solution (5 mgm/m .) of Rhodamin B in ethanol of ethylene glycol comes close to these demands. It works well from 250 nm to nearly 600 nm, except for a small region at 430 nm where the lower absorbance permits too large a penetration of the exciting light. Observation of a large number of substances shows that $q(\lambda)$ is a true constant independent of the exciting wavelength, within a few percent in most cases. Deviations can occur, particularly at the edge of the absorption spectrum.

Suppose in fact that the substance in question exists as two conformers A and B, with B representing a small fraction of the total, but absorbing at slightly longer wavelengths than A. Obviously in the region in which both A and B absorb, the latter contributes so small a fraction to the emitted intensity that a variation of $q(\lambda')$ with wavelengths will not be easily detected, even if the yields q_A and q_B are substantially different. However, at the long wave edge, where a large fraction of the total absorption is due to B a variation of $q(\lambda')$ can conceivably, and probably easily be detected. The same situation would apply if a fluorophore forms several ground state complexes with the solvent.

The constancy of $q(\lambda')$ has been found to be the general rule particularly for the aromatic hydrocarbons and their substituted derivative but is not without exception. Ferguson has shown that excitation of 9:10 dibromoauttracene at wavelength shorter than 320 nm, that is in the S_2 transition results in yield of about one half of that observed for excitation to the S_1 level. Teale observed a similar phenomenon in di-iodo fluorescein and Lippert in several nitro derivatives of aromatic compounds. In recent years has measured decreases of 10 - 25% in the relative fluorescence yield of indole in water but not in other solvents (alcohol, ethylene glycol) and Tatischeft and Klein have reported larger changes in tyronine and tryptophan in water solutions. The decreases in yield in the heavy atom derivatives, have been connected to the of dissociation of these groups with formation of radicals a process which can become competitive with the thermal deactivation for a sufficiently high excitation energy. Electron transfer to solvent in the excited state has been also proposed as competitive transition the probability of which increases with energy of excitation in the case of indole. Trustworthy measurements of the yield of electron transfer are still needed to decide whether the reported fluorescence yield decreases could admit this explanation.

where c is the molar concentration, k the molar absorption at wavelength and x the path length. The signal regitered by the detector is,

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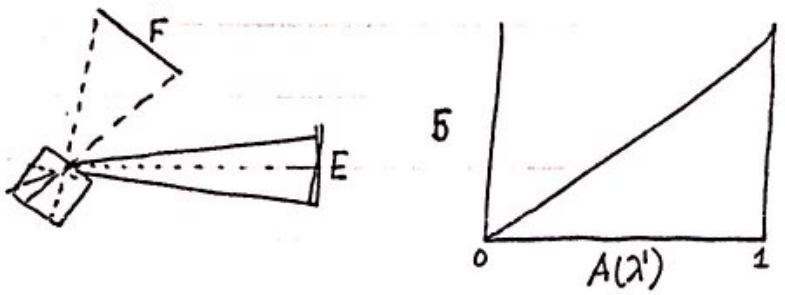
Suppose that the relative distribution of the intensity of the exciting light ~~along~~ among the different wavelengths is known. Then a plot of $S/I(\lambda')$ against λ' must reproduce the absorption spctrum if $q(\lambda')$ is a true constant independent of the exciting wavelength. Observation of a large number of substances shows that $q(\lambda)$ is a true constant independent of the exciting wavelength, within ~~probably~~ a few percent in most cases. Deviations can occur, particularly at the edge of the absorption spectrum. Suppose in fact that the substance in question exists as two conformers A and B, with B representing a small fraction of the total, but absorbing at slightly longer wavelengths than A. Obviously in the region in which both A and B absorbe, the latter contributes so small a fraction to the emitted intensity that a a variation of $q(\lambda')$ with wavelengths will not be easily detected, even if the yields q_A and q_B are are substantially different. However, at the long wave edge, where a large fraction of the total absorption is due to B a variation of $q(\lambda')$ can conceivably, and probably easily be detected. The same situation would apply if a fluorophore forms several ground state complexxes with the solvent.

(* — Front face collection of the fluorescent light provides an additional method of minimizing the difficulties caused by the variable extent of penetration of the exciting light into the sample. The geometry of the system is shown below:



If the fluorescence collecting lens is focused 1 to 3 mm below the front face of the cuvet containing the solution, the light is collected with almost equal efficiency at all low and high optical densities. Equation (19) takes the simple form

$$S(\lambda, \lambda') = \text{Const}\ A(\lambda')\, q(\lambda')\, f(A). \qquad (21)$$

where $f(A)$ is a function of the absorbance for the exciting light which, by judicious adjustment of the collecting lens F may be made to differ by less than 5% between low and high optical densities. $f(A)$ is determined by keeping the excitation wavelength constant and varying the concentration of fluorophore to obtain absorbances between 0.05 and 2. If this method is used a plot of $S(\lambda, \lambda') / I(\lambda') f(A)$ will reproduce the fractional absorption spectrum $A(\lambda)$. The relation of this to the absorption spectrum is given by eq. (18.) At a sufficiently high concentration of fluorophore virtually all the exciting light is absorbed at every wavelength, $A(\lambda')$ tends to 1 and $S(\lambda, \lambda')$ reproduces the profile of the exciting source, if $q(\lambda')$ is independent

of the wavelength of excitation. ~~Thus~~, Used under conditions of complete absorption, a fluorescent solution with a quantum yield independent of wavelength of excitation becomes a proportional quantum counter, since it is able to convert with uniform efficiency all the excitation quanta, irrespective of color, into quanta with a unique spectral distribution. Direct measurement of the light source after by a photomultiplier, after dispersion by a prism or grating, demands correction for the inevitable spectral differences in grating transmission and photomultiplier response. The proportional quantum counter does away with these two operations. An ideal quantum counter of this kind should emit fluorescence at the longest visible wavelengths and the ratio of maxima to minima of absorption should be as low as possible to prevent large differences in the penetration of light into the solution at the different wavelengths. Additionally it must be sufficiently soluble in a transparent solvent to reach a concentration at which all wavelengths are absorbed in a very thin layer without however forming molecular aggregates with different absorption spectra and fluorescence yield. A concentrated solution (5 mgm/m2.) of Rhodamin B in ethanol of ethylene glycol comes close to these demands. It works well from 250 nm. to nearly 600 nm, except for a small region at 430 nm where the lower absorbance permits too large a penetration of the exciting light.

The coincidence of the fluorescence-excitation spectrum with the absorption spectrum provides a means for identifying fluorescent substances at concentrations too small to produce appreciable absorption., and also in the presence of other compounds either non fluorescent, or ~~xxxx xxxxxxxxxx xxxxxxxxxxx~~ emitting at other wavelengths. Consider in fact the fractions of the exciting light x_f absorbed from the exciting beam by the fluorescent compound in the presence of other unidentified non-fluorescent components which will themselves be responsible for the absorption of a fraction x_o of the excitation.

$$x_f(\lambda) = \left(1 - 10^{-(A_f + A_o)}\right) \frac{A_f}{A_f + A_o}.$$

$$x_o(\lambda) = \left(1 - 10^{-(A_f + A_o)}\right) \frac{A_o}{A_f + A_o}.$$

where A_f and A_o are absorbances at wavelength λ, due to the fluorescent and non-fluorescent species respectively, when <u>alone in</u> solution.

If $A_o + A_f \ll 1$ $\quad 1 - 10^{-(A_f + A_o)} \sim 2.3\,(A_f + A_o)$

and $\quad x_f \longrightarrow 2.3\,A_f \qquad x_o \longrightarrow 2.3\,A_o$

so that each component does not interfere with the absorption of light by the other, so long as the total absorbance of the solution is small compared to unity.

