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Electron Spin Resonance of the Excited Triplet State of Biologically Important Molecules

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The excited triplet states of molecules play an important role in the mechanisms of any photochemical reaction. These states are responsible for the occurrence of two-quantum photsensitisation, photoionisation, and photoreduction reactions. The present review compiles and gives a systematic account of the principal results obtained in the application of ESR to the study of the excited triplet state of biologically important molecules.
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I. INTRODUCTION

The application of the method of electron spin resonance (ESR) in the study of the excited triplet state of molecules began with the pioneer work of Hutchison and Mangum[1,2] more than ten years ago and has yielded much information about its properties and role in the photochemistry of organic molecules. The ESR method revealed the splitting of the lowest triplet level in the absence of an external magnetic field and the effect of the size of the conjugated system, the substituents in the molecule, and the properties of the solvents on the zero-field splitting energy. Data were obtained on the direction of the transition moment, molecular symmetry, and the distribution of spin density in the lowest excited triplet state. The ESR method provided valuable information about the lifetimes, probabilities of transition to the triplet state, and quenching and energy transfer processes.

The successful application of the ESR method in the study of the mechanism of the photochemical reactions in solid solutions several years later led to the discovery of two-quantum photosensitisation, photoionisation, and photoreduction reactions[2-8] proceeding via the triplet state of aromatic compounds, including biologically important substances, such as porphyrins, chlorophyll[8-11], aromatic aminoacids[9,11-14], nucleic acid bases[15], phenoxazine, phenothiazine, and flavins[16]. The phenomenon of triplet–triplet annihilation, leading to the formation of one molecule in a highly excited singlet, triplet, or quintet state from two molecules in a phosphorescent triplet state was first observed at approximately the same time. It attracted the attention of investigators as a possible method of using low-energy quanta to achieve reactions requiring a considerable energy expenditure[17]. It was suggested that this phenomenon may play a role in photobiological reactions.

There are many biological processes where the first stage involves the transition of specific molecules to an excited electronic state. This is the photodynamic effect—inactivation of proteins by radiation, photodimerisation of nucleic acid bases, vision, and bioluminescence. Photosynthesis, which provides energy to virtually all living things on earth, takes place owing to the absorption, transfer, and storage of the energy of sunlight by the photosynthetic apparatus of plant and bacterial cells. At the molecular level this involves tetrapyrrole pigments—chlorophyll and bacteriochlorophyll in an excited state.

All these findings—the vast scope of the new research technique, the discovery reactions proceeding via the triplet state, and finally the suggested role of the triplet state in biological processes—stimulated in recent years a profound interest by photobiologists in the study of biologically important substances in an excited triplet state. Owing to the extreme complexity involved in the observation of the phenomenon at an electronic level in real biological structures, it still remains unknown whether primary photochemical reactions *in vivo* take place at a singlet or triplet electronic level. However, such information is undoubtedly important for the understanding of the mechanism of the phenomena.

To date, more than 100 publications have appeared on ESR studies of molecules in an excited triplet state, both experimental and theoretical. A systematic and critical review of these investigations is necessary for future work. This review cannot give a complete analysis of all the studies and therefore we shall consider only briefly the advances in this field of research and shall deal in greater detail with data concerning biologically important substances.

The properties of molecules in singlet and triplet excited states are significantly different. These differences concern primarily their electronic structure: the charge distribution, spin density, and the symmetry of the π-electron cloud, and hence the donor–acceptor properties of the molecules, their reactivity, lifetime, and other characteristics determining the rate of the photochemical process. The great importance of the excited triplet state in the initial stage of the photochemical process is due to the fact that it is long-lived compared with the excited singlet state.

Fig. 1*A* presents a typical scheme of the molecular energy levels originally proposed in a simplified form by Jablonski[18] to explain the prolonged afterglow of organic molecules in rigid matrices. The radiative $T_1 \rightarrow S_0$ transition is of an intercombination type and is forbidden by the spin selection rules. The lifetime of the molecule in the lowest triplet state is therefore longer than that of the excited singlet state (about 10^{-9} s), increasing to between 10^{-4} s and several seconds, particularly at low temperatures where the probability of the non-radiative $T_1 \rightarrow S_0$ transition, the $T_1 \rightarrow S_1$ thermal transition, and quenching processes of different kinds diminishes. Since the transition to the T_1 triplet state involves the inversion

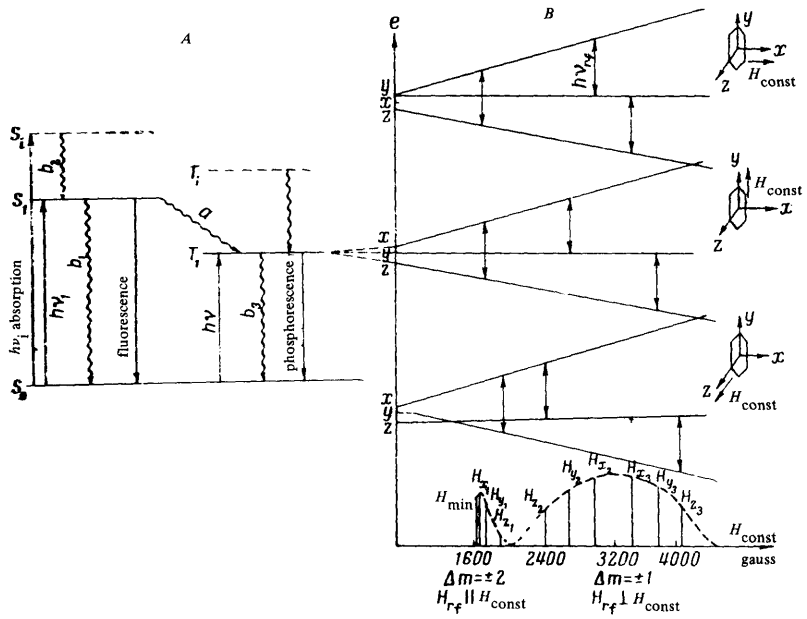


Figure 1. Schematic representation of the energy levels and transitions in an aromatic molecule. *A*—optical transitions; *B*—radio frequency ($h\nu_{rf}$) transitions in the presence of a constant magnetic field (H_{const}) and a radio frequency field (H_{rf}); S_0, S_1, S_i—the singlet energy levels; T_1, T_i—the triplet levels; *a*—intercombination non-radiative transition to a triplet state; b_1, b_2, b_3—non-radiative transitions to the corresponding lower state.

of the spin of the excited electron the overall spin of the system is unity and the multiplicity of the level is 3. Hence the term triplet is applied to this system of energy levels.

The mechanism of the transition to the phosphorescent state has been considered [19-21]. Terenin suggested that the metastable T_1 level be identified as a triplet level and put forward the hypothesis that the triplet state, characterised by unpaired spins, should play a significant role in photochemical reactions [22,23]. Lewis et al. [24] demonstrated for the first time by a static magnetic method the occurrence of light-induced paramagnetism.

We may note here also other characteristics of the excited triplet state. Firstly, as a result of the Coulombic repulsion of the unpaired electrons occupying the same orbital level, the T_1 level always has a lower energy than the first excited singlet level and therefore requires a lower activation energy. Secondly, since the lifetime in the triplet state is longer by a factor of 10^6–10^9 than the lifetime in the singlet state, favourable conditions are created for the involvement of the molecule in photochemical reactions, the rates of which are limited by diffusion processes at a low concentration of the reactants. Moreover, the energy of the triplet excitation may be readily transferred to another, unexcited molecule. Although the cross-section of the triplet–triplet transfer is lower than that of the singlet–singlet transfer, there is sufficient time for the energy of the long-lived triplet state to migrate over a long distance and therefore there is a high probability of its reaching the reaction centre.

We have already noted that the overall spin of the molecule in the triplet state S is unity and in a constant magnetic field the levels are split into three sublevels with $\Delta m = \pm 1.0$ projections of the spin on to the direction of the constant magnetic field. For molecules with a symmetry lower than spherical, the degeneracy is removed even in the absence of an external field. The zero-field splitting is due to dipole spin–spin interaction between the electrons in the triplet state, which is fairly well described by the two-electron spin Hamiltonian [1,2]

$$\hat{\mathcal{H}} = g\beta \mathbf{H}\hat{S} + DS_z^2 + E(S_x^2 - S_y^2). \qquad (1)$$

The first term defines the Zeeman energy of the interaction of the total spin S with the constant magnetic field **H** and the second, the dipole spin–spin interaction. S_X, S_Y, and S_Z are components of the overall spin S in the molecular system of coordinates, g is the spectroscopic splitting factor, and β the Bohr magneton. The zero-field splitting constant D defines the average dipole interaction between the spins along the molecular axis z and E takes into account the deviation of the spin–spin interaction tensor from axial symmetry [25,26]. The constants D and E can be readily determined from ESR spectra (the relevant equations are quoted below).

