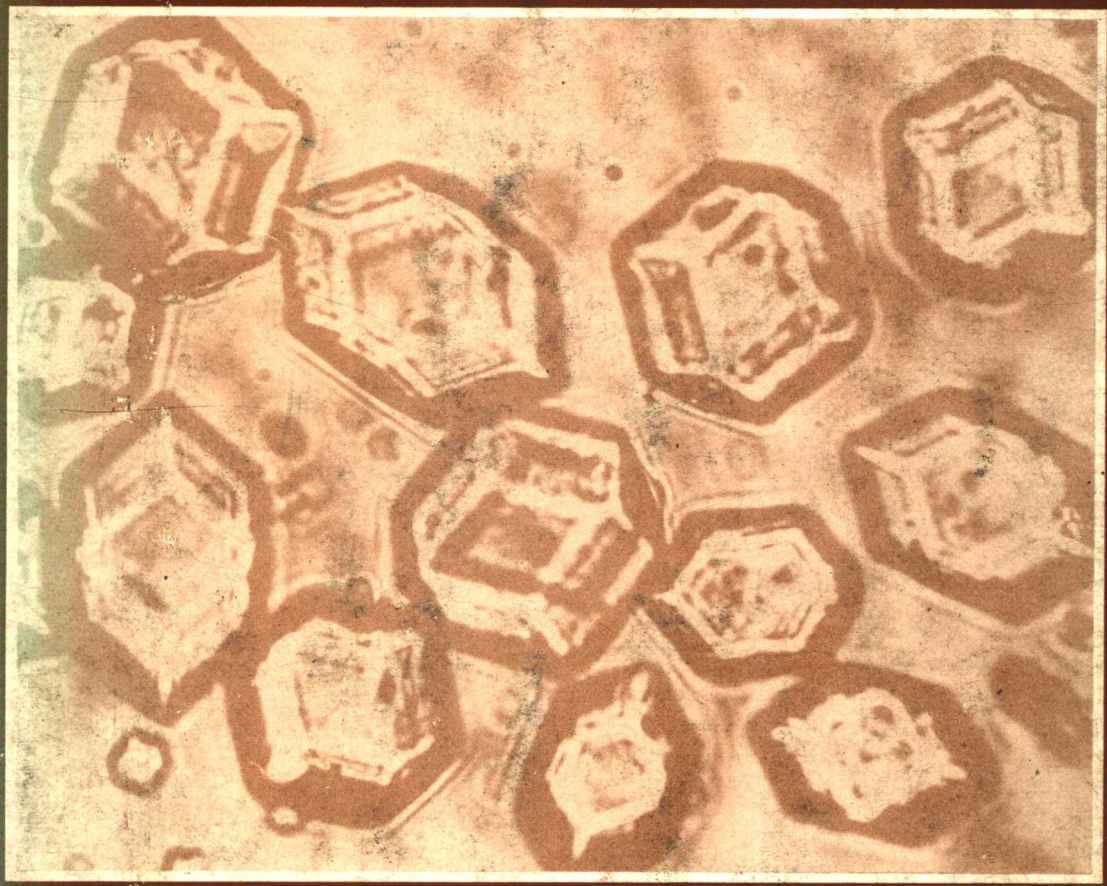
LUMINESCENCE of BIOPOLYMERS and CELLS



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Konstantin Konstantinovich Turoverov was graduated from Leningrad State University in 1962 with a degree in polymer physics. He is now associated with the Division of Marine Biology of the Far-Eastern Branch of the Academy of Sciences after having worked with the luminescence research group of the Institute of Cytology. His work has been focused mainly on the physical mechanisms of the influence of conformational transformations of biopolymers on their luminescence.

The original Russian text, published for the Institute of Cytology of the Academy of Sciences of the USSR by Nauka Press in Leningrad in 1966, has been thoroughly revised by the authors for the present edition.