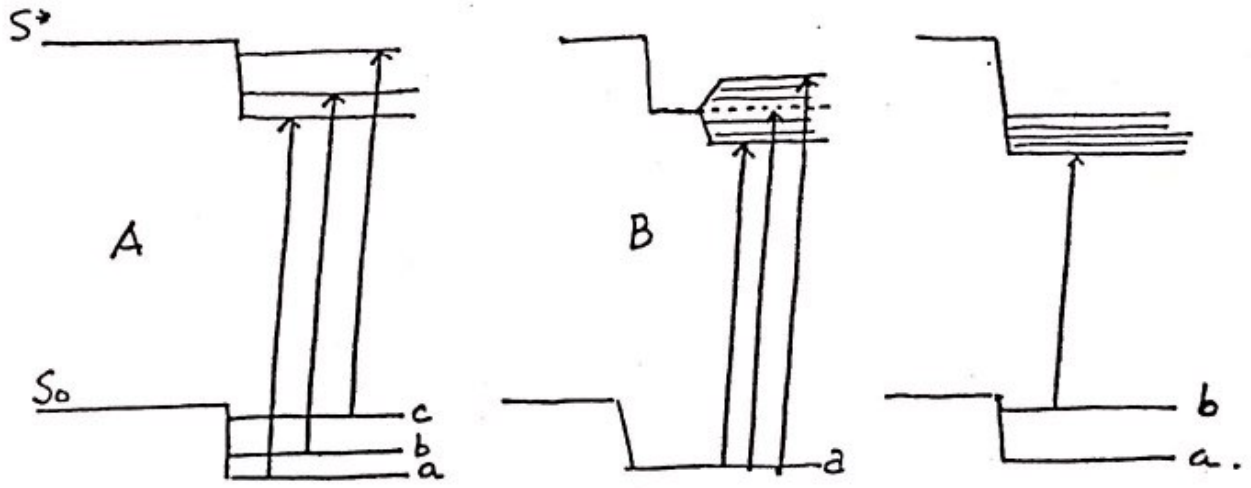
The constancy of $q(\lambda')$ has been found to be the general rule particularly for the aromatic hydrocarbons and their substituted derivatives, but is not without exceptions. Ferguson has shown that excitation of 9:10 dibromoanthracene at wavelength shorter than 320 mμ, that is in the S_2 transition. results in yield of about one half of that observed for the excitation to the S_1 level. Teale has observed a similar phenomenon in di-iodofluorescein and Lippert in several Nitro derivatives of aromatic compounds. In recent years Klein has measured decrease of 10-25% in the relative fluorescence yield in certain solvent of indole in water but not in other solvents (alcohol, ethylene glycol) and Tatischeff and Klein have reported larger changes in tyrosine and tryptophan in water solutions. The decrease in yield in the heavy atom derivatives, and the NO$_2$ groups have been connected to the energy of dissociation of these groups with formation of radicals a process which can which is higher happen to become competitive with the thermal deactivation for a sufficiently high excitation energy. A source to solvent Electron transfer in the excited state has been also proposed as the competitive transition the probability of which increases with energy of excitation in the case of indole. Trustworthy electron transfer yield measurements of the yield of electron ha are still needed to decide whether the reported fluorescence yield decreases could admit this explanation.

* The most common observation is of a spectrum consisting of a smooth fluorescent band closely of energies closely represented by a skewed Gaussian distribution

Solvent Effects Upon The Fluorescence Emission

The width of the electronic absorption bands in solution is similar to that observed in the gas phase but the resolution of the energy levels is much less complete. The highest resolution of the spectra is observed in the solutions of the unsubstituted aromatic hydrocarbons in non-polar solvents particularly in these non polar solvents which interact minimally with the fluorophore like the perfluorohydrocarbons (Lipsky). Even in these cases resolution is limited to the vibrational structure. The substituted aromatic hydrocarbons have much less resolved spectra than the unsubstituted ones. When atoms carrying lone pairs are directly attached to the aromatic rings hydrogen bonding with proton donor solvents results in efficient coupling of the electronic and vibrational energies permiting only small changes in the probability of transitions to nearby energy levels. The general result is that a classical analysis of the fluorescence spectrum of substituted aromatic molecules in solution in terms of energy levels is virtually impossible, in all but exceptional cases. The most common observation is of a spectrum consisting of a band of energies closely represented by a Gaussian distribution. (See Schpolskii) The influences already described are further complicated by the existence of multiple interactions, sometimes quite specific in nature, between solvent and fluorophore molecules. These result from dispersion forces, permanent dipole effects and hydrogen bonds. In this way a whole set of weak molecular complexes of solvent and fluorophore are generated so that the absorption and fluorescence spectra of molecules in solution are best described as belonging to a molecular population rather than to a single molecular species. It is not easy to divide in any particular case - let alone in general - the extent of spectral heterogeneity owing to weak complexes of solvent and fluorophore. The energies of interaction between a solvent with an appreciable dipole moment (e.g. water) and a fluorophore

with a permanent dipole moment of 2 - 5 Debye units is as large or larger than KT at room temperature. Being of the order of the thermal energy these interactions do not give rise to a single species of complexes with uniquely defined electronic ground states but to a virtually continuous set of such states. For dipole-dipole interactions the distribution of the states is prescribed by the Langevin equations:* Upon excitation each of these complexes will generate a Frank-Condon excited state of its own and the interconversion of these within the excited lifetime will depend upon the strength of the molecular interaction as well as the temperature and viscosity of the solvent. It would appear at first examination that because of the Frank-Condon restriction the set of absorption transition from the molecular complexes of ground-state fluorophore molecules would constitute a single degenerate transition as indicated in A in the scheme below:



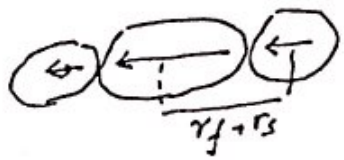
However, owing to changes in the electronic distribution in the fluorescent molecules in the ground and excited states the complexes with the maximum interaction and therefore lowest energy, will be different from the ground and excited states. In over simplified form the change in the electronic distribution may be associated with a difference in both magnitude, and direction of the dipole moments μ^o and μ^* corresponding to ground and excited state. The smaller energy difference, and therefore the longest wavelength absorption, will correspond to

a state that cannot be the lowest of the ground state complexes but must correspond to a less stable state (b) in the above scheme. In a fluid medium excitation at the longest wavelength will still result in a fluorescence spectrum entirely like that observed on excitation at other wavelengths. However, if the viscosity of the solvent is high and the equilibration with appearance of the several possible solvent fluorophore complex is impeded, only the emission from the lowest-energy excited complex is observed. This red shift of the fluorescence has been repeatedly observed in solvents at low temperatures when excited at the red edge of the absorption and is most marked in polar solutions of those fluorophores that differ most in their ground and excited state dipole moments. In agreement with expectations the observed red shifts corresponds to energies of the order of 1 kcal.

The Effect of temperature upon the fluorescence emission

Following in more quantitative fashion the ideas sketched above, the complete process of absorption and emission will take the following course:

1. Absorption takes place from a manifold of states corresponding to discrete solvent-fluorophore complex. For a semi quantitative analysis we consider the interactions to be those between dipoles of strength μ_s (solvent) and μ^o or μ^* (fluorophore in ground or fluorescent state respectively). Maximal interaction between fluorophore and solvent may be assured as result of anti-parallel arrangement of three dipoles, the fluorophore and two solvent molecules placed at distances $\gamma s + \gamma f$ where γs and γf are the radii of solvent and fluorophore. The energy of interaction between the two solvent dipoles may be



neglected as it would contribute at best a few percent to the total energy, and assuring independence in the two remaining interactions, the energy is

$$u = -\frac{\mu_s \mu^o}{r^3} \quad ; \quad r = r_s + r_f \qquad (1)$$

Similarly $$u^* = -\frac{\mu_s \mu^*}{r^3} \qquad (2)$$

The <u>average</u> energy of interaction would be smaller because of the disorienting effect of temperature. The Boltzman average for this case is given by the Langevin equation

$$\langle u \rangle = u\, L(u/KT)$$
$$\langle u^* \rangle = u^* L(u^*/KT) \qquad (3)$$

where

$$L(u/KT) = \coth\left(\frac{u}{KT}\right) - \frac{KT}{u}; \quad L(u^*/KT) = \qquad (4)$$

The average level from which absorption takes place has an energy

$$\mathcal{E}_a = \mathcal{E}_a^o - u\, L(u/KT) \qquad (5)$$

where ε_a^o the zero vibrational level of the ground state in the absence of dipole interactions.