The Hamiltonian (1) transformed relative to the principal molecular axes is [26]

$$\hat{\mathcal{H}} = \mathbf{g}\beta \mathbf{HS} - (XS_x^2 + YS_y^2 + ZS_z^2), \qquad (2)$$

where X, Y, and Z are the principal values of the spin–spin interaction tensor diagonalised in the molecular

system of coordinates. They are related to the constants D and E in the following way:

$$X = {}^1/_3D + E,\ Y = {}^1/_3D - E,\ Z = -{}^2/_3D\ . \qquad (3)$$

If the constant magnetic field is directed along one of the molecular axes, for example x, eigenvalues for the triplet levels become [28]

$$e_1 = X,\ e_2 = e_3 = \frac{Y+Z}{2} \pm \sqrt{\frac{(Y-Z)^2}{4} - (g\beta H)^2}\ . \qquad (4)$$

Similar expressions can be found for the case where the field is directed along the y and z axes. Magnetic dipole transitions are allowed between two adjacent sublevels of the triplet level and involve a change in the magnetic quantum number: $\Delta m = \pm 1$. The transition moment, for example $x \rightarrow y$, is perpendicular to the xy plane (see Fig. 1*B*). When a variable magnetic field at a frequency ν such that

$$h\nu = g\beta H\,\Delta m \qquad (5)$$

is applied at right angles to the constant magnetic field, resonance absorption of electromagnetic energy takes place and is recorded by the ESR method. As a result of the occurrence of the zero-field splitting, the resonance absorption for different orientations of the constant magnetic field H_{const} relative to the three molecular axes will be observed for different values of H_{const} (Fig. 1*B*). If a single crystal is placed in the cavity and excited, being successively oriented so that the x, y, and z axes are parallel to H_{const}, three pairs of lines may be observed in the ESR spectrum—H_{x_2}, $H_{x_3}(H_{const} \parallel x)$, H_{y_2}, $H_{y_3}(H_{const} \parallel y)$, H_{z_2}, $H_{z_3}(H_{const} \parallel z)$ respectively (Fig. 1*B* below). However, the $\Delta m = \pm 1$ selection rules hold rigorously when the interaction between the spins and the external magnetic field is much greater than the intrinsic interaction between the spins, and the component of the spin angular momentum of the electrons $m(h/2\pi)$ in the direction of the field remains constant. Such transitions in a single crystal of a dilute solid solution of naphthalene in durene were observed by Hutchinson and Mangum [1,2]. However, the possibilities of growing diluted single crystals are limited. Van der Waals and de Groot [27,28] deduced theoretically and observed experimentally for the first time the forbidden transitions between the lowest and highest sublevels of the triplet level corresponding to a change in the magnetic quantum number $\Delta m = \pm 2$. The transition is observed at much weaker fields when the intrinsic interaction of the electrons is comparable to their interaction with the external magnetic field. The component of the spin angular momentum $m(h/2\pi)$ is not a constant of motion and the selection rules break down. The $\Delta m = \pm 2$ transitions are polarised parallel to the constant magnetic field and are observed in parallel fields (Fig. 1*B*). In particular, it follows from Eqn. (4) that the transitions between the sublevels take place for values of H_{const} defined by the equation

$$(e_2 - e_3)^2 = (h\nu)^2 = \frac{1}{4}(Y-Z)^2 + (g\beta H)^2\ . \qquad (6)$$

We shall employ as an example the data of Hutchison and Mangum [2] for naphthalene:

$$\frac{X-Z}{hc} = 0.0863\ см^{-1},\ \frac{Y-Z}{hc} = 0.1143\ см^{-1},\ \frac{h\nu}{hc} = 0.3221\ см^{-1} = 2.0033.$$

On substituting the numerical values in Eqn. (6), we find that the resonance absorption for naphthalene with $\Delta m = \pm 2$ will be observed for $H_{x_1} = 1602$ G, $H_{y_1} = 1650$ G, and $H_{z_1} = 1707$ G. Thus, whereas the $\Delta m = \pm 1$ transitions are recorded in the range of H_{const} from 2354 to 4490 G and the overall line width is 2196 G, for $\Delta m = \pm 2$ transitions the latter value is 105 G, i.e. the anisotropy of the transitions is smaller by a factor of 20. [27,28] Therefore,

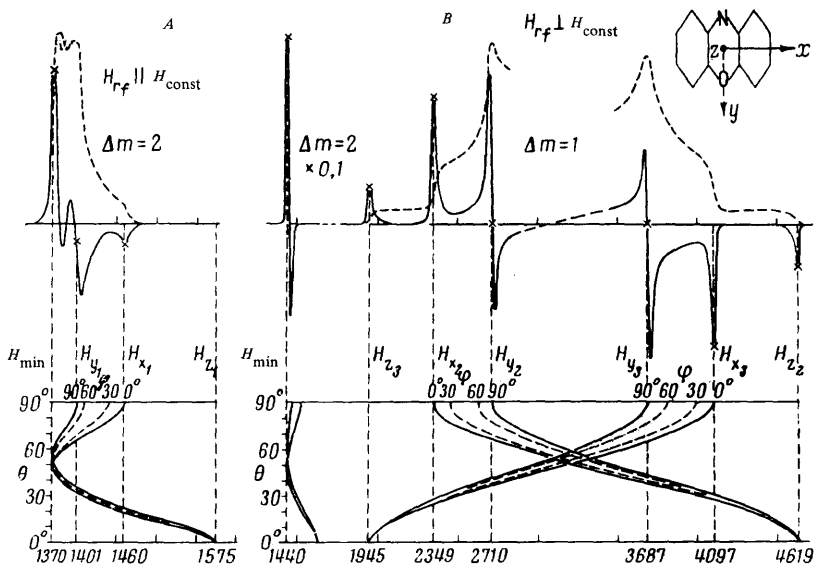


Figure 2. ESR spectrum of phenoxazine in the triplet state (molecular symmetry less than that for three axes: $x \neq y \neq z$). Below: distribution of resonance fields $F(\delta, H)$ as a function of $f(\theta, \varphi)$ for the specified ν = const.

despite the fact that the intensity of these transitions is lower by two powers of ten than that of the $\Delta m = \pm 1$ transitions, the ESR signal may be observed in specimens with a random orientation—solid solutions [28].

For an arbitrary orientation of H_{const} relative to the molecular axes, Kottis and Lefevre [29,30] used the operator (2) to find the distribution function of the resonance fields in terms of the spatial orientation of the x, y, and z axes relative to the constant magnetic field (the angles φ and θ, Fig. 2B). For a fixed value of the microwave quantum ($h\nu$), such a function makes it possible to determine the resonance fields for specified X, Y, and Z parameters. Figs. 1 and 2B show that, for $h\nu > D$, three resonance values of the field correspond to each molecular orientation. Two of these correspond to the $\Delta m = \pm 1$ transitions ($H_{const} \perp H_{rf}$) and the third to $\Delta m = \pm 2$ ($H_{const} \parallel H_{rf}$). Finally, there is resonance absorption for a minimal value of H_{const} (H_{min}) at the specified frequency (Fig. 2B). In this case the intensity of ESR absorption is independent of the relative orientations of H_{const} and H_{rf}:

$$H_{min} = (2g\beta)^{-1}[(h\nu)^2 + 4(XY + XZ + YZ)]^{1/2} = (2g\beta)^{-1}\left\{(h\nu)^2 - 4\left[\frac{D^2}{3} + E^2\right]\right\}^{1/2}. \quad (7)$$

Expressions for the calculation of the resonance fields or, if their values are known from ESR spectra, of the zero field splitting constants and energies are:

$$H_{x_1} = (2g\beta)^{-1}[(h\nu)^2 - (D-E)^2] = (2g\beta)^{-1}[(h\nu)^2 - (Y-Z)^2]^{1/2},$$
$$H_{x_2} = (2g\beta)^{-1}[(2h\nu - D - 3E)^2 - (D-E)]^{1/2} = (2g\beta)^{-1}[(2h\nu - 3|X|)^2 - (Y-Z)^2]^{1/2},$$
$$H_{x_3} = (2g\beta)^{-1}[(2h\nu + D + 3E)^2 - (D-E)^2]^{1/2} = (2g\beta)^{-1}[(2h\nu + 3|X|)^2 - (Y-Z)^2]^{1/2}. \quad (8)$$

H_{X_1} corresponds to the $\Delta m = \pm 2$ transition and H_{X_2} and H_{X_3} to the $\Delta m = \pm 1$ transition. The expressions for the H_{Y_1}, H_{Y_2}, H_{Y_3}, H_{Z_1}, H_{Z_2}, and H_{Z_3} resonance fields are similar to Eqn. (8) and can be obtained by a cyclic interchange of X, Y, and Z.

Subsequently it was found that the $\Delta m = \pm 1$ transitions can be observed not only for single crystals but also specimens with a random orientation. In a solid solution the molecules are fixed relative to one another and magneto-selection takes place. Molecules whose corresponding axes lie within 5° of the constant magnetic field contribute to the intensity of the canonical peak [27]. This was observed experimentally for the first time by Yager et al. [31] for naphthalene, coronene, and triphenylene. Similar spectra were recorded for biologically important compounds: magnesium-aetioporphyrin [32] and later for other porphyrins [33,34], chlorophyll [34], orotic and polyadenylic acids, adenosine and guanosine monophosphates [35-37], aromatic aminoacids, and certain proteins [38].

ESR absorption in a field corresponding to a g-factor of 2.0023 has been observed for certain aromatic substances [33,34,39]. This is the so called two-quantum transition ($H_{t.q.}$) caused by the simultaneous absorption of two radiofrequency quanta. The following relation must hold for a two-quantum transition to be observed:

$$(g\beta H_{t.q.})^2 = (h\nu)^2 - \frac{1}{3}D^{*2} \quad (D^* = \sqrt{D^2 + 3E^2}). \quad (9)$$

The molecular orientations for which the energy differences between two pairs of adjacent sublevels are similar or identical make a dominant contribution to the $H_{t.q.}$ absorption line.

Thus for molecules with a lower than third-order symmetry, the ESR spectrum in perpendicular fields consists of eight peaks. Such line shape corresponds to the static case where during the radiofrequency transition the nuclei of the atoms in the molecule do not undergo significant conformational, rotational, or other displacements. However, de Groot and van der Waals [40] showed that the line shape of the ESR spectra of the triplet state of certain aromatic compounds, for example benzene, triptycene, and aromatic ions, can vary significantly with temperature owing to different dynamic effects. The line shape of the ESR spectra of substances whose triplet state is orbitally degenerate varies in the same way [40]. In this case the Jahn–Teller effect leads to the instability of the fully symmetrical non-linear molecule and to the removal of the orbital degeneracy due to nuclear displacements. The high molecular symmetry breaks down, which leads to a change in the line shape of the ESR spectrum and in the zero-field splitting constants. The dynamic effect has been treated theoretically by Kottis and Lefevre [30].

Having examined the principal characteristics of the line shape of the ESR spectra common to all aromatic substances, we shall proceed to consider the characteristics of the ESR and the properties of the excited triplet state of biologically important compounds.