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ЛЮМИНЕСЦЕНЦИЯ БИОПОЛИМЕРОВ И КЛЕТОК
LYUMINESTSENTSIYA BIOPOLIMEROV I KLETOK

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PREFACE

During the last 15 years, the specialized roles played by tryptophan residues in proteins have gradually become evident. The unique structure of tryptophan, so unlike that of any other naturally occurring amino acid, is responsible for its ability to form charge-transfer complexes, make up parts of the active-site regions of dehydrogenases and other enzymes, form sections of "hydrophobic" regions of proteins, and probably play a key role in the binding regions of antibodies and serum albumins. The indole nucleus endows tryptophan with the ability to act in these roles and is responsible for the ability to emit fluorescence in the ultraviolet and phosphorescence in the visible region. At the same time, it has become clear that tryptophan residues are by far the most important groups responsible for the luminescence of proteins and intact cells. The importance of tryptophan is one of the main reasons why fluorescence and other forms of luminescence spectroscopy are rapidly developing into powerful investigative tools of the molecular biologist.

Preeminent as it is in the study of biological macromolecules, fluorescence is but one aspect of luminescence. The work of Barenboim and his colleagues has contributed not only to fluorescence spectroscopy but also to other areas where light emission is encountered. These areas include phosphorescence, biological chemiluminescence, light emission following activation by high-energy radiation, as well as ultraviolet fluorescence microscopy of cells and subcellular particles. The breadth of interest of the authors, as evidenced by this book, seems entirely natural, since all these phenomena should be discussed together. Indeed, it is the arbitrary classification of luminescence into separate categories which is somewhat artificial.

The general reader will find the basic principles stated in straightforward terms, and the review of luminescence research is an excellent introduction to work in this field. One notes some subjects described here which are not, to any extent, being pursued outside the USSR. Of particular interest are the studies on the "ultraweak" ultraviolet chemiluminescence of cells and organelles, and the use of ultraviolet fluorescence microscopy to observe cellular structure using the intrinsic protein fluorescence. In addition, the serious worker will find the last

two chapters, which deal with instrumentation, quite valuable. There are few, if any, comparable descriptions of Soviet photometric apparatus available in English.

For many years the contributions of Russian scientists to the understanding of luminescence phenomena have been most impressive. The already classical studies of Vavilov, Sveshnikov, Terenin, Feofilov, Neporent, and others are examples. Several laboratories in the USSR have carried this long tradition of luminescence research into the biological area and have been most active since the discovery of protein fluorescence in 1956. The contributions of groups headed by S. V. Konev and Yu. A. Vladimirov are numerous and well known, and the importance and originality of work by Barenboim's laboratory should also be appreciated. The present book reviews much Soviet research including that of the authors, which may not be familiar to scientists in the West.

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Contents

INTRODUCTION

LUMINESCENCE OF SOLUTIONS OF POLYATOMIC ORGANIC MOLECULES

The physical basis of luminescence is treated in a large number of books and reviews. These include, in increasing degree of complexity, the books by Konstantinova-Shlezinger (1961), Udenfriend (1962), West (1956), Terenin (1947), Levshin (1951), Pringsheim (1949), El'yashevich (1962), Vol'kenshtein (1955), Stepanov (1955), and Stepanov and Gribkovskii (1963). This introduction is a summary of the fundamentals of molecular luminescence which the reader may find useful in reading this book. It is based in part on the monographs listed above, but also on various reviews published in the last few years, mainly those devoted to the luminescence of complex, and above all, heteroatomic molecules.

0.1. MOLECULAR SPECTRA AND BASIC CONCEPTS OF LUMINESCENCE

The energy E of a molecule consists of its kinetic energy E_{kin} the energy E_{el} associated with the motion of the electrons, the vibrational energy E_{vib} of the nuclei, and the rotational energy E_{rot} of the molecule as a whole:

$$E = E_{kin} + E_{el} + E_{vib} + E_{rot} \qquad (0.1)$$

This formula is valid only in the first approximation since it does not take into account the interaction between the three forms of internal motion, which is particularly important for complex molecules. However, Eq. (0.1) enables us to construct energy diagrams in which an energy level term corresponds to each electronic state. Moreover, each electronic level has a number of vibrational sublevels, and each vibrational sublevel, a number of rotational sublevels (Fig. 0.1).