2. Because of Franck-Condon restrictions the average state reached in absorption has an additional energy - $u^* L(u/KT)$ since the dipole moment of the fluorophore has instantly changed to μ^*, but the dipole distributions are those corresponding to μ^o. The fluorescent state reached has an energy

$$\mathcal{E}_f = \mathcal{E}_f^o - u^* L(u/KT) \qquad (6)$$

3. If the temperature and viscosity of the solvent permits the rearrangement of the solvent cage during the fluorescent lifetime, the transition $L(u/KT) \rightarrow L(u^*/KT)$ takes place. The effect of this is to decrease the

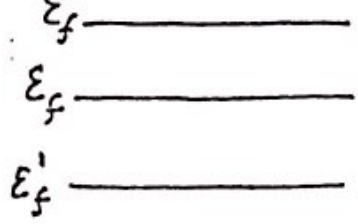
of the system at the expense of the electronic energy which reaches now a level

$$\mathcal{E}_f' = \mathcal{E}_f^o - u^* L(u^*/KT) \qquad (7)$$

4. Emission takes place from this state consisting in a transition to a ground state level given by

$$\varepsilon_a' = \varepsilon_a^\circ - u\,L(u^*/kT) \tag{8}$$

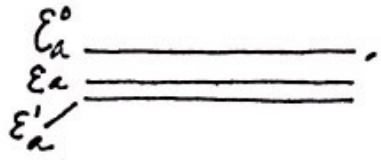
This state lies evidently below the starting ground state, corresponding as it does to a more perfect alignment of the dipole; The final step is the conversion of the electronic energy $u(L(u^*/KT) - L(u/KT))$ (9) into eutropy with a corresponding rise in the electronic energy level. The general disposition of the levels is shown in the diagram. The energy of the o-o' transition is given by



$$A = \varepsilon_a^\circ - \varepsilon_f^\circ - u\,L\left(\frac{u}{KT}\right) + u^*\,L\left(\frac{u}{KT}\right).$$

$$A = (\varepsilon_a^\circ - \varepsilon_f^\circ) + L\left(\frac{u}{KT}\right)*(u^* - u) \tag{10}$$

and the fluorescence energy on the assumption of equilibration of the dipole interactions in the excited state is



$$F = \left(\varepsilon_f^\circ - \varepsilon_a^\circ\right) - u^*\,L\left(\frac{u^*}{KT}\right) + u\,L\left(\frac{u^*}{KT}\right)$$

$$F = (\varepsilon_f^\circ - \varepsilon_a^\circ) - L\left(\frac{u^*}{KT}\right)*(u^* - u) \tag{11}$$

and the shift to the solvent-fluorophore interaction is

$$S = A - F = -(u^* - u)\left[L\left(\frac{u^*}{KT}\right) - L\left(\frac{u}{KT}\right)\right] \tag{12}$$

If $u^* > u$ we have $L(u^*/KT) > L(u/KT)$ and the Stokes shift is negative (red shift). The last equation is valid provided the solvent shell can undergo during the fluorescence lifetime the rearrangement of the dipoles to satisfy the interaction with μ^*. Considering such a rearrangement of the fluorophore solvation as a simple two-state process, it will be accomplished during the excited state to a fractional extent given by

$$\frac{\tau}{\tau + \rho_s}$$

where τ is the fluorescence lifetime and P_s the correlation time for the

change in the solvent shell. The average state reached at the time of the emission will correspond then to an average energy decrease.

$$L\left(\frac{u^*}{KT}\right)\frac{P_s}{P_s+\tau} + L\left(\frac{u^*}{KT}\right)\frac{\tau}{P_s+\tau} \qquad (13)$$

which gives when introduced in eq. (11)

$$F = \mathcal{E}_f^o - \mathcal{E}_a^o - (u-u^*)\left[L\left(\frac{u}{KT}\right)\frac{P_s}{P_s+\tau} + L\left(\frac{u^*}{KT}\right)\frac{\tau}{P_s+\tau}\right] \qquad (14)$$

Eq. (14) permits calculation of the fluorescence spectral shift as a function of temperature, rather than the Stoke shift itself. The latter can be difficult to determine experimentally when transitions of higher energy overlap appreciably the first excited . This difficulty of separating the $S_o \rightarrow S_1$ transition from others, does not ex in the fluorescence emission which in the vast majority of cases represent the single $S_1 \rightarrow S_o$ transition.

Multiply hydrogen bonded solvents like the glycols forms viscous glasses at low temperatures. Under these conditions $P_s \gg \tau$ and equation (14) simplifies to

$$F \equiv F_o = \mathcal{E}_a^o - \mathcal{E}_e^o - L\left(\frac{u}{KT}\right)(u^*-u) \qquad (15)$$

As the temperature is increased $L(\frac{u}{KT})$ is progressively replaced by $L(\frac{u^*}{KT})$ and the fluorescence shifts rapidly to the red approaching a limit when $\tau/(\tau+P_s)\rightarrow 1$. At still higher temperatures the red shift reverses itself slowly as $L(\frac{u^*_S}{KT})$ is a decreasing function of the temperature, though it changes much more slowly with temperature than P_s, which is proportional to the ratio of solvent viscosity to temperature (η/T). The figure below shows a theoretical calculation for a 'typcial' solvent, in which the thermal coefficient of the viscosity depends upon the viscosity itself in the manner,

$$\Delta\eta/\Delta T = -0.02\,(1 + \log_e \eta_{cp}) \qquad (16)$$

where η_{cp} is the viscosity coefficient in centipoises. This 'typical' solvent would show a thermal coefficient of viscosity of 2%/°C at a viscosity of 1 cp. and 16%/°C at a viscosity of 1,000 cp. The graph shows that at the lowest temperatures there is no appreciable change in the fluorescence spectrum as the temperature is raised. This is followed by a stretch of higher temperatures at which there is a rapid red shift with temperature, and a final zone with slow blue shift. The first zone corresponds to the region where $\tau/(\tau+P_s) >> 1$, the second to the region where $\tau/(\tau+P_s)$ changes rapidly with temperature from a value near zero to a value near one. In the third zone $\tau/(\tau+P_s)$ is effectively one and the blue shift follows from the progressive decrease in $L(u^*/KT)$ with increase in T. Physically the first zone is one of inappreciable motion of the dipole during the fluorescence lifetime. In the second zone the dipole motions can increasingly follow the change in the dipole strength of the fluorophore upon excitation. The blue shift zone reflects specifically the progressive disorder of the dipole and the consequent decrease in their interactions as the temperature is raised.

The figure below shows a plot of the spectral displacement employing the actual temperatures and viscosities, and the dipole moment (2.8D) of acetone in the interval of temperatures of 30°C to -90°C. The unknown P_s may be fairly replaced by the rotational relaxation time of the solvent molecules calculated by the equation.

$$P_s = 3\eta V/RT \qquad (17)$$

where V is the molar value of acetone. The values of P_s, thus calculated will be expected to yield $\tau/(\tau+P_s)$ between the extremes of 0.995 and 0.95 if τ is set equal to 4 ns. The region of rapid red shift is therefore not accessible in these observations. On the other hand the reverse blue shift is quite noticeable.

Notice that equation (14) refers to the shift of the fluorescence spectrum, not to the Stokes shift. Equations (14) and (10) can be combined to give this quantity,

$$S = A - F = (u^* - u)\left[L\left(\frac{u^*}{KT}\right) - L\left(\frac{u}{KT}\right)\right]\frac{\tau}{\tau + \rho_s} \qquad (18)$$

A plot of the Stokes shift calculated from the latter equation shows the same three zones depicted in Fig. It will be noticed that the inversion of the direction of the Stokes shift is confined to values of the temperature which would not be reached experimentally. The reason is clear on comparison of eq. (14) and (10). In the region in which $\tau/(\tau+P_s)\to 1$ both absorption and emission shift to the blue with increase in temperature, with the absorption approaching its limit more rapidly than the emission because $L(\frac{\mu}{KT}) < L(\frac{\mu^*}{KT})$ and dL/dt decreasing with increasing value of L. The absence of inversion of the Stokes shift can be used as a further criterion to gauge the realism of the proposed physical picture where α is the thermal coefficient of the viscosity of the solvent. Taking into account the order of magnitude of the practical units of dipole moment (Debye 10^{-18})) and distance (Å, 10^{-8}) and setting

$$hc\Delta V = 2(A-F).$$

we can compute ΔV for a given value of T/To for a fluorophore of known lifetime in a solvent of defined viscosity.