II. ELECTRON SPIN RESONANCE OF THE TRIPLET STATE OF BIOLOGICALLY IMPORTANT MOLECULES

1. Porphyrins

The first data on the excited triplet state of porphyrins observed by the ESR method were published in 1963. [32] Evacuated solutions of aetioporphyrin, mesoporphyrin, and protoporphyrin and their magnesium complexes, zinc-aetioporphyrin, haematoporphyrin and its mercury complex, and tetraphenylporphyrin(TPP) and its zinc complex, frozen at 77°K, were investigated [42-46]. In fields of about 1600 G (klystron frequency ν = 9260 MHz), ESR absorption corresponding to the $\Delta m = \pm 2$ transition was observed. The ESR signal consisted of a single slightly asymmetric line with a half-width ΔH = 10–12 G between the points of maximum slope. The intensity of the ESR signal did not vary with time on illumination with visible light ($\lambda \geq 400$ nm).

Among the porphyrins investigated Mg-aetioporphyrin proved to have the highest concentration of paramagnetic centres (about 10^{16} spins in a 0.03 cm^3 specimen at a porphyrin concentration of 2×10^{-3} M). For other porphyrins, the number of paramagnetic centres in the specimen at a concentration of 10^{-3} M was 5×10^{14}–7×10^{15} spins, in agreement with the shorter lifetime $\tau_{phosph} = 5 \times 10^{-2}$–$10^{-2}$ s compared with τ_{phosph} of Mg-aetioporphyrin (2×10^{-1} s). [41] The ESR absorption of the triplet states of the copper complexes of aetioporphyrin and haematoporphyrin could not be observed although it is known that the intensity of the phosphorescence of such copper complexes greatly exceeds that of the magnesium complexes and the bases. Since τ_{phosph} for the copper complexes is 10^{-4}–5×10^{-5} s, the steady-state concentration of the excited triplet molecules is lower by 2–3 powers of ten than that of the bases and magnesium complexes. Within the limits of the concentration range investigated (5×10^{-5}–10^{-3} M), the amplitude of the ESR signal is directly proportional to the concentration of the pigment [42]. The concentration-induced quenching for various porphyrins begins when a concentration of 10^{-3} M is reached. The ESR signal amplitude then falls [42,46].

For reactions proceeding via an excited triplet state, it is important to know the characteristics of molecules in the triplet state such as τ and the steady-state concentration n_{st}. Indeed, in the porphyrin series magnesium complexes are the most active, compared with the bases and zinc complexes, while the copper complexes are the least active[32,42]. This applies in particular to two-quantum reactions, since in the case of high values of τ there is a considerable population of the triplet level and hence a high probability that a second energy quantum will be absorbed.

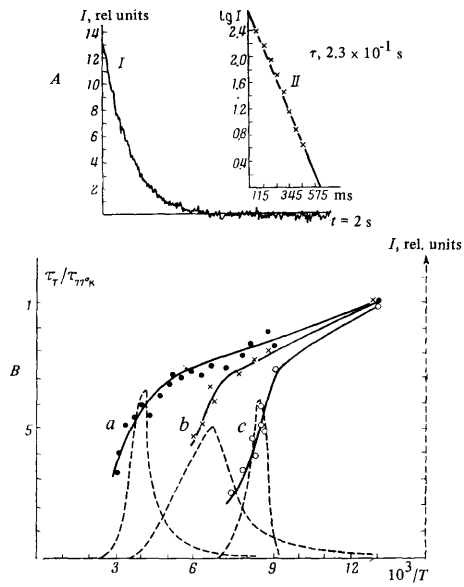


Figure 3. *A*) Kinetics of the decay of the ESR signal of the triplet state ($\Delta m = \pm 2$) of Mg-aetioporphyrin in ethanol at 77°K (I) and the same plot on a logarithmic scale (II); *B*) variation of $\tau_T/\tau_{77°K}$ with $1/T$ for Mg-aetioporphyrin in poly(methyl methacrylate) (a), in highly viscous methyl methacrylate (the initial stage of polymerisation) (*b*), and in ethanol (*c*). Lower curves —variation of the yield of radicals with temperature.

The authors of the review developed a method for the measurement of τ by ESR using a spectrum accumulator and a mechanical shutter[47]. The variation of τ and n_{st} with the concentration of the pigment and oxygen, the properties of the matrix, and temperature was investigated[47-49]. τ was determined from the time required for the amplitude of the $\Delta m = \pm 2$ ESR signal to diminish by a factor e after switching off the illumination. Fig. 3*A* presents a typical decay curve for the ESR signal of Mg-aetioporphyrin at 77°K. For all the porphyrins investigated, the decay curves are exponential. The values for τ for the triplet state are listed in Table 1. We may note that the variation of the pigment concentration in the range 5×10^{-5}–10^{-3} M, the nature of the solvent [ethanol, cyclohexanol, and poly(methyl methacrylate) (PMT)], and the presence of oxygen in the specimen at 77°K do not affect the value of τ for the triplet state.

With increase of temperature the lifetime diminishes. Table 2 presents the values of τ for Mg-aetioporphyrin dissolved in ethanol and PMT at different temperatures.

Table 1. Zero-field splitting constants and the values of τ for the triplet state of porphyrins at 77°K and 320°K.

Compounds	D/hc, cm^{-1} $\Delta m=\pm1$ 77° K	D^*/hc, cm^{-1} $\Delta m=\pm2$ 77° K	D^*/hc, cm^{-1} $\Delta m=\pm2$ 320° K	$10^{-2}\tau$, s 77° K	Solvent
Metal complexes					
Mg-aetioporphyrin	0.036	0.036	0.033	23.5	Ethanol, PMT
Zn-aetioporphyrin	0.036	0.038	—	—	Ethanol, ethanol + pyridine
Mg-protoporphyrin	—	0.036	—	—	Isobutyl alcohol
Zn-protoporphyrin	—	0.036	—	—	Ethanol + isobutyl alcohol
	—	0.038	—	—	Ethanol + pyridine
Mg-mesoporphyrin	0.036	—	—	—	Ethanol
Zn-tetraphenylporphin	—	0.031	0.025	6.2	Ethanol, PMT
Mg-phthalocyanine		0.027	—	—	Ethanol + pyridine
Dications					
$2H^+$-aetioporphyrin	0.033*	0.036	—	—	Ethanol + HCl
$2H^+$-mesoporphyrin	0.033	0.036	—	3.0	Ethanol + HCl
$2H^+$-haematoporphyrin	0.033	0.033	—	—	Ethanol + HCl
Bases					
Aetioporphyrin	0.048	0.047	0.042	3.2	Ethanol + pyridine, PMT
Mesoporphyrin	0.048	0.047	0.041	2.5	Ethanol + pyridine, PMT
Protoporphyrin	—	0.046	—	—	Ethanol + pyridine
Haematoporphyrin	—	0.047	—	—	Ethanol

*For freshly prepared specimens, $D^*/hc = 0.033$ cm^{-1}; for specimens stored for a long time in solutions with HCl, $D/hc = 0.036$ cm^{-1}.

Table 2. Variation of τ for Mg-aetioporphyrin in the temperature range 77–335°K.

Ethanol						
Temperature, °K	77	108	120	127	131	135
10τ, s	3.35 ±0.03	1.75 ±0.06	0.95 ±0.1	0.8 ±0.1	0.6 ±0.1	0.55 ±0.1
Poly(methyl methacrylate)						
Temperature, °K	77	120	180	250	320	333
10τ, s	2.35 ±0.03	1.85 ±0.06	1.55 ±0.06	1.4 ±0.06	1.0 ±0.1	0.75 ±0.1

The steady-state concentration of the triplet molecules varies with τ. The parallel variation of these quantities with temperature is evidence that the factors responsible for the temperature-induced quenching are the same. A deviation from the parallel variation is observed in ethanol solutions. In all cases the $\tau_T°K/\tau_{77°K} = f(1/T)$ curves are complex and have two sections. The variation along the slow section is close to exponential[47,48], which corresponds to purely temperature-induced quenching[51] (Fig. 3B). In addition, the effectiveness of the quenching depends on the viscosity of the medium and proves to be highest in ethanol and lowest in PMT. The presence of oxygen also sharply increases the effectiveness of the quenching in ethanol and cyclohexanol, but does not affect the quenching in a polymeric matrix when there is no diffusion of oxygen. The sharp inflection in curves describing the variation of τ (and also n_{st}) (Fig. 3B) is noteworthy. The temperature at which the inflection occurs is associated with the onset of formation of free porphyrin radicals. It has been

shown[48-50] that the formation of free radicals has a two-quantum mechanism. Together with pigment radicals, the polymeric matrix stabilises the radicals of methyl methacrylate. It is noteworthy that temperature-induced quenching of the triplet molecules of the photochemically less active Zn-tetraphenylphorphin is less effective, owing to the formation of free radicals, than for Mg-aetioporphyrin. Thus the temperature-induced quenching of τ and n_{st} is analogous to the temperature-induced quenching of the phosphorescence of aromatic compounds[51] and is determined, apart from the effect purely due to temperature and associated with the redistribution of the vibrational energy of the molecule, by the structural properties of the medium, the oxygen effect, and quenching due to photochemical reactions.

The ESR spectra of $\Delta m = \pm 1$ transitions. The ESR spectra in high magnetic fields (2900–3800 G) due to $\Delta m = \pm 1$ transitions were first observed for Mg-aetioporphyrin[8,43] (as already mentioned, this substance gives rise to the highest ESR signal in low fields). Later the same investigators[33] and also Lhoste et al.[52] (for haematoporphyrin) recorded $\Delta m = \pm 1$ transitions for bases, dications, and other metal complexes of porphyrins dissolved in ethanol, pyridine, cyclohexanol, and their mixtures and also in PMT. The ESR spectra at room temperature have been recorded exclusively in PMT. The cationic forms of porphyrins have been investigated in acid ethanol solutions. The method of signal accumulation has been used to record the spectra of molecules with low values of n_{st}, particularly at room temperature[47,48].