The lowest possible electronic energy (E_0 in Fig. 0.1) corresponds to the ground (unexcited) state of the molecule. Electronic states with higher energy ($E_1, E_2, \ldots, E_n$) are called excited states.

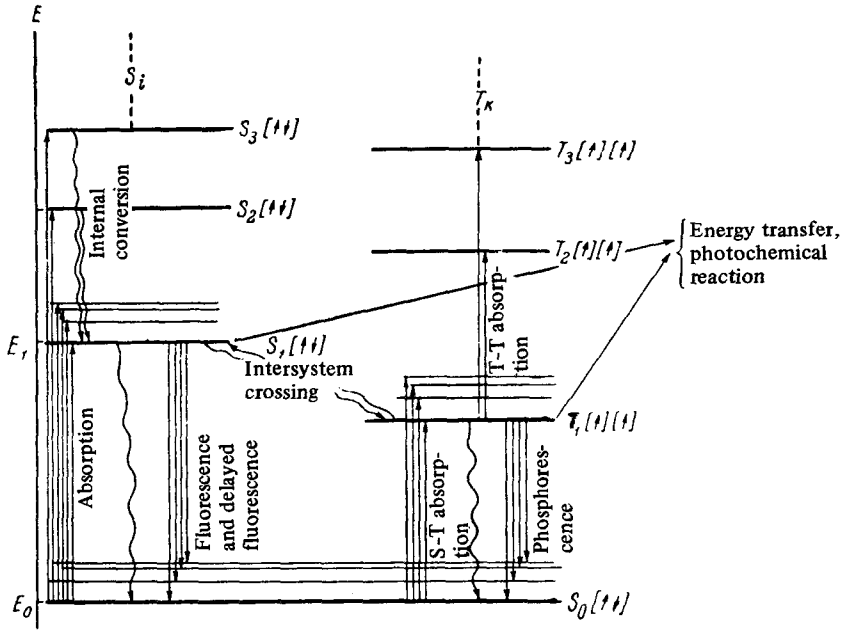

Fig. 0.1. Molecular energy levels and transitions between them. The level energy E is marked on the vertical axis. S_0 is the ground state, $S_1, S_2, \ldots, S_i$, and $T_1, T_2, \ldots, T_k$ are singlet and triplet levels, respectively. Thick horizontal lines represent electronic levels, thin horizontal lines represent vibrational sublevels. Vertical lines indicate transitions between the electronic levels accompanied by the absorption or emission of light. Wavy lines represent radiationless transitions. Spin directions of electrons in the outer orbitals of a molecule in the corresponding electronic states are shown schematically in square brackets.

If the frequency ν of a photon incident on the molecule satisfies the relation

$$h\nu = E_1 - E_0 = \Delta E \tag{0.2}$$

where $h = 6.625 \times 10^{-27}$, erg·sec (Planck's constant), the molecule can absorb the quantum $h\nu$ and undergo a transition from the ground state E_0 to a higher-lying level E_1. (One often speaks of electronic transitions in molecules because the transition of a molecule from one electronic state to another is connected with a change in the electron-cloud configuration.)

The reverse transition, i.e., the transition from E_1 to E_0 may be accompanied by the emission of a photon of energy $h\nu$, i.e., the emission of luminescence.* Depending on the type of excitation, the emission is called

*A molecule can, of course, undergo a transition to an excited state not only as a result of absorption of a photon, but also in the course of chemical reaction, or under the action of various types of radiation.

photo-, chemi-, or radioluminescence or is characterized by some other suitable prefix. In this introduction we shall consider luminescence which is excited by light.