$$\Delta\nu = 10,086\,\frac{D_s(D^*-D_o)}{\gamma^3}\left\{L(\quad etc\quad)\right\}$$

The graph shows a computation for solvents with $\alpha = 0.06°C^{-1}$ and a fluorophore of 5 ns lifetime as a function of To. It can be clearly seen that there are three distance regions of change of ΔV. For a solvent with normal viscosity changes $\Delta V\to o$ for T<To/2. Between To/2 and 2/To ΔV increases rapidly reaching a maximum value which is approximately 2/3 of that predicted for maximum dipole

interaction. At higher temperatures when $\tau/(\tau+P_s)$ is practically unity an increase in temperature produces a shift to the blue. This effect is due to the difference $L(\frac{u^*}{KT}) - L(\frac{u}{KT})$ which diminishes progressively with T. It can be experimentally demonstrated with PRODAN in alcohol or ethylene glycol solutions. The graphs shows very clearly that it is not possible to make significant comparisons between different solvents at a single temperature in order to compute $\mu^*-\mu^o$. In the various attempts made in this fashion it is always noticeable that low viscosity solvents like acetone, or DMF produce considerably smaller shifts than would be expected from their polarity. It seems much more reasonable to attempt a calculation of $\mu^*-\mu$ by observation on one or two solvents, employing the variation of the Stokes shift with temperature. The results derived are valid for the pure electronic transitions which are difficult or impossible to point out in the broad absorption and emission spectra characteristic of the fluorescence of solutions. In a broad band we should expect all levels to be similarly influenced by the solvent interactions, and because these reflect themselves in the energies involved in the transitions it seems reasonable to measure them by a quantity that reflects the effects upon the whole spectrum. We shall therefore redefine the Stokes shift to refer to the wavenumber internal between the center of mass of the absorption and fluorescence bands. The center of mass νg will be defined as

$$\nu_g = \frac{\sum F(\bar{\nu}) \cdot \bar{\nu}}{\sum F(\bar{\nu})}$$

where $F(\bar{\nu})$ represents the fluorescence emission at wavenumber ν in units proportional to the number of emitted photons.

Fluorescence Lifetime.

Statistical Character of the emission.

Although we speak of the properties of atomic and molecular oscillators as if they could be studied in isolation, this is not the actual case. Absorption and emission processes can only be studied in populations of atoms and molecules, and the properties of the supposed typical members of the population deduced from the macroscopic properties of the process. The emission from a molecular population results from the superpossition of the emissions from the elementary oscillators. If they emit independently of each other, so that the phases of the individual emissions are distributed at random the results may be very different than in the case in which rigorous phase relations exist among the individual emissions. We speak in the first case of 'incoherent' and in the second of 'coherent' radiation. The spontaneous emission from atoms or molecules gives always rise to incoherent radiation unless a very small part of the source, containing only a few emitting elements is seen. Coherent radiation can be produced as a result of stimulated emission, as it occurs in Lasers and masers. Because of the strict phase relations the properties from a source of coherent radiation are those from an independent oscillator, the emission as a whole posessing then the damped oscillator character that we have already described.

On the other hand, in the case of incoherent radiation the random character of the phases erases any possibility of defining an amplitude for the emitted radiation. The intensity $I^{(x,y,z)}$ of the ~~emitted waves is given~~ radiation as received by a detector at a point with coordinate, x, y, z equal.

$$\cancel{I = \left(\sum_i A^2 \sin^2(\omega t + \varphi_i)\right)^2}$$

Better $$I(xyz) = \left(\sum_i A \sin(\omega t + \varphi_i(x,y,z)\right)^2.$$

where $\varphi_i(x,y,z)$ is the phase of the ith emission at time t at the point ~~with coordinate~~ x, same point

The phases being chosen at random we have $\overline{\sin\phi_i \cos\phi_i} = 0$, $\overline{\sin^2 \phi}_i = \overline{\cos^2 \phi}_i = \frac{1}{2}$. and,

$$I = A^2 N/2 \qquad (2)$$

The emitted intensity is simply the sum of the individual intensities Incoherent emission may be considered therefore as the average emission from isolated elements. The behaviour of the excited population is described by the equation,

$$dn^*/dt = - n^* \Gamma + f(t) \qquad (3)$$

whre n^* is the number of excited elements at time t and f(t), the rate of excitation is the usual pumping function of 'forcing function' already encountered in other conexion. The cathode of the photomultiplier tube responds proportionally to the square of the amplitude of the electric field. Because of this property the response is proportional to the number of incoherent emissions.

Experimental determination of the fluorescence lifetime.

If f(t) in quation (3) is an infinitely narrow pulse of exciting light, at all times after such pulse has fallen to zero, we can set f(t) = 0 and solve (3) as a homogeneous differential equation of first order.

$$dn^*/dt + n^* \Gamma = 0 \qquad (4)$$

the solution of which is the simple exponential decay,

$$n^* = n_o \exp(-\Gamma t) \qquad (5)$$

the number of emissions per unit time equals $n^* \Gamma$ and therefore the intensity registered as function of time is

$$F(t) = F(0) \exp(-\Gamma t) \qquad (6)$$

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$$-\left.\frac{dE}{dt}\right)_{ideal} = AE \qquad (1)$$

The constant A has the value

$$A = \frac{8}{3}\frac{n\pi^2e^2}{mc}\bar{\nu}^2 \qquad (2)$$

where e and m are the charge and mass of the electron, n the refractive index of the medium surrounding the oscillator and $\bar{\nu}$ the wavenumber of the emitted radiation. A real oscillator radiates according to the equation

$$-\left.\frac{dE}{dt}\right)_{real} = AEf_e \qquad (3)$$

where fe is the oscillator strength of emission. Because of the probability of reciprocal transitions f_e can be replaced in the equation by f_a where the absorption considered is that reciprocal to the fluorescence transition, that is the $S_o \rightarrow S_1$ absorption. Integration of the last equation for an oscillator that ceases to absorb radiation at ~~true~~ time zero gives for the energy remaining in the oscillator at time t:

$$E(t) = E(0) \exp(-A f_a t) \qquad (4)$$

The characteristic damping time is $(A f_a)^{-1} = t^*$

Computing the value of the universal constants in eq.(2)

$$t^* = \left(0.222 \times \bar{\nu}^2 n f_a\right)^{-1} \qquad (5)$$

Thus for an oscillator of unit strength emitting light in the center of the spectrum ($\bar{\nu} = 2 \times 10^4$), in water ($n = 1.33$)

$$t^* = 1.5 \times 10^{-8} \text{ s} \qquad (6)$$

The equation permits the calculation of the fluorescence lifetime of a monochromatic radiator, on the assumption that radiation is the only process that removes energy from the excited state. The fluorescence lifetime corresponding to this condition, which must be carefully distinguished from the experimental fluorescence lifetime τ, is called the <u>emission lifetime</u>. [The effect of additional process is always to increase the rate of energy dissipation so that $t^* \leq \tau$.] There have been several attempts to modify equation (5) to obtain the emissive lifetime of molecules emitting broad fluorescent bands with arbitrary Stokes shifts. Ladenburg (1921) introduced the oscillator strength of the $S_o \rightarrow S_1$ transitions; Perrin and Forster proposed modified equations. Stickler and Berg have modified the original equation to provide for averaging the oscillator strengths for each wavelength of emission.