Fig. 4 presents high-field ESR spectra of porphyrins in an excited triplet state. For the metal complexes of porphyrins with D_{4h} symmetry (Mg-aetioporphyrin, Zn-aetioporphyrin, and Zn-tetraphenylporphin), the ESR spectrum has the expected line form, i.e. four canonical peaks: H_{x,y_2} and H_{x,y_3} for the x and y molecular axes directed along the constant magnetic field and H_{z_2} and H_{z_3} for $z \parallel H_{const}$. The intensity ratio corresponds to the expected inequality $I_{H_{x,y}} > I_{H_z}$. Indeed, in the absence of an external magnetic field the triplet level splits into two sublevels separated by a distance D. Since the molecular symmetry is D_{4h}, one of the sublevels remains doubly degenerate (the molecular x and y axes in the plane of the molecule are physically equivalent. We may note that, at room temperature in PMT, the $H_{x,y}$ lines are considerably narrowed (Fig. 4*b*, curve 2). Two tetrapyrrole pigments—Zn-aetioporphyrin and chlorophyll (discussed in greater detail below)—exhibit two-quantum transitions in the region of $\Delta m = \pm 1$ (Fig. 4*c*).

For porphyrin bases with D_{2h} symmetry (according to spectroscopic data[53]), one should expect the appearance of a six-line spectrum with H_x, H_y, and H_z canonical peaks. However, the experimental ESR spectrum consists of four lines, which corresponds to cylindrical symmetry of the molecule in the triplet state. In the light of the latest theoretical concepts and X-ray diffraction data[54], the porphyrin bases may be represented by a superposition of structures *A*, *B*, and *C* (Fig. 4*f*) with statistical weights of 19, 32, and 49% respectively. Structures *B* and *C* with D_{4h} symmetry then represent the porphyrin bases to the extent of 80%. This finding is satisfactorily consistent with ESR data.

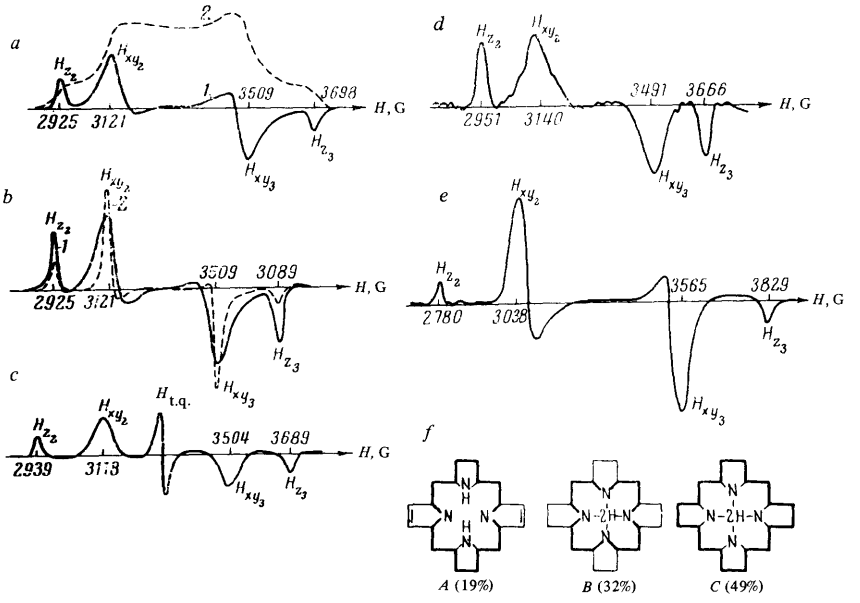


Figure 4. ESR spectra ($\Delta m = \pm 1$) of aetioporphyrins in an excited triplet state: *a*) Mg-aetioporphyrin in ethanol at 77°K [1) first derivative of the ESR spectrum; 2) the spectrum in the integral form]; *b*) Mg-aetioporphyrin in poly(methyl methacrylate) [1) at 77°K; 2) at 330°K]; *c*) Zn-aetioporphyrin in ethanol at 77°K; *d*) $2H^+$-aetioporphyrin in ethanol solution of hydrochloric acid at 77°K; *e*) aetioporphyrin in a pyridine—ethanol mixture at 77°K; *f*) π-electron structure of porphyrins.

The ESR spectra of the $\Delta m = \pm 1$ and $\Delta m = \pm 2$ transitions were also recorded at higher temperatures (up to 350°K)[33, 45, 55]. It must be emphasised that, with increase of temperature, the spectrum as a whole is displaced towards higher fields (the constant D^*/hc diminishes), the number of peaks does not change, but the lines themselves become markedly narrower and their intensity ratios change (Fig. 4*b*, curve *2*).

The zero-field splitting constants calculated from the ESR spectra are summarised in Table 1. The average values of D^*/hc calculated from the $\Delta m = \pm 1$ spectrum agree satisfactorily with the D^*/hc values calculated from the $\Delta m = \pm 2$ spectra. Analysis of the data in Table 1 leads to the following findings: (1) lateral substituents of the porphyrin ring such as CH_3, CHO, C_2H_5, and CH_2COOH do not affect the constant within the limits of experimental error, i.e. they do not affect significantly the dimensions and symmetry of the π-electron cloud of the molecule; (2) all metal complexes with aliphatic substituents also have the same zero-field splitting constants. On the other hand, phenyl substituents in Zn-tetraphenylporphin and Mg-phthalocyanine increase the size of the π-electron system and diminish the D/hc constant; (3) the values of D/hc for the metal complexes are somewhat higher than those for the dications; this finding is probably associated with some contraction of the π-electron cloud by the metal atom towards the centre of the molecule; (4) on passing to porphyrin bases, the constants increase significantly. This anomalous effect may be attributed either to the orbital splitting of the lowest triplet level or to the fact that in the porphyrin molecule in an excited triplet state the electrons spend most time in the 18-membered small ring of the molecule (Fig. 4*f*). This leads to a marked decrease of D/hc, since this structure predominates over all others under conditions of dynamic equilibrium.

Table 3. Characteristics of the ESR spectra of the triplet state of porphyrins at 77° and 320°K (ν = 9260 MHz).

Porphyrin	H_{min}, G 77°K	H_{min}, G 320°K	ΔH 77° K	ΔH 320° K	D/hc, cm^{-1} 77°K	D/hc, cm^{-1} 320°K	Symmetry
Aetio-	1626	1631	11.5	11.0	0.047	0.042	D_{2h}
Mg-aetio-	1637	1639	9.5	10.5	0.036	0.033	D_{4h}
Meso-	1627	1632	11.5	10.0	0.046	0.041	D_{2h}
Proto-	1626	1629	11.0	11.0	0.047	0.044	D_{2h}
Zn-TPP	1640	1644	11.0	12.5	0.031	0.025	D_{2h}

The values of D^*/hc are almost insensitive to the solvent (ethanol, cyclohexanol, PMT, isopentane, and pyridine). However, pyridine, which is known to exhibit complex-forming properties, causes an appreciable broadening of the ESR line. The introduction of magnesium into the centre of the porphyrin molecule narrows the ESR line compared with the spectra of the bases and dications. When the temperature is raised from 77° to 320°K, the values of D^*/hc decrease by 7–19% for various porphyrins (Table 3), which is probably associated with an increase of the size of π-electron cloud owing to the population of high vibrational sublevels of the triplet state or to dynamic effects leading to the predominance of more symmetrical porphyrin structures [40].

As already mentioned in the introduction to the review, the main contribution to the canonical peaks of the ESR spectra of the $\Delta m = \pm 1$ transitions in a system of molecules oriented at random comes from molecules for which the principal axes of the spin–spin interaction tensor are oriented in the direction of the external magnetic field H_{const} or nearly so (±5°);[27,28] other orientations give rise to an almost constant absorption background. Thus there is a selection of molecules with respect to canonical orientation—magnetoselection. When such a system is excited by polarised light (magnetophotoselection), only the canonical peaks for which the direction of the $S_0 \rightarrow S_1$ transition moment and the electric vector of the incident light waves (E and H_{const}) coincide appear in the spectrum [56,57]. The authors of the review [58] investigated the variation of the line form of the ESR spectra ($\Delta m = \pm 1$ transitions) as a function of the polarisation of the exciting light relative to the direction of the external magnetic field. Fig. 5A presents the ESR spectra of Mg-aetioporphyrin and Zn-aetioporphyrin in the triplet state excited

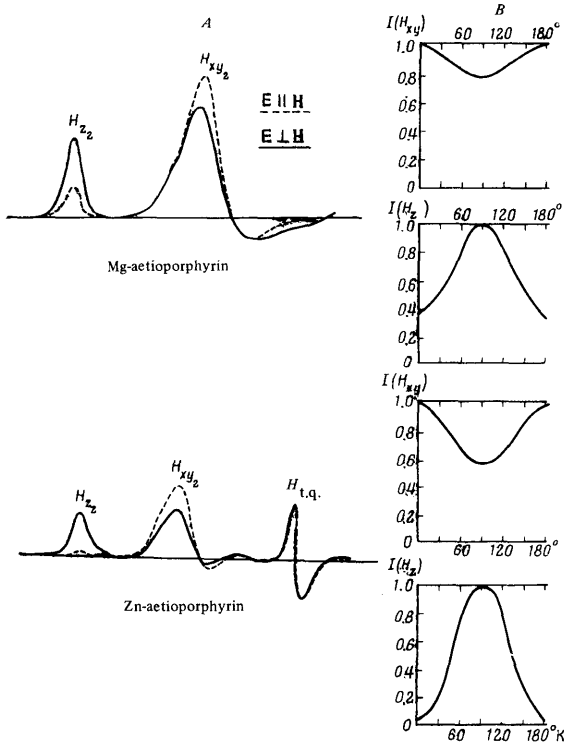


Figure 5. *A*) ESR spectra of Mg- and Zn-aetioporphyrins for different polarisations of the exciting light; *B*) variation of the height of the canonical peaks as a function of the angle between H_{const} and E.

by polarised light. On the right-hand side of the same figure, the amplitudes of the canonical peaks H_Z and H_{XY} are plotted against the angle between the directions of the polarised light vector E and of H_{const}. Evidently the limiting heights of different canonical peaks occur for parallel and perpendicular orientations. For the parallel orientation ($E \parallel H_{const.}$), the H_{XY} peak is a maximum and

the H_Z peak a minimum; for the perpendicular orientation, the situation is reversed. For intermediate orientations of E and H_{const}, the amplitude of the H_Z peak varies as $\sin^2 \alpha$ and that of the H_{xy} peak, as $1 + \cos^2 \alpha$. The amplitude of the $H_{t.q.}$ peak is independent of polarisation.