Depending on the nature of the excited state and the nature of the transition to the ground state, the emitted luminescence may be classified as fluorescence or phosphorescence. The distinction between these two types of luminescence will be discussed in greater detail in Section 0.2. Relaxation from the excited state to the ground state occurs in a very short but measurable interval of time. For many organic molecules in solution this time is not less than 10^{-8} to 10^{-9} sec (fluorescence), which is considerably longer than the period of the light waves. This is the most characteristic feature of luminescence and distinguishes it from Rayleigh and Raman scattering, Vavilov–Cerenkov emission, refraction and reflection of light, and so on. These are not associated with transitions of molecules to discrete excited states and involve time intervals comparable with the period of the exciting radiation (10^{-14} sec) [Vavilov, (1950); Stepanov, (1955)].

Luminescence may be characterized by its integrated intensity and spectral composition. The dependence of the intensity I on wavelength λ is called the *luminescence spectrum*.

The fluorescence spectrum is always shifted toward longer wavelengths relative to the absorption spectrum (Stokes' law). However, the long-wavelength part of the absorption spectrum overlaps the short-wavelength part of the fluorescence spectrum to some extent. This is particularly characteristic of complex molecules. Fluorescence at wavelengths shorter than the wavelength of the exciting radiation is called anti-Stokes fluorescence. The additional energy necessary for its emission comes from an excess of vibrational energy in the excited molecules.

The dependence of the integrated intensity of luminescence, or the intensity of luminescence at a given wavelength, on the wavelength of the exciting radiation is called the luminescence excitation spectrum. The ratio of the number of emitted photons to the number of absorbed photons is defined as the quantum yield q. It has been shown experimentally for a number of substances that the quantum yield of fluorescence remains constant over a broad range of wavelengths; this is frequently referred to as Vavilov's law.

In general, an electronic transition will also involve a change in the vibrational and rotational energies of the molecule. The electronic spectrum is therefore in reality an electronic-vibrational-rotational spectrum. Since the energy levels have finite widths and tend to overlap in complex molecules, forming a continuous sequence, the profile of the electronic spectra of complex molecules has practically no vibrational structure. The shape of the absorption and emission bands is determined by the transition probabilities between different vibra-

tional levels of the ground and excited states, and by the function describing the distribution of the vibrational levels over the energies of the upper and lower electronic states for absorption and emission respectively.

Shpol'skii Effect

The absence of a clearly defined vibrational structure in the electronic-vibrational spectra of complex molecules is a serious disadvantage in practical applications of molecular spectroscopy. Many attempts have therefore been made to find the conditions under which the vibrational structure of the spectrum can be made apparent. It is possible to reduce the number of degrees of freedom of molecules by freezing them in a solution. However, the solutions of many organic substances form molecular crystals in the solid state, and these have a number of specific absorptive properties leading to a broadening of the spectral emission band.

Intermolecular interaction can be suppressed almost completely by studying the substance under investigation in the gaseous phase. In this phase, however, there are many vibrational and rotational states forming a practically continuous sequence. This again leads to a broadening of the structure of the luminescence spectrum. Moreover, many complex molecules cannot be investigated in the gaseous phase without producing dissociation.

It is possible, however, to combine the advantages associated with cooling and vaporization, and at the same time avoid the complications introduced into the spectrum by the particular properties of these phase states. This can be achieved by placing the medium under investigation in another medium, known as the matrix or host, which ensures that the molecules are isolated from each other. The system is then frozen with the molecules fixed in definite orientations. The medium under investigation is thus really in the form of an "orientated gas" in the host medium.