Their equation is

$$\tau_{em}^{-1} = 2.88 \times 10^{-9} n^2 \langle \bar{\nu}^3 \rangle \int_{\Delta\bar{\nu}_a} \varepsilon(\bar{\nu}) d\ln \bar{\nu} \qquad (7)$$

where

$$\langle \bar{\nu}_f^{-3} \rangle = \frac{\int_{\Delta\bar{\nu}_e} F(\bar{\nu}) d\nu}{\int_{\Delta\bar{\nu}_e} F(\bar{\nu}) \cdot \bar{\nu}^{-3} d\nu}. \qquad (8)$$

In these equations $\Delta\bar{\nu}_a$ and $\Delta\bar{\nu}_e$ correspond to the experimental limits of the absorption and emission bands ($S_o \leftrightarrow S_1$ transitions) ~~and~~, $E(\nu)$ is the molar absorption and $F(\nu)$ describes the spectral distribution of the emission in photons per wavenumber interval. The effect of competing radiationless processes in the excited state may be introduced in straight forward fashion. If the rate of emission from the real oscillator is A_γ and the rate of energy decrease due to a competiting radiationless process is k_d

$$dE/dt = A_r + K_d \qquad (9)$$

and

$$\tau = (A_r + K_d)^{-1} \qquad (10)$$

giving

$$\tau/t^* = A_r/(A_r + K_d) \qquad (11).$$

Evidently the ratio of the mean energy radiated by the oscillator in the presence and absence of competitive processes is also

$$q = A_r/(A_r + K_d) \qquad (12)$$

so that

$$\tau = qt^* \qquad (13)$$

τ and q, the fluorescence lifetime and absolute yield may be experimentally measured, so that t^* may be calculated and compared with t^* computed from the spectroscopic quantities. A rough agreement may be anticipated even by the use of an equation as simple as eq (5). f_a = -.22 for NADH $\bar{\nu} = 2.2 \times 10^4$ giving t^* = For example for NADH in water (f_a = 0.22) the

A classical oscillator cannot store the absorbed energy but radiates it as monochromatic radiation at a rate given by the relation

$$-\left.\frac{dE}{dt}\right|_{ideal} = AE \qquad (1)$$

The constant A has the value

$$A = \frac{8}{3}\frac{n\pi^2e^2}{mc^3}\nu^2 = \frac{8}{3}\frac{n\pi^2e^2}{mc}\bar{\nu}^2. \qquad (2)$$

where e and m are the charge and mass of the electron, n the refractive index of the medium surrounding the oscillator and $\bar{\nu}$ the wavenumber of the emitted radiation. A real oscillator radiates according to the equation

$$-\left.\frac{dE}{dt}\right|_{real} = AEf_e. \qquad (3)$$

where f_e is the oscillator strength of emission. Because of the equal probability of reciprocal transitions f_e can be replaced in the equation by f_a where the absorption considered is that reciprocal to the fluorescence transition, thus f is the $S_0 \leftrightarrow S_1$ absorption. Integration of the last equation for an oscillator that ceases to absorb radiation at time zero gives for the energy remaining in the oscillator at time t as

Einstein $f_a A = f_e B$

$$E(t) = E(0)\exp(-Af_at). \qquad (4)$$

The ~~damping~~ characteristic damping time is $(Af_a)^{-1} = t^*$

Computing the value of the universal constants in eq. (2)

$$t^* = (0.222 \times \bar{\nu}^2 n f_a)^{-1} \qquad (5)$$

of unit
Then for an oscillator strength ~~of 1~~ emitting light in the center of the spectrum ($\bar{\nu} = 2 \times 10^4$), in water ($n = 1.33$)

$$t^* = 1.5 \times 10^{-8} \text{ s.} \qquad (6)$$

~~Several~~ The equation permits the calculation of the fluorescence lifetime of a monochromatic radiator, on the assumption that radiation is the only process that removes energy from the excited state. The fluorescence lifetime corresponding to this condition, which must be carefully distinguished from the experimental fluorescence lifetime τ, is called the emissive lifetime. [The effect of additional process is always to increase the rate of energy dissipation so that $t^* \leq \tau$.] There have been several attempts to modify the equation to obtain the emissive lifetime of molecules emitting broad fluorescent bands with arbitrary Stokes shifts. Ladenburg (1921) proposed simply to introduced the oscillator strength of the $S_0 \rightarrow S_1$ transitions, Perrin and Forster proposed unmodified equations. Strickler and Berg have modified the original equation to take account of provide for an averaging of the oscillator strengths for each wavelength of emission. Their equation is

$$\tau_{em}^{-1} = 2.88 \times 10^{-9} n^2 \langle \bar{\nu}^3 \rangle \int_{\Delta \bar{\nu}_a} \varepsilon(\bar{\nu}) \, d\ln \bar{\nu} \qquad (7)$$

where

$$\langle \bar{\nu}_f^{-3} \rangle = \frac{\int_{\Delta \bar{\nu}_e} F(\bar{\nu}) \, d\nu}{\int_{\Delta \bar{\nu}_e} F(\bar{\nu}) \cdot \bar{\nu}^3 \, d\nu}. \qquad (8)$$

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In these equations $\Delta\bar{\nu}_a$ and $\Delta\bar{\nu}_e$ correspond to the experimental limits of the absorption and emission bands ($S_0 \leftrightarrow S_1$ transitions) and, $E(\nu)$ describes the molar absorption and $F(\nu)$ describes the spectral distribution of the emission in photons per wave number interval. ~~It is of interest~~

The effect of competing radiationless processes in the excited state may be introduced in straightforward fashion. If the rate of emission from the real oscillator is A_r and the rate of energy decrease due to a competing radiationless process is k_d.

$$-dE/dt = A_r + K_d. \tag{9}$$

and

$$\tau = (A_r + K_d)^{-1}. \tag{10}$$

giving

$$\tau/t^* = A_r/(A_r + K_d). \tag{11}$$

Evidently the ratio of the mean energy radiated by the oscillator in the presence and absence of competitive processes is also.

$$q = A_r/(A_r + K_d). \tag{12}$$

So that

$$\tau = qt^* \tag{13}$$

τ and q, the fluorescence lifetime and absolute yield may be experimentally measured, so that t^* may be calculated ~~to~~ and compared with t^* computed from the spectroscopic quantities.* For example for NADH in water ($f_a = 0.22$) the equation of Strickler and Berg gives $t^* = 76$ ns. Direct measurements yield $\tau = 0.4$ ns. $q = 0.025$ which combined give $\tau_e \sim 16$ ns. (Kosh. et al.)

with precise methods of determination of the fluorescence lifetime and the absolute fluorescence yield it should be possible to accurately determine the emissive lifetime and to compare it with the value given by the Stickler and Berg's formula, or the others proposed for this purpose. The methods of measurement of fluorescence lifetime, as used today, have the necessary accuracy but this is not the case with the measurements of fluorescence yield. In a few cases in which systematic comparisons have been attempted (Stickler and Berg, Baldwin and Ware, Scott et al.) the agreement has been satisfactory, probably within 20% in the worst cases. However it is not known to what extent these discrepancies reflect the imperfection of the fluorescence yield measurements or those of the Stickler and Berg equation.

Fluorescence emission as a quantum statistical process.

Looked upon as the result of incoherent emission by an excited molecular population, the ~~process of~~ kinetics of the emission of light by a photo excited population are described by an equation of the form.

$$\frac{dn^*}{dt} = -\Gamma n^* + f(t). \qquad (14)$$

where n^* is the number of molecule in the excited state at time t and Γ the rate constant of emission. The dimensions of Γ are sec^{-1} (transitions per molecule per unit time). $f(t)$ is an arbitrary function of the time, describing the time course of the excitation.

If excitation ceases at $t=0$, the last equation, in the form.

$$\frac{dn^*}{dt} = -\Gamma n^* \qquad (15)$$

describes the decay of the excited state at decrease in the number of excited molecules at all for the times. Integration gives

$$n^*(t) = n^*(0)\exp(-\Gamma t). \qquad (16).$$

The equivalence of this equations (6) with that and (4). corresponding to the loss of energy of an excited oscillator should be noted. Despite the formal similarity they ~~desc~~ physical process that they describe are very different. The decrease in energy in the excited state according to eq (4) arises from a continuous loss of energy of the oscillator as it radiates. According to eq. (16) this loss is due to the ~~co~~ disappearance of a fraction of the excited oscillators. Eq. (4) describes a continuous process, while eq. (16) describes the loss of the population by a statistical transitions from ~~in a~~ molecular population between well defined energy levels. For all practical experimental purpose the description given by (16) is in agreement with the physical picture, and we shall use it exclusively in discussing the properties of the excited population. The value of the radiative oscillator picture is useful in establishing the relations of reciprocity with the absorption and in deriving an estimate of the ~~absolute~~ emissive fluorescence lifetime of the molecules.