This behaviour of the canonical peaks can be readily explained if it is assumed that an oscillator oriented in the plane of the molecule is responsible for the triplet excited state. This conclusion is also consistent with optical data[53,59]. The line shape for porphyrin dications varies similarly on excitation with polarised light (Table 4).

Table 4. Effect of polarised light on the intensity of the canonical peaks in $\Delta m = \pm 1$ transitions.

Porphyrin	$\frac{I(H_z)_\parallel}{I(H_z)_\perp}$	$\frac{I(H_{xy})_\parallel}{I(H_{xy})_\perp}$
Mg-aetio-	0.23±0.05	1.44±0.1
Zn-aetio-	0.15±0.05	1.7±0.1
$2H^+$-aetio-	0.42±0.05	1.2±0.1
$2H^+$-meso-	0.7±0.1	1.5±0.2

Table 4 lists the ratios of the amplitudes of peaks 1 (H_1), for the parallel orientation of E and H_{const}, to the amplitude corresponding to the perpendicular orientation. Theoretically these ratios are given by the following formulae:

$$\frac{I(H_z)_\parallel}{I(H_z)_\perp} = 0; \quad \frac{I(H_{xy})_\parallel}{I(H_{xy})_\perp} = 2^{57}.$$

However, Table 4 shows that they do not hold exactly for porphyrins, or incidentally, for the other compounds investigated[56]. This deviation from theoretical values is associated with depolarisation effects of different types, which have been discussed in detail by Kottis and Lefevre[57].

2. Photosynthesising Pigments

The ESR signal of the triplet state of chlorophyll b was observed for the first time in a frozen ethanol solution[60]. The same investigators[61] succeeded in observing the ESR absorption of the excited triplet state using a spectrum accumulator with alcoholic and pyridine chlorophyll a, chlorophyll a + b, and phaeophytin a + b solutions at pigment concentrations of about 10^{-3} M. Transitions were observed in the range 1636–1640 G (ν = 9260 MHz) between the lowest and highest triplet sublevels ($\Delta m = \pm 2$). The amplitude of the ESR signal of the chlorophyll b triplet state is proportional to its concentration, but, starting with a 10^{-3} M pigment concentration, the amplitude falls owing to concentration-induced quenching[46,60]. When films of chlorophyll a + b, in which the pigment is in a highly aggregated state, were investigated, no ESR absorption was observed, which is also due to concentration-induced quenching[61]. The intensity of the ESR signal of the triplet state of chlorophyll *b* is two and a half times greater than that of the signal of chlorophyll **a**. This might have been expected, since τ_{phosph} for chlorophyll b is almost three times higher than for chlorophyll a.[59]

The ESR spectra of $\Delta m = \pm 2$ transitions consist of singlet slightly asymmetrical lines (Fig. 6*A*). At the temperature of liquid nitrogen, oxygen does not influence the signal intensity. Table 5 lists the ESR parameters of the triplet state of the pigments investigated.

Using a spectrum accumulator, Lhoste[34] observed the ESR signal of the triplet state of chlorophyll b in high fields ($\Delta m = \pm 1$ transitions). The ESR signal consists of seven peaks (Fig. 6*D*): H_{x_2}, H_{x_3}, H_{y_2}, H_{y_3}, H_{z_2}, H_{z_3}, and the $H_{t.q.}$ peak corresponding to the two-quantum transition. This spectrum corresponds to C_{2v} molecular symmetry. The zero-field splitting constants were estimated from the spectra: $D/hc = 0.0306 \pm 0.0010$ cm^{-1} and $E/hc = 0.0034 \pm 0.0006$ cm^{-1}. The additional peaks (IV and V) were not assigned by the authors (Fig. 6*D*), but they may be due to the presence of an admixture of chlorophyll a or phaeophytin.

Table 5. Characteristics of the ESR spectra of chlorophyll a and b in the triplet state (ν = 9260 MHz).

Pigment	H_{min}, G	ΔH, G	D^*/hc, cm^{-1}	Solvent
Chlorophyll a	1640	13	0.032	Ethanol
Chlorophyll b	1636	13	0.037	Ethanol
	1638	13	0.035	Pyridine

From the standpoint of the understanding of the role of the excited triplet state of chlorophyll in photosynthesis *in vivo*, its direct investigation in photosynthesising systems (chloroplasts, chromatophores, and leaves turning green) is of greatest interest. An attempt has been made[61] to observe the ESR signal due to photosynthesising pigments in chloroplast and "greening" leaf preparations. A study was made of pea chloroplasts. In native preparations, ESR absorption due to the triplet state was not observed. The light-induced ESR signals due to the triplet state of chlorophyll were observed in chloroplast preparations containing between 10 and 50 vol.% of pyridine (Fig. 6*C*). Pyridine is known to have a deaggregating effect on the pigment system of chloroplasts and in the given case there was a possibility of the extraction of chlorophyll into pyridine. As pointed out earlier, in studies on pigment films ESR signals of the triplet state were not observed either. Therefore one may suppose that at high pigment concentrations there is an effective transfer of excitation energy via triplet levels resulting in a sharp decrease of τ and hence also n_{st} for chlorophyll. In this connection one might have expected the appearance of an ESR signal in leaves during the process of acquisition of the green colour, which involves the formation of the monomeric components of the pigment system. A study has been made[61] of the ESR absorption of etiolated corn leaves subjected to illumination for different periods (between 1 and 30 h). However, the ESR signals due to the triplet state were not observed in these systems either. The extraction of the pigments from the leaves to a pyridine solution makes it possible to record the ESR absorption. Probably, during the first stages of the generation of the green colour in the leaves, the deactivation processes of the triplet molecules are already fairly effective. Such deactivation may be a consequence of an effective transfer and employment of the energy of the triplet excitation in the reaction centre, so that in native chloroplasts measurable steady-state concentrations of chlorophyll molecules in an excited triplet state are not observed.

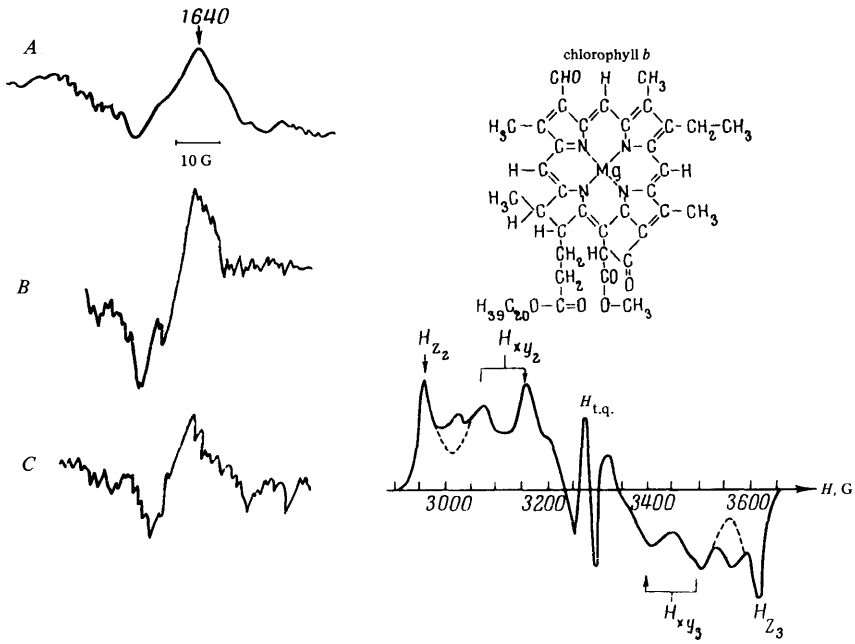


Figure 6. The structure and ESR signal of the excited triplet state of chlorophyll a (*A*), phaeophytin a + b (*B*) in ethanol and in chloroplasts treated with pyridine (*C*) ($\Delta m = \pm 2$ transitions, $\nu = 9260$ MHz), and of chlorophyll b (*D*) in ethanol ($\Delta m = \pm 1$ transition, $\nu =$ 9210 MHz). The spectrum *B* is probably the sum of two signals, due to phaeophytin a and phaeophytin b, as shown by the reproducible splitting of the line.

3. Aromatic Aminoacids, Di- and Poly-peptides, and Proteins

The observation of the excited triplet state of aromatic aminoacids by the ESR method has been reported by a number of workers, starting in 1963.[9,11,13,32,38,62-68] Ethanol and aqueous ethanol solutions of aromatic aminoacids (tyrosine, tryptophan, and phenylalanine) were excited by ultraviolet light at 77°K. $\Delta m = \pm 2$ transitions were recorded. The ESR signals consist of single asymmetric ESR lines (Fig. 7*A* and 7*B*) with the parameters listed in Table 6. After the illumination has been switched off, the ESR signal of tryptophan in ethanol decayed with the period $\tau = 2.4$ s (decrease of the initial ESR signal amplitude by a factor e), which is consistent with optical data[69]. When acetic acid was added to an ethanol solution of tryptophan, the signal intensity fell approximately by a factor of ten[32,42]. Ptak and Douzou[65] observed the ESR signals of the excited triplet state of aromatic aminoacids in methanol solutions and obtained results analogous to those of Gribova and coworkers[32,42], in particular they showed that the decay of the ESR signal is strictly parallel to the decay of phosphorescence (Fig. 7*B*). The times required for the decrease of the ESR signal by a factor of 2 ($\tau_{1/2}$) in a phosphate buffer are respectively 6.7 s for tryptophan, 6 s for phenylalanine, and 3 s for tyrosine. It is noteworthy that the ESR and luminescence characteristics of the triplet state of aromatic aminoacids depend on the nature of the matrix, the temperature, and the pH of the solution. In particular Shiga and Piette[66] noted that the nature of the solvent (water–methanol, phosphate buffer) does not influence the ESR signal line form, but significantly alters the signal decay time.