Shpol'skii and co-workers [Shpol'skii and Il'ina (1951); Shpol'skii *et al.* (1952)] were the first to show that the luminescence spectra of frozen solutions of polyacenes, polyenes, and polyphenyls in normal paraffins as host media exhibit a vibrational structure, due to electronic transitions, which resembles line structure. (They referred to it as "quasi-line structure.") This phenomenon is sometimes called the Shpol'skii effect. According to Shpol'skii (1959, 1960, 1962, 1963), paraffin hosts can be used to prepare the medium under investigation (activator) in a state of molecular dispersion because the host and the activator are geometrically similar.

To ensure that the emitting molecules form an "oriented gas" the host must hold these molecules quite rigidly and must not be deformed by them. It must not interact with the luminescing molecules and must form with them a homogeneous solid solution. Finally, the host must be transparent throughout the region in which the luminescing molecules are excited.

By using *n*-paraffins as the host media, it has been possible to obtain quasi-line spectra for a broad range of materials, including, for example, the heterocyclic compounds [Nurmukhamedov *et al.* (1960); Shigorin *et al.* (1962)], some dyes of biological origin, e.g., porphyrins [Litvin and Personov (1961); Lyalin and Kobyshev (1963)]. However, quasi-line spectra have not thus far been obtained for protein, nucleic acids, and their components, although the appearance of such a spectrum would give information on the spatial relationships between the host and the embedded molecule, which is of particular importance for many aspects of molecular biology [Vol'kenshtein (1965)].

It is important to note that, apart from normal carbohydrates, it is also possible to use other solvents as host media, e.g., dibenzylaminoethanol [Val'dman and Sheremet'ev (1963)], cyclohexane [Kanda and Shimada (1961)], and benzene [Kanda *et al.* (1963)].

Extensive applications of the Shpol'skii effect in qualitative and quantitative analysis, in the study of the structure of polyatomic organic molecules, in crystal chemistry, and in solid state physics, were reviewed by Shpol'skii (1965*a*, 1965*b*).

Effect of the Solvent on the Electronic Spectra of Molecules

The intensity, the quantum yield, and the luminescence spectra are affected by the properties of the ambient medium. In this section we shall briefly review the effect of the solvent on the position of the spectra, a subject of considerable interest in connection with the main subject matter of this book.

Neporent and Bakhshiev (1960) have suggested the subdivision of molecular interactions causing shifts of electronic spectra in solutions into (1) universal interactions due to the effect of the properties of the solvent on the solute molecule, and (2) specific interactions between the solute molecule and one or a few of the surrounding solvent molecules due to the donor–acceptor interaction, association formation of hydrogen bonds, and so on.

The theory of the effect of universal interactions on the position of the electronic spectra of molecules [Bakhshiev (1961, 1964)] can be used to relate the shift of the spectra with the macroscopic parameters of the solvent, e.g., refractive index and dielectric constant, and the properties of the solute molecule, e.g., its volume and dipole moments in the ground and excited states.

Theory predicts the existence of four electronic levels (Fig. 0.2) for a molecule in solution: two equilibrium (A and B) levels and two nonequilibrium (A' and B') ones differing from the corresponding levels (E_0 and E_1) in an isolated molecule by the energy of interaction of the molecule with the surrounding solvent. Calling this energy W_i, we have:

$$W_i = \vec{\mu}_i \vec{F}_i \tag{0.3}$$

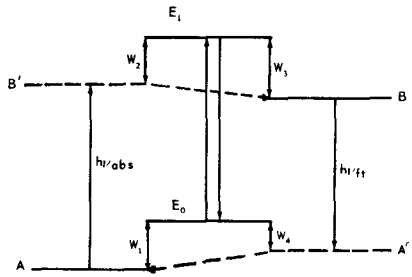

Fig. 0.2. Schematic illustrations of the electronic levels of a molecule in a solvent. Explanation in text.

In this expression $\vec{\mu}_i$ is the (permanent or induced) dipole moment of the molecule under consideration in the corresponding electronic state, and F_i is the electric field due to the whole set of surrounding solvent molecules in this state.