In the statistical picture the average fluorescence lifetime is not simply the reciprocal of a damping constant A, but the mean time spent by the molecule in the excited state. This must equal

$$t^* = \int_0^\infty t\, n^*(t)\, dt \Big/ \int_0^\infty n^*(t)\, dt \qquad (17)$$

Introducing the value of $n^*(t)$ from (16) and carrying out the integrations

$$t^* = \Gamma^{-1} \qquad (18)$$

Thus equating the damping time of the oscillator with the rate of radiative emission by the population of excited molecules. Similarly, the effect of non-radiative ~~Equation Ex~~ transitions in competition with the emission is taken into account by setting

$$\frac{dn^*}{dt} = -(\Gamma + k_r)\, n^* + f(t). \qquad (19)$$

which evidently gives

$$\tau = (\Gamma + k_r)^{-1}; \quad q = \frac{\Gamma}{\Gamma + k_r} \qquad (20)$$

Notice that q in equation (20) refers to the ratio of the number of molecules leaving the excited state with, or without emission of a photon, ~~and that~~ Therefore q equals the ratio of photons emitted / photons absorbed. defining a photon or quantum yield. Eq. (12) refers to the ratio of the energy dissipated by radiative and by non radiative path. The two values of q would be equal only for monochromatic oscillators emitting monochromatic

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Fluorescence lifetime measurements

We shall discuss the methods of measurement of the fluorescence lifetime by reference to eq. (14). Equation (16) corresponds to the experimental conditions in which the solution is illuminated by a light pulse and the decay of the fluorescence is recorded after excitation has ceased. Ideally the exciting pulse must have appreciable intensity for only a fraction of the lifetime of the excitation and an 'infinitely narrow' pulse would be, in practice, one that disappears at a time at which virtually 99% of the molecules are still excited, therefore persisting for only 1% of the fluorescence lifetime. For $\tau \simeq 10$ ns it presupposes excitation with an effective pulse width of 0.1 ns. Such narrow pulses are difficult to generate. Pulse widths of 0.2–0.4 ns have been practically achieved by gas-discharge lamps, and more reliably and with much higher repetition rate by using synchrotron radiation. Lasers are capable of producing pulses of ~~a width~~ of a few picoseconds width and a high repetition rate. Originally the fluorescence emitted under repetitive excitation was recorded directly as an oscilloscopic trace. In more recent years this procedure has been replaced by the more accurate 'single photon technique' in which the exciting light is attenuated so that each exciting pulse produces at most one photoelectron. The time delay between the photoelectric pulses due to excitation and fluorescence is obtained by the so-called 'time to amplitude conversion'. A voltage that increases linearly with time is

initiated on arrival of the photoelectric exciting pulse and stopped on arrival of the fluorescence photoelectric signal. Time-to-amplitude conversion permits time discriminations on the order of 50 ps. The time ~~to amplitude~~ delays are of the individual photons are recorded as unit increases in a multichannel analyzer. After a large number of pulses the curve drawn by plotting the number of counts in each channel against the respective time delay approaches the time course of the fluorescence emission. A reasonable definition of the decay curve requires some 10^4-10^5 pulses, which in common practice require 10-100 minutes. The fluorescence lifetime is obtained with a precision that depends upon the number of counts, and may be estimated as follows: Let the number of counts in the channel corresponding to the actual lifetime be N_τ. ~~and~~ To obtain the lifetime with a relative precision $\Delta\tau/\tau$ by measuring ~~only~~ the counts in this channel only will require

$$\frac{\Delta\tau}{\tau} = \frac{\sqrt{N_\tau}}{N_\tau} = \frac{1}{\sqrt{N_\tau}}. \qquad (21)$$

If the number of channels is optimized for the required precision we need $\tau/\Delta\tau$ channels, and the ratio of N_τ, the total counts collected to N_τ is

$$N_\tau/N = \frac{\int_{\tau-\Delta\tau/2}^{\tau+\Delta\tau/2} \exp(-t/\tau)\,dt}{\int_0^\infty \exp(-t/\tau)\,dt} = \frac{\Delta\tau}{\tau} e^{-1} \qquad (22)$$

The last two equations give

$$N = c\left(\frac{\tau}{\Delta\tau}\right)^3 \qquad (23)$$

This is the total number of counts required if we use only the number N_τ for the determination. If the numbers in many channels are used the averaging process will improve precision by the square root of the number of values averaged. If the first half of the total number of channels is averaged,

$$N = c\left(\frac{\tau}{\Delta\tau}\right)^3 \Big/ \frac{1}{\sqrt{2}}\left(\frac{\Delta\tau}{\tau}\right)^{\frac{1}{2}} = c\sqrt{2}\left(\frac{\tau}{\Delta\tau}\right)^{3/2}. \qquad (24)$$

The single photon technique is straightforward in conception but the experimental realization is quite complex and the analysis of the results is complicated by the finite width of the exciting pulses, the non-instantaneous response of electronic detection system and drifts and variations of the conditions during the necessarily long time of data collection. As at presently used pressures of 0.1–0.2 ns. can be achieved in the measurements of fluorescence lifetimes 2 ns. or longer. The literature contains only very few measurements of subnanosecond lifetimes by this method indicating that there are outside the routine application of the technique.

A second method of measurement – phase fluorometry – furnished the first direct lifetime measurements when Gaviola introduced it in 1926. In this method excitation is by a sinusoidally modulated light beam, which can be represented by the equation

$$f(t) = a + b \sin \omega t. \qquad (25)$$

ω, the circular modulation frequency, ~~equals $2\pi\nu$ where~~ corresponding to $\omega/2\pi$ ~~Has the~~ frequency in Hertz. Introduction of (25) into (14) yields the linear inhomogeneous equation

$$\frac{dn^*}{dt} = \Gamma n^* + a + b \sin \omega t \qquad (26).$$

When a dynamic system is subjected to periodic impulses, after a short transient time, in our case of order Γ^{-1}, a state is reached in which the system response varies with the frequency of the periodic impulses, but with an amplitude and phase determined by its own characteristics (Lord Rayleigh, the theory of sound). Thus we expect that the solution of eq (26) will be of the form.

$$n^* = A + B \sin(\omega t + \phi) \qquad (27).$$

where A, B and ϕ are to be determined. If dn^*/dt is computed in (27), substitution of n^* and dn^*/dt in (26) gives a solution in terms of the new parameters. By equating the coefficients of $\sin \omega t$, $\cos \omega t$ and the absolute term on both members of the equation we find.

$$\left.\begin{aligned} A &= a/\Gamma \\ B'(\Gamma\cos\phi - \omega\sin\phi) &= b. \\ B(\Gamma\sin\phi + \omega\cos\phi) &= 0. \end{aligned}\right\} \quad (28).$$

These equations give

$$\tan\phi = -\omega/\Gamma = -\omega\tau. \qquad (29)$$

$$B/A = (b/a)\cdot\frac{\Gamma}{(\Gamma^2+\omega^2)^{1/2}} = (b/a)/\sqrt{1+\omega^2\tau^2} \qquad (30)$$

The minus sign in (29) indicates that the response lags behind the stimulus ($\phi < 0$). b/a is the modulation ~~ratio~~ depth of the excitation and B/A the modulation depth of the fluorescence. The ratio $(B/A)/(b/a) \equiv M$, is the relative modulation of the fluorescence with respect to the excitation. ϕ is the phase difference of the photocurrent owing to fluorescence from that owing to the exciting light. The minus sign in (29) indicates that the former lags behind the latter. Eqs (29) and (30) show that the fluorescence lifetime of a homogeneous population (single value of Γ) may be obtained from either the phase delay

$$\phi = \tan^{-1}\omega\tau. \qquad (31)$$

or from the relative modulation

$$M = 1/\sqrt{1+\omega^2\tau^2} \qquad (32).$$

Figure shows a plot of ϕ and M against $\log\omega\tau$. The region of steepest change is at $\omega\tau = 1$. If $\tau \sim 10$ ns a frequency of $1/2\pi\tau \approx 6$ MHz is optimal for excitation. When $\omega\tau = 0.1$ $\phi \sim 9°$ and $M = 0.99$. This may be taken as the lowest practical limit for a phase measurement

Modulation measurements at such low frequencies would not be a practical proposition. At $\omega\tau = 10$ the phase measurements would be very imprecise on account of the rapid variation of $\tan\phi$ with ϕ. Modulation measurements would be close to this practical limit as the fluorescence would then be largely demodulated. Because of technical limitations present day phase fluorometers have only two or three frequencies of excitation but these are usually sufficient to cover the range of lifetimes from about 0.2 to 50 ns. with good precision. Employing high frequency of excitation (30 MHz) measurements of lifetimes of 1/4 to 1/2 ns can be done routinely with ~~errors of the order of 10%~~. coefficients of variation of the order of 10%.