Among the three aromatic aminoacids, the highest ESR signal ($\Delta m = \pm 2$) was recorded for tryptophan and the lowest for phenylalanine in solutions at the same concentration. Shiga and Piette[66] noted that the yield of tyrosine molecules in an excited triplet state is one third of that for tryptophan. Zuclich[38] found that the intensity ratios of the ESR signals ($\Delta m = \pm 2$) for tryptophan, the tyrosinate ion, tyrosine, and phenylalanine are respectively 8 : 2 : 1 : 1. The characteristics of the ESR spectra determined from the $\Delta m = \pm 2$ transitions are listed in Table 6.

Hélène et al.[70] investigated the dependence of the parameters of the ESR spectra on the aggregation of aminoacids in solution. As for the variation of phosphorescence intensity, the authors observed an increase in the ESR signal on deaggregation when deaggregating additives (ethanol and sodium chloride) were added to the aqueous solutions of the aminoacids before freezing. They explained this effect by a change in the probability of an intersystem crossing and in the probability of energy transfer in the microcrystals.

Malig et al.[67] made a detailed study of the effect of the pH of the medium on the properties of the ESR signal of L-tyrosine in the crystalline state. They observed two ESR signals and the corresponding absorption bands of the

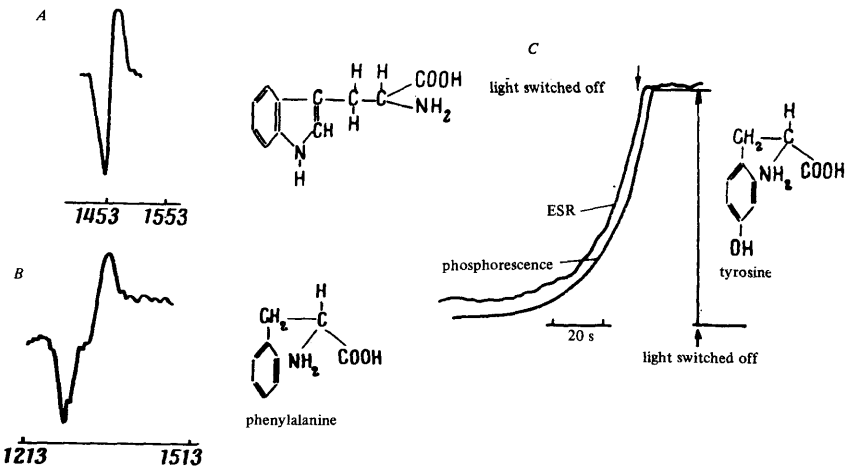


Figure 7. ESR spectrum of the triplet state of tryptophan (*A*) and phenylalanine (*B*) at 77°K in ethanol solutions ($\Delta m = \pm 2$ transitions). Curves *C* represented the decay of the ESR signal and phosphorescence of L-tyrosine under the same conditions.

two forms of tyrosine: unionised and ionised. The parameters of the spectra are presented in Table 6. The signal intensity depends on the pH of the medium. At pH 6.9 the ionic form is absent and a peak at 280 nm, due to the non-ionic form, is observed in the absorption spectrum together with the corresponding ESR signal at 1287 G ($\nu = 9138$ MHz). At pH 10 only the ESR signal at 1390 G and the corresponding absorption maximum of the ionic form at 295 nm (Fig. 8) persist. On raising the temperature from 77° to 295°K, the equilibrium is displaced towards the formation of the unionised form; $\tau_{1/2}$ for the decay of the ESR signal is about 2 s for the ionic form and about 3 s for the non-ionic form.

Table 6. Parameters of the ESR spectra of aromatic aminoacids in the triplet state ($\Delta m = \pm 2$ transitions; 77°K).

Compound	D^*/hc, cm^{-1}	ΔH, G	$\tau_{1/2}$, s	Solvent
Tryptophan	0.126	15	2.4	Ethanol (Ref.32)
	0.119	22	4.1	Methanol + 6 *N* HCl phosphate buffer (Ref.66)
Tyrosine	0.112	32	1.9	Ditto
Non-ionised L-tyrosine	0.161	32	3	H_2O + propylene glycol
Ionised L-tyrosine	0.141	47	2	H_2O + propylene glycol + HCl
Phenylalanine	0.161	63	6.4	Methanol + HCl phosphate buffer

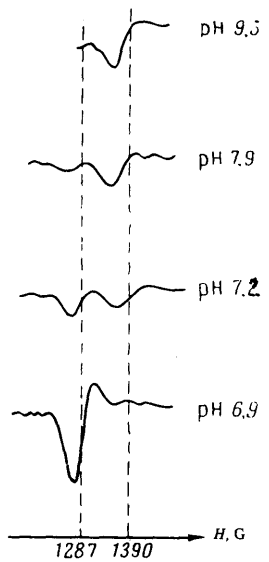


Figure 8. ESR spectrum of L-tyrosine in the triplet state as a function of pH (pH established with a tris buffer and HCl at 295°K).

Similar studies were made by Shiga et al.[68] for L-tryptophan, glycyl-L-tryptophan, and poly-L-tryptophan. The decay curves for the ESR signal and phosphorescence are identical. At an L-tryptophan concentration in excess of 2×10^{-4} M, concentration-dependent quenching is observed; we then have $\tau = 4.4$ s regardless of concentration. The intensity of the ESR signal depends non-linearly on the light intensity and saturation is attained at a fairly high intensity.

The ESR signal of the triplet state of poly-L-tryptophan is the same as that of L-tryptophan with $\tau_{1/2} = 4.2$ s, but the number of paramagnetic centres per unit optical density is smaller by a factor of 2. The authors explained the decrease of the yield by concentration-induced quenching.

$\Delta m = \pm 1$ transitions. $\Delta m = \pm 1$ transitions in tyrosine were observed for the first time by Bosch et al.[35] Recently Zuclich[38] reported the observation of the $\Delta m = \pm 1$ ESR spectra for three aminoacids. Tryptophan was

excited in pure ethanol, tyrosine in 0.1 M HCl solution in ethanol, and phenylalanine and tyrosinate in 0.25 M NaOH solution in ethanol. The zero-field splitting energies X_1, X_2, X_3 and the zero-field splitting constants ($D = -3/2$ and $Z = -\frac{3}{2}X_\perp$) are listed in Table 7. Zuclich[38] adopted X_1, X_2, and X_3 (instead of X, Y, and Z) as the symbols for the zero-field splitting energies because in a specimen with a random orientation they cannot be assigned to specific molecular axes. $X_\perp$ is the zero-field splitting energy associated with the direction perpendicular to the plane of the molecule. $X_\perp$ values were determined by studying the line form of the ESR spectrum in polarised light.

Table 7. Parameters of the zero-field splitting of aromatic acids (cm^{-1}).

Compound	$\Delta m=\pm1$						$\Delta m=\pm2$
	X_1/hc	X_2/hc	X_3/hc	D/hc	E/hc	D^*/hc	D^*/hc
Tryptophan	0.0082	0.0656	0.0738	0.0984	0.0410	0.1213	0.1210
Tyrosine	0.0124	0.0867	0.0991	0.1301	0.0558	0.1621	0.1635
Tyrosinate ion	0.0180	0.0720	0.0900	0.1080	0.0540	0.1429	0.1432
Phenylalanine	0.0053	0.0930	0.0983	0.1475	0.0439	0.1659	0.1652

The transfer of triplet excitation energy in mixtures of aminoacids. This made it possible to identify the ESR signal of the triplet state in proteins and to understand the mechanism of the interaction of photoexcited molecules *in vivo*. The transfer of triplet excitation energy in a mixture of tyrosine and tryptophan in 6 M HCl has been investigated[68,96]. During the first seconds of illumination, two ESR signals of tyrosine and tryptophan were recorded ($\Delta m = \pm2$ transitions). The intensity of the signal due to tryptophan increased with time and that due to tyrosine fell compared with the one-component system under continuous illumination. The behaviour of aromatic dipeptides in the presence of tryptophan is similar[68]. According to Tables 6 and 7, the zero-field splitting constants for various aromatic acids differ markedly and therefore the ESR signals can be identified readily and unambiguously. The ESR data for the triplet state of individual aromatic aminoacids and particularly di- and poly-peptides made it possible to identify the triplet state of proteins.

Shiga et al.[66,68] were the first to investigate the excited triplet state of proteins by the ESR method and the energy transfer in proteins was discussed by Lhoste[52] and Zuclich[38]. Bovine serum albumin, egg albumin, β-lactoglobulin, D-aminoacid oxidase (the apo- and holo-enzymes), and native oxy- and deoxy-haemocyanines were investigated. In order to record ESR signals of low intensity, a spectrum accumulator was employed. In all cases[66,68] $\Delta m = \pm2$ transitions were observed. For all the above proteins, ESR absorption gave rise to the same H_{min} and line form as for the ESR signal of L-tryptophan. Thus the triplet signal originates from the tryptophan residues. The behaviour of D-aminoacid oxidase proved to be somewhat different. It is suggested[68] that in this protein, containing flavin-adenine dinucleotide (FAD), the ESR signal is caused not only by the triplet excitation of the aromatic aminoacid but also by a considerable interaction between the aromatic aminoacid in the excited triplet state and FAD. The holoenzyme gives rise to two different signals with $H_{min} = 1450$ and 1560 G, which are respectively due to tryptophan and FAD. Proteins have a significant influence on the triplet state of FAD, but the converse effect is not shown. While the characteristics of the ESR spectra of proteins are similar, the decay time $\tau_{1/2}$ depends on the nature of the protein and on the composition of the solvent. The values of $\tau_{1/2}$ are compiled in Table 8 on the basis of the data of Shiga et al.[68]

Table 8. The $\tau_{1/2}$ values for the triplet state of proteins at 77°K.