On excitation the electron density of the molecule undergoes a redistribution and its dipole moment is changed. The configuration of the solvent molecules has no time to change during the electronic transition, and the new state B' into which the molecule passes on absorption of a photon is a nonequilibrium one. Relaxation processes associated with a change in the configuration of the solvent molecules relative to the molecule in question then occur during the lifetime of the excited state. In the simplest case, when the relaxation time τ_r is much shorter than the lifetime of the excited state τ_{fl} (liquid solution), the molecule after emission passes into the equilibrium excited state B.

In the same way emission of a luminescence quantum brings the molecule into nonequilibrium ground state A'.

It should be noted that if $\tau_r \approx \tau_{fl}$ or $\tau_r \gg \tau_{fl}$ then the state B from which the emission takes place can also prove to be a nonequilibrium one. This case can occur in solid frozen solutions. The energy difference between the ground and excited states ΔW governs the relative shift of the absorption and fluorescence spectra. In general, this shift may be different from the analogous shift for fluorescence spectra in unfrozen solutions.

Polarized Fluorescence

The condition given by Eq. (0.2) is necessary but not sufficient to ensure that the molecule will absorb the incident light energy. It is also necessary that the electric vector in the light wave has a component parallel to the absorbing oscillator.

In practice, one has to deal not with an individual molecule but with an enormous number of such molecules. Since the molecules are not preferentially oriented in solution, absorption of light will be independent of its polarization. However, polarized luminescence can be observed even in the case of excitation

by natural (unpolarized) light (spontaneous polarization). Luminescence with maximum polarization is then observed at right angles to the direction of propagation of the exciting radiation, because molecules whose oscillators are parallel to the direction of propagation of the incident beam (A_2 in Fig. 0.3) do not absorb the incident radiation. However, when luminescence is observed in the direction of propagation of the incident radiation, the polarization is not evident.

In addition to spontaneous polarization, one can investigate the polarization of luminescence excited by plane-polarized light. In this case polarized luminescence can be observed even in the direction of propagation of the incident radiation. Additional information about the position of the oscillators in space can be obtained by investigating the polarized luminescence of oriented molecules, e.g., in stretched films excited by plane-polarized light.

The degree of polarization can be defined by

$$P = \frac{I_1 - I_2}{I_1 + I_2}, \tag{0.4}$$

where I_1 and I_2 are the intensities of components with perpendicular polarizations. The degree of polarization of luminescence depends on the mutual orientation of the absorbing and emitting oscillators in the molecule. Since emission usually occurs from a single excited state, the degree of polarization is usually constant throughout the luminescence spectrum.

The dependence of the degree of polarization on the wavelength of the exciting radiation is called its polarization spectrum. If the excitation of the molecule involves a single electronic transition the degree of polarization is independent of the wavelength of the incident radiation. When absorption involves the participation of a number of oscillators, the analysis of polarization spectra and comparison of such spectra with absorption spectra will provide information about the relative orientation of the absorption and emission oscillators. An analysis of this kind will show whether or not several electronic

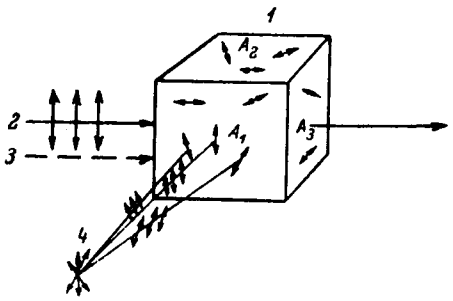

Fig. 0.3. Emission of polarized luminescence. (1) Container with solution; (2) linearly polarized exciting radiation; (3) natural light; and (4) partly polarized luminescence.

transitions take part in producing the absorption spectrum. Polarization spectra are frequently more specific for a given medium than are the fluorescence spectra.