It should be remarked that in practice the electro-optical modulation of the light results in excitation which is not purely sinusoidal, as required by (26) but has a certain harmonic content. If the detection system is frequency sensitive, rejecting effectively the harmonics that appear in the fluorescence the results obtained are those predicted by (28). While it is difficult to generate quasi sinusoidal excitation frequencies of 100 MHz or higher, repetitive short pulses of a few tenths of a nanosecond width have the necessary harmonic content to permit, in principle, the determination of the response fluorescence response at such high frequencies, similar to the GHz region. The feasibility of the method for the

lower frequencies has been experimentally demonstrated by Bartels and co-workers. Recently the use of the Kerr shutter in this fashion with high repetition synchronous pulses has been proposed and it is estimated that lifetimes of a few p.s. could be measured by such method.

It is of interest that the limits of precision and accuracy in the determination of the fluorescence lifetime by either of the two methods described result largely from drift and irregular behaviour of the light sources employed for excitation.

Comparison of pulse and phase fluorometry

These two methods of measurement exploit the classical 'impulse response' and 'harmonic response' of a dynamic system respectively. Simple relations exist between these two. In general the impulse response $I(t)$ is an arbitrary function of the time $I(t)$ with Fourier sine and cosine transforms defined by the equations:

$$Q = \int_0^\infty \sin \omega t \cdot I(t)\, dt$$

$$P = \int_0^\infty \cos \omega t \cdot I(t)\, dt \qquad (33).$$

The phase delay and the relative modulation of the harmonic response at frequency ω are given by (Solodovnikov)

$$\tan \beta = -Q/P \; ; \quad H = \sqrt{P^2 + Q^2}. \qquad (34)$$

* For a unique decay ~~(33) gives~~

$$I(t) = e^{-\Gamma t}$$

and (33) gives

$$Q = \frac{\omega}{\Gamma^2+\omega^2} \quad ; \quad \rho = \frac{\Gamma}{\Gamma^2+\omega^2}.$$

Application of (34) reproduces the values given in (29) and (30).

The response to a light impulse of an arbitrary molecular population is a sum of exponentials of the time with independent weights, so that in general

$$I(t) = \sum_i a_i e^{-m_i t} \qquad (35)$$

Q and P are then the sum of Laplace transforms of sin ωt and cos ωt of the explicit forms,

$$Q = \int_0^\infty \sum a_i \sin \omega t \, e^{-m_i t} dt = \sum_i \frac{a_i \omega}{m_i^2 + \omega^2} \qquad (36)$$

$$P = \int_0^\infty \sum a_i \cos \omega t \, e^{-m_i t} dt = \sum_i \frac{a_i m_i}{m_i^2 + \omega^2} \qquad (37)$$

*

The equations (33) – (37) show the complete equivalence of the information obtained by the two methods, and the choice between them is only a matter of the technical advantages of one or the other in any particular case. With appreciable fluorescence signals phase fluorometry is by far the speedier method by a factor of 10-100, & is of comparable accuracy in the measurement of long life times, but the phase method is capable of routine subnanosecond measurements, while the pulse method can only achieve these with far more effort and less precision. The greatest advantage of pulse fluorometry is in the possibility of direct demonstration of complex decays, though almost limited in practice to cases in which I(t) consists of only two terms.

This is the case when a solution containing two fluorescent compounds is excited. If both compounds emit with comparable intensity and the lifetimes differ by at least 50% the presence of two decays is obvious enough, even on simple inspection of the decay curve. As the fluorescence lifetimes become closer and/or one of the components becomes greatly predominant over the other the resolution becomes increasingly difficult.

In phase fluorometry agreement between lifetimes measured by phase and modulation in pure compounds is of the order of ± 0.1 — ± 0.25 ns. Disagreements that are two to three times as large indicate heterogeneity of the emitting population, but it is easy to see that even under the assumption that two components alone are responsible for the bulk of the emission one would not be able to determine uniquely the two amplitudes and phases. To obtain an unequivocal answer one requires two further measurements, one of phase and another of modulation ~~of the lifetime~~ for a different exciting light frequency. The method of resolution of N components employing phase and modulation measurements at N frequencies is valid, in theory for any N, but confined in practice to $N = 2$. It is described in detail in an Appendix.

Experimental fluorescence lifetimes.

A crude estimate of the maximum lifetime to be expected for a given compound can be obtained by the use of eq. (5). This predicts that compounds with strong absorption ($f_a > 0.5$) in the $S_0 \rightarrow S_1$ transition will have fluorescence lifetimes under 10 ns. and that this value will increase as the absorption transition weakens. Further shortening of τ can take place on account of competitive radiationless transitions so that the lifetime predicted from the absorption strength will be particularly applicable to those cases with high fluorescence yields, and therefore weak competitive non-radiative transitions. Compounds with intense fluorescence seldom show lifetimes under 3 ns. while subnanosecond lifetimes are confined to compounds with low fluorescence yield like the water solutions of ANS and NADH (Table). The longest fluorescence lifetimes in aromatic compounds are due to weak, quasi forbidden L transitions, as observed in chrysene ($\tau \sim 45$ ns) and pyrene ($\tau \sim 400$ ns). Measurements of this very long lifetimes is often complicated by the presence of oxygen in the solutions. At room temperature air saturated water is 0.25 mM in oxygen and air saturated ethanol is 1.5 mM and other organic solvent contain 5-7 times as much oxygen. The characteristic time for nearly every collision of the fluorophore with oxygen leads to nonradiative deactivation and the characteristic

deactivation time is 400 ns for water and 20–30 ns. for organic solvents (see under Quenching). In consequence measurements of fluorescence ~~water~~ in lifetimes in water seldom requiring degassing or an atmosphere of nitrogen but these precautions are necessary when measuring the lifetime of many fluorophors in organic solvents.

In general the lifetime is independent or almost independent of the ~~exciting wavelength~~ the wavelength of fluorescence emission. One would expect in general ~~that~~ emissions from a vibrationally excited state and those from the zero level to have different lifetime, which would show up on examination of the blue edge of the emission, though minimized by the small relative contribution of the excited-levels emission to the total emission. In compounds with quasi forbidden transitions fluorescence transitions like chrysene one observes appreciable variations as the lifetimes for emission from the different vibrational levels of the ground state are recorded. In these cases one is compelled to look at the vibrational peaks as representing independent electronic transitions coupled to each other in absorption and/or emission. Variation of the fluorescence lifetime within the emission is seen always in the course of solvent relaxation processes in the excited state. They will be discussed under this topic.

Because of the rapid thermalization following excitation to the higher electronic states one can expect only a lengthening of the fluorescence lifetime of at most

a few picoseconds as the wavelength of excitation is decreased. Thus, in practice, any reproducible variation of the fluorescence lifetime with excitation wavelength, except those at the very red end of the ~~spec~~ absorption spectrum, indicates the existence of a heterogeneous emitting population.