Protein	$\tau_{1/2}, s^{-1}$					
	I	II	III	IV	V	VI
Egg albumin	3.3	4.1	4.3	4.4	—	—
Bovine serum albumin	3.7	4.6	4.0	4.7	—	—
β-Lactoglobulin	3.9	4.0	4.7	4.8	—	—
D-aminoacid oxidase						
apoenzyme	2.4	—	—	—	—	—
holoenzyme	2.8	—	—	—	—	—
Native haemocyanin						
oxy-	—	—	—	—	1.7	3.4
deoxy-	—	—	—	—	4.8	4.4
Apohaemocyanin						
deoxy-	—	—	—	—	4.8	3.8

The following notation is employed in the Table: solvent I—buffer (pH 7; 0.1 M); solvent II—1 : 1 glycerol–buffer; solvent III—buffer + 8 M urea; solvent IV—1 : 1 glycerol–buffer + 8 M urea; solvent V—tris buffer (pH 6.8; 0.05 M; containing 0.01 M Ca^{2+}); solvent VI—1 : 1 glycerol–tris buffer.

Table 8 shows that $\tau_{1/2}$ increases in the presence of urea and when glycerol is added. The authors suggest that the nature of the environment of the tryptophan residues in the protein has a significant influence on $\tau_{1/2}$. On denaturation the environment changes and some of the tryptophan residues may pass to the solution. The activity spectra and the intensities of the ESR signals of various proteins are also different. It is believed[68] that these differences are due to the following causes:
(a) different distances between the tryptophan residues and their different orientations in different proteins;
(b) different distribution of tyrosine and phenylalanine residues between the tryptophan residues; (c) different degrees of dissociation of the protons in the peptide chain relative to the tryptophan residues; (d) different environments of the tryptophan residues.

However, Zuclich's latest measurements[38] showed that in human and bovine serum albumin (in an ethylene glycol–phosphate buffer at pH 7) two ESR signals of the triplet state ($\Delta m = \pm2$ transitions) are observed. The signals undergo neither broadening, nor displacement, nor a change in the zero-field splitting constant. The relative intensities of the tryptophan signals in the three proteins investigated (human and bovine serum albumin and ovalbumin) were respectively 1 : 1 : 2. The tyrosine signal was four times more intense in human serum albumin than in bovine albumin and was completely absent from ovalbumin. This is consistent with the finding that the degree of tyrosine → tryptophan energy migration increases with the relative concentration of tryptophan. Since the tyrosine content in the albumins is the same, the author attributes the differences between the intensities to the differences between the contents of tryptophan residues

(one and two tryptophan molecules per molecule of human and bovine serum albumin respectively). In this case at least 75% of the tyrosine molecules in bovine albumin are quenched by the two tryptophan residues. The author [38] calculated the minimum radius R_0 of the region of energy transfer from tyrosine to tryptophan in bovine serum albumin and obtained $R_0 = 20$ Å. The $\Delta m = \pm 1$ ESR spectrum was observed only for ovalbumin [38] and proved to be wholly due to tryptophan residues.

4. Flavins and Their Derivatives

Flavosemiquinones are known as intermediate electron-transfer agents in the respiratory chain of mitochondria and chloroplasts. The study of the triplet state by the ESR method in solid solutions and the determination of the zero-field splitting constants made it possible to describe qualitatively the properties and distribution of electron density in the triplet state of these complex molecules [16,52,71].

Table 9. Characteristics of the ESR spectra of flavins and pteridines in the triplet state at 77°K ($\Delta m = \pm 2$ transitions; $\nu = 9210$ MHz).

Compound	H_{min}, G	D^*/hc, cm^{-1}	Solvent
Isoalloxazine compounds			
Riboflavin	1602	0.062	Ethanol
Lumiflavin	1602	0.062	ditto
Tetra-acetylriboflavin	1603	0.061	»
3-Benzyl-lumiflavin	1602	0.062	»
o-(2)-Methyl-lumiflavin	1596	0.066	»
2-Thiolumiflavin	1596	0.066	»
4-Phenyliminolumiflavin	1599	0.064	Ethanol + dimethylformamide
8-Morpholino-8-norlumiflavin	1585	0.072	Ditto
Neutral FMN	1602	0.062	Propylene glycol + H_2O
Anionic FMN	1602	0.062	Propylene glycol + NaOH
Cationic FMN	1582	0.074	Propylene glycol + HCl
Alloxazine compounds			
Lumichrome	1578	0.077	Ethanol
1,3-Dimethyl-lumichrome	1581	0.075	ditto
1,3-Dimethylalloxazine	1575	0.078	»
Pteridine derivatives			
Lumasine	1564	0.086	»
1,3-Dimethyl-lumasine	1569	0.083	»
8-Methyl-lumasine	1578	0.079	»
6,7-Dimethyl-4-oxopteridine	1535	0.098	Ethanol + dimethylformamide
2-Amino-6,7-dimethyl-4-hydroxypteridine	1559	0.088	ditto
2-Ethylthio-4-hydroxypteridine	1557	0.089	»
2,4-Diamino-6,7-dimethylpteridine	1537	0.097	»
2,4,7-Triamino-6-phenylpteridine	1538	0.097	»

The ESR spectra of the excited triplet state associated with the $\Delta m = \pm 1$ and $\Delta m = \pm 2$ transitions were recorded for substituted, tautomeric, and reduced forms of flavins and pteridines, including flavin mononucleotide (FMN), FAD, and riboflavin. The most complete data about the properties of the ESR spectra of the triplet states of flavins and peridines were obtained by Lhoste et al. [52] Table 9 gives the characteristics of the ESR spectra associated with the $\Delta m = \pm 2$ transitions. A typical ESR spectrum of the triplet state ($\Delta m = \pm 2$) for $H_{const} \perp H_{rj}$ is presented in Fig. 9*A*. The distribution of resonance fields in the region 1500–1700 G ($\nu = 9210$ MHz) is shown below (Fig. 9*B*).

At 77°K, $\Delta m = \pm 1$ transitions were observed in a concentrated ethanol solution or a propylene glycol—water-hydrochloric acid mixture. The spectrum of the FMN cations is presented in Fig. 9*A*. The parameters of the ESR spectra are listed in Table 10. The line shape of the triplet absorption in the region of $\Delta m = \pm 1$ is unusual and corresponds to equal energies of the two sublevels of the triplet level, which are comparable in absolute magnitude but have different signs. For this reason the corresponding ESR lines merge. The narrow $H_{t.q.}$ line in the spectrum corresponds to a two-quantum transition. The $\Delta m = \pm 1$ spectrum of the cation exhibits a low symmetry of the triplet state owing to the effect of the charge.

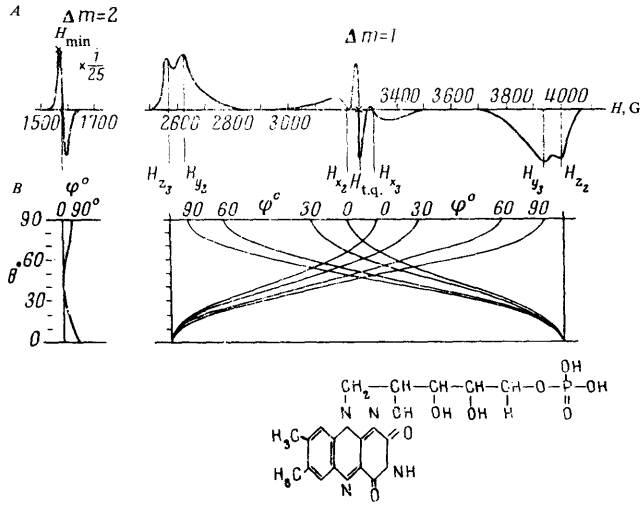


Figure 9. *A*) ESR spectrum of the triplet state ($\Delta m = \pm 2$, ± 1 transitions; $\nu = 9210$ MHz) of the cationic form of flavin mononucleotide in water–propylene glycol–6 *N* HCl solution at 77°K; *B*) distribution of resonance fields.

It is significant that the triplet state of the FMN anions does not differ from that of the neutral form either in the properties of its phosphorescence or in the parameters of its ESR spectrum. The triplet state of the cationic form is significantly different from the two preceding ones. In particular H_{min} is displaced by 20 G towards higher fields and the spin–spin interaction energy is higher (Tables 9 and 10).

Compared with other bicyclic (naphthalene, $D^* = 1.0030$ cm^{-1}) and polycyclic aromatic compounds [28], the zero-field splitting constants of pteridines and flavins are lower, which indicates a greater delocalisation of the excited-triplet electrons in the molecule. Another consequence of the lower values of the constants is that the triplet state is due to a $\pi-\pi^*$ transition and not $n-\pi^*$. For the $\pi-\pi^*$ triplet state, the energy of the molecule associated with the axis perpendicular to the molecular plane is negative. This highest value of the energy determines D/hc (Table 10). Two other values of the energy of the splitting of the triplet level in the absence of a field, associated with the axes in the molecular plane, determine E/hc. E/hc is small for pteridines and flavins.

Analysis of the data in Tables 9 and 10 leads to the following findings: (a) the nature of the substituent in the 10-position of flavins does not influence the zero-field

splitting constants, with the sole exception of tetraacetylriboflavin; (b) alkylation (3-benzyl-lumiflavin) or ionisation in the N_3 position (anionic form of FMN) do not alter the constants but alkylation in the 2-position, corresponding to the flavoquinone form, increases D^*/hc. The introduction of sulphur in the 2-position (2-thiolumiflavin) does not affect D^*/hc, but decreases the lifetime of the triplet state; (c) the alloxazine–isoalloxazine tautomerism decreases D^*/hc. A similar effect obtains with pteridine derivatives, compared with, for example, 1,3-dimethyllumazine and 8-methyl-lumazine; (d) the benzenoid ring plays a definite role in the delocalisation of electrons. D^*/hc for flavins is smaller by approximately 0.2 cm^{-1} than for pteridines. This shows that electrons in the triplet state are delocalised over the benzenoid ring.