The absolute value of the polarization is reduced by Brownian rotation of the molecules. The relationship between the degree of polarization, the properties of the luminescing molecules, and the properties of the ambient medium is given by the Perrin formula

$$\frac{1}{P} = \frac{1}{P_0} + \left(\frac{1}{P_0} - \frac{1}{3}\right)\tau \frac{RT}{V\eta}, \tag{0.5}$$

where R is the gas constant, T is the absolute temperature, η is the viscosity of the medium, V is the molecular volume of the luminescing molecules, τ is the lifetime of the excited state, and P_0 is the limiting polarization corresponding to $T/\eta \to 0$. Equation (0.5) can be used to determine the lifetimes of excited states if the molecular volumes of the luminescing molecules are known, and vice versa.

More detailed information about polarized luminescence can be found in the review and monograph by Feofilov (1948, 1961).

0.2. OPTICAL TRANSITIONS BETWEEN SINGLET AND TRIPLET STATES

It is well known that the electron has an intrinsic angular momentum (spin) which can assume one of the two values $\pm\frac{1}{2}(h/2\pi)$. According to the Pauli principle, a given orbit cannot contain more than two electrons with opposite spins. If all the spins in a molecule are antiparallel in pairs, the resultant spin S of the electrons is zero. If one of them changes its direction of spin, the resultant spin becomes equal to unity.

The quantity $2S + 1$ is the multiplicity of a given level, i.e., the number of possible orientations of the electron spin relative to the resultant angular momentum. When $S = 0$ we have $2S + 1 = 1$, and the corresponding energy level is called a singlet. When $S = 1$, so that $2S + 1 = 3$, the energy level is called a triplet. The ground (unexcited) state of a molecule is usually a singlet state. According to the Pauli principle, a change in the spin direction of one of the electrons is accompanied by a transition of the molecule to a triplet state. The appearance in the molecule of two unpaired electrons in the triplet state gives the molecule the properties of a chemically active biradical [Terenin (1947)].

It will be convenient to use the following designation of molecular energy levels: S_i for singlet and T_k for triplet; and $i = 0, 1, 2, 3,...$ and $k = 1, 2, 3,...$ to denote the position number of a level of given multiplicity (the state T_1 can be regarded as the ground state of the molecule with altered spin). Transitions between electronic levels are governed by the so-called selection rules, and can be divided into allowed and forbidden transitions. Allowed transitions are those in which there is no change in the multiplicity ($S_0 \leftrightarrow S_1$, $T_1 \leftrightarrow T_2$ in Fig. 0.1):

forbidden transitions are those in which there is a change in the multiplicity ($S_O \nleftrightarrow T_1, S_1 \nleftrightarrow T_2$).

Singlet–singlet transitions are readily observed both in absorption and in emission. Radiation emitted as a result of transitions of the molecules from excited to ground singlet states is called fluorescence. In solutions, radiative transitions usually occur between the lowest excited singlet or triplet levels, even when higher-lying electronic levels are excited. This is often referred to as the Kasha rule [Kasha (1950)].*

Triplet–triplet transitions have been found in studies of the absorption of light by many different molecules [see, for example, McClure (1951)]. However, triplet–triplet fluorescence has been detected only in triphenylene solutions [Dupay *et al.* (1964)]. The lifetime of a molecule in a level T_2 is comparable with the lifetime in the first singlet excited level S_1.

Singlet–triplet transitions in solutions of polyatomic organic molecules have been observed both in absorption [West (1956)] and in emission [Parker (1964)]. Emission accompanying $T_1 \rightarrow S_0$ transitions is called phosphorescence. Optical transitions between terms of different multiplicity are forbidden in dipole emission (intercombinations are forbidden). The lifetime of a molecule in the triplet state for the triplet–singlet transition, which are forbidden, is considerably greater than the lifetime for allowed transitions.