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$$n^* = A + B \sin(\omega t + \phi) \qquad (27)$$

where A, B and ϕ are to be determined. If dn^*/dt is computed in (27), substitution of n^* and dn^*/dt in (26) gives a solution in terms of the ~~same~~ new. parameters. On equating the coefficients of sin wt, cos wt and the absolute term on both members of the equation we find

$$\left.\begin{aligned} A &= a/\Gamma \\ B(\Gamma \cos\phi - \omega \sin\phi) &= b. \\ B(\Gamma \sin\phi + \omega \cos\phi) &= 0. \end{aligned}\right\} \qquad (28)$$

These equations give

$$\tan \phi = -\omega/\Gamma = -\omega\tau. \tag{29}$$

$$B/A = (b/a)\cdot \frac{\Gamma}{(\Gamma^2+\omega^2)^{1/2}} = (b/a)/\sqrt{1+\omega^2\tau^2} \tag{30}$$

~~The minus sign in (29) indicates that the response lags behind the stimulus ($\phi<0$).~~ b/a is the modulation depth of the excitation and B/A the modulation depth of the fluorescence. The ratio (B/A)/(b/a) ≡ M, is the relative modulation of the fluorescence with respect to the excitation. ϕ is the phase difference of the photocurrent owing to fluorescence from that owing to the exciting light. The minus sign in (29) indicates that the former lags behind the latter. Eqs (29) and (30) show that the fluorescence lifetime of a homogeneous population (single value of Γ) may be obtained from either the phase delay

$$\phi = \tan^{-1} \omega\tau \tag{31}$$

or from the relative modulation

$$M = 1/\sqrt{1+\omega^2\tau^2} \tag{32}$$

Figure shows a plot of ϕ and M against log $\omega\tau$. The region of steepest change is at $\omega\tau = 1$. If $\tau \sim 10$ ns a frequency of $1/2\pi\tau$ 6 MHz is optimal for excitation. When $\omega\tau = 0.1$ $\phi \sim 9°$ and M = 0.99; this may be taken as the lower practical limit for a phase measurement. Modulation measurements at such low frequencies would not be a practical proportion. At $\omega\tau = 10$ the phase measurements would be very imprecise on account of the rapid variation of tan ϕ with ϕ. Modulation measurements would be close to the practical limit as the fluorescence would then be largely demodulated. Because of technical limitations, present day phase fluorometer have only two or three frequencies of excitation but these are usually sufficient to cover the range

of lifetimes from 0.2 to 50 ns. with good precision. Employing high frequency of excitation (30 MHz) measurements of lifetimes of 1/4 to 1/2 ns can be done routinely with coefficients of variation of the order of 10%.

It should be remarked that in practice the electro-optical modulation of the light results in excitation which is not purely sinusoidal, as required by (26) but has a certain harmonic content. If the detection system is frequency sensitive, rejecting effectively the harmonics that appear in the fluorescence, the results obtained are those predicted by (28). While it is difficult to generate quasi sinusoidal excitation frequencies of 100 MHz or higher, repetitive short pulses of a few tenth of a nanosecond width have the necessary harmonic content to permit, in principle, the determination of the fluorescence response at frequencies, extending to the GHz region. The feasibility of this method for the lower frequencies has been experimentally demonstrated by Märkelo and co-workers. Recently the use in this fashion of the very short-high repetition synchrotron pulses has been proposed and it is estimated that lifetime of a few ps. could be measured by such methods.

It is of interest that the limits of precision and accuracy in the determination of the fluorescence lifetime by either of the two methods described results today from drift and irregular behaviour of the light sources employed for excitation.

Comparison of pulse and phase fluorometry

These two methods of measurement exploit the classical 'impulse response' and 'harmonic response' of a dynamic system respectively. Simple relations exist between these two. In general the impulse response I(t) is an arbitrary function of the time I(t) with Fourier sine and cosine transforms defined by the equations:

$$S = \int_0^\infty \sin \omega t \cdot I(t)\, dt$$

$$F = \int_0^\infty \cos \omega t \cdot I(t)\, dt \qquad (33)$$

The phase delay and the relative modulation of the harmonic response at frequency ω are given by (Solodovinikov)

$$\tan\phi = -Q/P \; ; \qquad M = \sqrt{P^2+Q^2} \tag{34}$$

The response to a light impulse of an arbitrary molecular population is a sum of exponentials of the time with independent weights, so that in general

$$I(t) = \sum_i a_i e^{-m_i t} \tag{35}$$

Q and P are then the sum of Laplace transforms of sin wt and cos wt of the explicit forms,

$$Q = \int_0^\infty \sum a_i \sin\omega t\, e^{-m_i t}\, dt = \sum_i \frac{a_i \omega}{m_i^2+\omega^2} \tag{36}$$

$$P = \int_0^\infty \sum a_i \cos\omega t\, e^{-m_i t}\, dt = \sum_i \frac{a_i m_i}{m_i^2+\omega^2} \tag{37}$$

For a unique decay

$$I(t) = e^{-\Gamma t}$$

and (33) gives

$$Q = \frac{\omega}{\Gamma^2+\omega^2} \; ; \qquad P = \frac{\Gamma}{\Gamma^2+\omega^2}$$

Application of (34) reproduces the values given in (29) and (30). The equation (33)-(37) show the complete equivalence of the information obtained by the two methods, and the choice between them is only a matter of the technical advantages of one or the other in any particular case. With appreciable fluorescence signals phase fluorometry is by far the speedier method by a factor of 10-100. They are of comparable accuracy in the measurement of long lifetimes, and the phase method is capable of routine subnanosecond

measurements, while the pulse method can only achieve these with far more effort and less precision. The greatest advantage of pulse fluorometry is the feasibility of direct demonstration of complex decays, though almost limited in practice to cases in which I(t) consists of only two terms. This is the case when a solution containing two fluorescence compounds is excited. If both compounds emit with comparable intensity and the lifetimes differ by at least 50% the presence of two decays is obvious enough, even on simple inspection of the decay curve. As the fluorescence lifetimes become closer and/or one of the components becomes greatly predominant over the other the resolution becomes increasingly difficult. In phase fluorometry agreement between lifetimes measured by phase and modulation in pure compounds is of the order of I0.1 - ±0.25 ns. Disagreements that are two to three times as large indicate heterogeneity of the emitting population, but it is easy to see that even under the assumptions that two components alone are responsible for the bulk of the emission one would not be able to determine uniquely the two amplitudes and phases. To obtain an unequivocal answer one requires two further measurements, one of phase and another of modulation for a different exciting light frequency. The method of resolution of N components employing phase and modulation measurements at N frequencies is valid in theory for any N, but confined in practice to N = 2. It is described in detail in an Appendix.

Experimental fluorescence lifetimes

A crude estimate of the maximum lifetime to be expected for a given compound can be obtained by the use of eq. (5). This predicts that compounds with strong absorption ($f_a > 0.5$) in the $S_o \rightarrow S_1$ transition will have fluorescence lifetimes under 10 ns. and that this value will increase as the absorption transition weakens. Further shortening of τ can take place on account of competitive radiationless transitions so that the lifetime predicted from the absorption strength will be particularly applicable to cases with high fluorescence yields, and therefore weak competitive non-radiative transitions. Compounds with intense fluorescence seldom show lifetimes under 3 ns. while subnanosecond lifetimes are confined to compounds with low fluorescence yields like the water solutions of ANS and NADH (Table). The longest fluorescence lifetimes in aromatic compounds are due to weak, quasi forbidden L transitions, as observed in chrysene ($\tau \sim 45$ ns) and pyrene ($\tau \sim 400$ ns). Measurements of this very long lifetimes is often complicated by the presence of oxygen in the solutions. At room temperature air-saturated water is 0.25 mM in oxygen and air-saturated ethanol and other organic solvents contain 5-7 times as much oxygen. Virtually every collision of the fluorophore with oxygen leads to radiationless deactivation and the characteristic deactivation time is 400 ns for water and 20-30 ns for organic solvents (see under quenching). In consequence measurements of fluorescence lifetimes in water seldom require degassing or an atmosphere of nitrogen, but these precautions are necessary when measuring the lifetimes of many fluorophores in organic solvents.

In general the lifetime is independent or almost independent of the wavelength of fluorescence emission. One would expect ~~in general~~ emissions from a vibrationally excited state and those from the zero level to have different lifetime, which would show up on examination of the blue edge of the emission, though minimized by the small relative contribution of excited-level emission

to the total emission. In compounds with quasi-forbidden fluorescence transitions like chrysene one observes appreciable variations as the life-times for emission to the different vibrational levels of the ground state are recorded. In these cases one is compelled to look at the vibrational peaks as representing independent electronic transitions coupled to each other in absorption or emission. Variation of the fluorescence lifetime within the emission is seen always in the course of solvent relaxation processes in the excited state. They will be discussed under this topic. Because of the rapid thermalization following excitation to the higher electronic states one can expect a lengthening of the fluorescence lifetime of at most a few picoseconds as the wavelength of excitation is decreased. Thus, in practice, any reproducible variation of the fluorescence lifetime with excitation wavelength, except those at the very red end of the absorption spectrum, indicates the existence of a heterogeneous emitting population.