In spite of the fact that they are forbidden, $S_0 \rightarrow T_1$ transitions occur because of the spin–orbital interaction. The level T_1 is not a pure triplet, but is in fact a superposition of triplet and singlet states. Spin–orbital interaction increases with increasing atomic number of the elements in the chromophoric group. The probability of $T_1 \rightarrow S_0$ transitions increases at the same time: for some compounds the probability of a phosphorescent transition is approximately proportional to the square of the spin–orbital interaction. This effect is readily seen by introducing heavy substituents, e.g., the halogens, into the molecule [West (1956); McGlynn *et al.* (1964)]. When spin–orbital interaction is strengthened, e.g., by the paramagnetic O_2 or NO molecules, this also leads to an increase in the probability of singlet–triplet transitions [Terenin (1947); Karyakin (1961); Barenboim (1963*a*)].

In spite of the fact that the probability of $S_0 \nleftrightarrow T_1$ transitions can be increased for a number of materials, this probability is still small in comparison with the probability of allowed transitions. However, intersystem crossing results in a high population of the triplet level T_1 through the level S_1 (Fig. 0.1).†

*There are, however, a number of exceptions to this rule; e.g., azulene and diphenylene [McGlynn *et al.* (1964)]. Emission from S_2 as well as the main emission from S_1 has been found by Terenin and Ermolaev (1952) in chlorophyll excited by ruby laser.

†The levels S_1 and T_1 also have different multiplicities, but for a number of quantum-mechanical reasons [McGlynn et al. (1964)] the rate constants for intersystem crossing; e.g., $S_1 \nleftrightarrow T_1$, are much greater than the rate constant for the $S_0 \rightarrow T_1$ electronic transition.

It is convenient to study $S_0 \leftrightarrow T_1$ transitions by investigating the associated phosphorescence because of the high population of the long-lived level T_1 (there is a large number of molecules in this state), the fact that phosphorescence continues even after fluorescence has decayed completely, and the fact that the two spectra are quite different.

Emission from the singlet excited state which is reached by the molecule after occupation of the triplet state ($T_1 \leftrightarrow S_1$, $S_1 \leftrightarrow S_0$) is called delayed fluorescence. This emission has the same spectrum as fluorescence, but has a different time constant. Detailed information about delayed fluorescence can be found in the review by Parker (1964). The account of delayed fluorescence given below is based on Parker's review.

Depending on the mechanism of the $T_1 \ldots \rightarrow S_1$ transition preceding delayed fluorescence, the latter can be divided into two types. In the first type, the transition $T_1 \ldots \rightarrow S_1$ occurs as a result of thermal activation of the intercombinational process $T_1 \rightsquigarrow S_1$. Accordingly, the emitted intensity is given by

$$I = Ae^{-E/kT}, \tag{0.6}$$

where T is the absolute temperature, k is Boltzmann's constant, and E is the activation energy necessary to take the molecule from the triplet state to the excited singlet state.

Since the intensity of this emission depends on the population of the triplet state, it is proportional to the first power of the intensity of the exciting radiation (monomolecular process). Other things being equal, the decay constant of delayed fluorescence is equal to the phosphorescence decay constant. This type of emission is occasionally referred to as *a* phosphorescence [Pringsheim (1949)].

The second type of delayed fluorescence occurs as a result of the so-called triplet–triplet annihilation in which the collision of two molecules of the same type in the triplet state results in an exchange of energy such that one of the molecules ends in an excited singlet state and the other in the ground state. A theoretical treatment of triplet–triplet annihilation has been given by Sternlicht *et al.* (1963). Triplet–triplet annihilation may be accompanied by the formation of triplet dimers and excimers, i.e., complexes consisting of an excited molecule and a molecule in the ground state [Parker, (1964)].

Since triplet–triplet annihilation is a bimolecular process, the intensity of the delayed fluorescence in this case is proportional to the square of the intensity of the exciting radiation. This is why the intensity decreases with decreasing concentration and increasing viscosity.

In addition to the above types of delayed fluorescence this phrase is occasionally also used to designate emission produced in recombinational processes following ionization. Further details will be found in Chapter 3.