Table 10. Characteristics of the ESR spectra of the triplet state of flavins and alloxazine compounds in concentrated solutions at 77°K ($\Delta m = \pm 1$ transitions; $\nu = 9210$ MHz).

Compound	X/hc	Y/hc	Z/hc	D/hc	E/hc	D^*/hc
	cm^{-1}					
3-Benzyl-lumiflavin	±0.0034	±0.0336	±0.0369	±0.0553	∓0.0150	0.0611
Cationic FMN	±0.0034	±0.0404	±0.0440	±0.0660	∓0.0185	0.0733
Tetra-acetylriboflavin	±0.067	±0.0309	±0.0376	±0.0564	∓0.0121	0.0602
Lumichrome	±0.0035	±0.0424	±0.0460	±0.0690	∓0.0194	0.0768
1,3-Dimethyl-lumichrome	±0.0026	±0.0415	±0.0440	±0.0660	∓0.0194	0.0741
1,3-Dimethylalloxazine	±0.075	±0.0406	±0.0481	±0.0721	∓0.0165	0.0776

Substituents in the 6- and 7-positions of the pteridine rings do not influence D^*/hc. The marked effect of various substituents in the 7- and 8-positions of the flavin ring (1,3-dimethyl-lumichrome has a smaller D^*/hc than 1,3-dimethylalloxazine) is evidence of an appreciable spin density in these positions. The introduction of CH_3 or Cl substituents in the 7- and 8-positions affects significantly the photochemical behaviour of flavins. Different substituents in the N_5 position quench the triplet state (5-acetyl-leucoflavin and 5-isopropyl-leucoflavin). A similar effect is observed for the 10-substituted derivatives Leucoflavins phosphoresce but do not give rise to ESR absorption. Reduced FMN does not show an ESR absorption either. According to Lhoste[52], the molecular skeleton of these species is non-planar in the lowest triplet state. This leads to a change in hybridisation of the N_5 orbitals. The triplet state can have a lower symmetry, which increases the spin–orbital coupling, reduces τ, and at the same time increases D^*/hc, making it impossible to observe the triplet absorption in the 3-cm range.

Thus the spin density of the triplet state of flavins is mainly localised in the quinoxaline part of the molecule. Characteristic features of these substances are a low spin density at the N_3 atom, a high density at N_5, N_1, and N_{10}, and the non-equivalence of the methyl groups in the 7- and 8-positions. These data are important in a consideration of the photochemical properties and biological functions of flavins.

5. Nucleic Acids, Purine and Pyrimidine Bases, and Nucleotides

Nucleic acids are high-molecular-weight compounds consisting of purine and pyrimidine bases, carbohydrate residues, and phosphate groups. Purine and pyrimidine bases enter into the composition of not only nucleic acids but also of a number of low-molecular-weight compounds which play an important role in vital processes, ranging from respiration to muscular contraction. Adenine play a special role in biology. In particular, adenine is a component of adenosine triphosphate (ATP), a kind of energy accumulator in a living system.

The ESR of the triplet state of nucleic acids and their constituent purine and pyrimidine bases, nucleotides, and nucleosides was investigated by Rahn, Shulman, Eisinger, and coworkers [35,36,72–77] and also by Hélène and coworkers [15,78,79]. At a neutral pH in an ethylene glycol–water solution, adenine, adenosine, guanine, guanosine, and their monophosphates give rise to powerful ESR signals ($\Delta m = \pm 2$ transitions)[72]. Under these conditions, cytosine monophosphate gives rise to only a weak phosphorescence, while thymine monophosphate does not yield either phosphorescence or an ESR signal. The zero-field splitting constants and the values of τ are listed in Table 11.

Table 11. ESR characteristics of the triplet state of certain nucleic acid derivatives.

Compound	D/hc, cm^{-1} $\Delta m=\pm1$	E/hc, cm^{-1} $\Delta m=\pm1$	D^*/hc, cm^{-1} $\Delta m=\pm2$	$\tau_{1/2}$, s
Adenine			0.126	2.6
Adenosine			0.126	2.6
Adenosine monophosphate, pH 7	0.121	0.027	0.126	2.5
Guanine			0.145	1.26
Guanosine			0.145	1.26
Guanine monophosphate	0.141	0.0127	0.145	1.25
pH 7				
pH 1			0.126	0.37
Thyminate ion, pH 12			0.198	0.50
Thymine monophosphate			0.198	0.45
Uridinate ion, pH 11			0.163	0.40
Orotic acid				
pH 7	0.176	0.016	0.184	0.30
pH 12			0.175	
Poly-dAT, pH 7			0.201	0.032
DNA			0.200	0.29

The triplet state ESR signal was not observed for native DNA at pH 7, although small signals due to the triplet state of guanine and adenine were recorded for denatured and degraded DNA [37,76]. At pH 12 (under these conditions thymine monophosphate ionises with a loss of a proton from the N_3 atom) the ESR signal of ionised thymine monophosphate (T^-) was observed at $H_{min} = 1067$ G for a decay time of 0.45 s, which is close to τ_{phosph} for DNA (0.3 s). The T^- signal is separated from the adenine signal by approximately 300 G and can be readily identified. A signal, analogous to that of thymine, was observed in poly-dAT at $H_{min} = 1046$ G ($\nu = 9104$ MHz) and with $\tau = 0.32$ s (see Table 11).[73]

A dilute mixture of guanine and cytosine monophosphate gives rise to an ESR signal of the triplet state of guanine monophosphate and a mixture of adenine and thymine monophosphates gives a triplet signal due to adenine monophosphate only. However in DNA there are no signals of the triplet state of the purine bases. Their triplet state is either not populated or it is populated and quenched. It is also possible that the energy of the triplet state is effectively transferred to thymine. An ESR signal was observed for native DNA from bovine thymus on ultraviolet irradiation in the same fields ($H_{min} = 1054$ G, $\Delta H = 50$ G, and $\tau = 0.29$ s) [37,72,73] as for the ionised form of thymine and poly-dAT.

$\Delta m = \pm 1$ transitions were observed together with $\Delta m = \pm 2$ transitions in frozen solutions of orotic acid and guanosine and adenosine monophosphates. The calculated constants are listed in Table 11, the analysis of the data of which shows that the zero-field splitting constants for purine and pyrimidine derivatives are significantly different. On the other hand, the constants also differ for the ionic and non-ionic forms of the bases. Rahn and coworkers [75,76] suggested that the DNA triplet is localised at a thymine residue, which is ionised when its labile proton is transferred via the hydrogen bond to adenine. The equality of the ESR absorption fields serves as an argument in favour of this hypothesis. The small difference between the values of τ is more likely to be due to a slight difference in the environment. That the DNA triplet originates from thymine was also confirmed by a detailed study of various synthetic nucleic acids and model polymers [72,73]—in all cases triplet absorption was shown by polymers containing thymine. Such unambiguous identification could not be made on the basis of spectroscopic data, since the phosphorescence of DNA is different from the phosphorescence of both individual nucleotides and their sum in solution.

6. Phenoxazine and Phenothiazine

Phenoxazine and phenothiazine belong to the class of photosynthesising dyes and also find wide application in pharmacology. They are tricyclic aromatic compounds with C_{2v} symmetry. Phenoxazine and phenothiazine readily form mixed single crystals with biphenyl; they have been investigated not only in solutions but also in single crystals.

Table 12. Parameters of the ESR spectra of the triplet state of phenoxazine and phenothiazine.

ESR constants	Phenoxazine		Phenothiazine	
	biphenyl crystal	ethanol glass	biphenyl crystal	ethanol glass
D/hc	± 0.0990	0.1249	± 0.1230	0.1237
E/hc	± 0.0154	0.0119	± 0.0142	0.0135
X/hc	± 0.0482	0.0535	± 0.0270	0.0278
I/hc	± 0.0175	0.0297	± 0.0553	0.0547
Z/hc	$+0.0659$	0.0833	± 0.0820	0.0825
g_{xx}	2.0037	—	2.0042	—
g_{yy}	2.0037	—	2.0045	—
g_{zz}	2.0020	—	2.0014	—

A study of the triplet state of phenoxazine and phenothiazine by the ESR method was made comparatively recently by Lhoste and coworkers [52,80]. The ESR spectra associated with the $\Delta m = \pm 1$ and $\Delta m = \pm 2$ transitions are presented in Fig. 2. As expected, the spectrum for a molecule with C_{2v} symmetry has six canonical peaks and an $H_{t.q.}$ line in the region of $\Delta m = \pm 1$. The line width of the H_X and H_Y canonical peaks is about 25–30 G, while that of the H_Y peak is about 15 G (measured between the points of maximum slope). They study of a single crystal of phenoxazine in biphenyl made it possible to assign the peaks unambiguously to the molecular system of axes, as shown in Fig. 2. In parallel fields, the canonical H_{X_1} and H_{Y_1} peaks observed were displaced from H_{min} (Fig. 2A). Owing to its low intensity, the H_{Z_1} peak was not recorded. The parameters of the ESR spectra are listed in Table 12. The signs of the constants given are arbitrary, but according to McLachlan [81], D/hc for the triplet $\pi-\pi^*$ state is positive.

The surprising difference between the zero-field splitting energies of phenoxazine in the biphenyl and ethanol matrices is noteworthy. It may be due to different polarities of the solvents, their capacity to form hydrogen bonds, and finally to small differences in their geometry in solid solutions. In particular, in a biphenyl crystal these molecules can remain planar, while in ethanol they may become slightly bent. The latter leads to a change in the geometry of the π-electron cloud and the corresponding values of the D^*/hc constants. Phenothiazine has a high tendency towards bending and therefore its zero-field splitting energy changes little on passing from one matrix to another (in a single crystal it is also bent) [52]. The bending of the benzene rings of the molecule is deduced in particular from calculations of the dipole moment and the electronic structure of phenothiazine free radicals [80].

The study of the hyperfine structure of the ESR spectrum of the triplet state of phenoxazine and phenothiazine single crystals made it possible to assign the H_X peak to the long molecular axis. Of the other two peaks, the peak with a lower energy (H_Y in Fig. 2) was assigned to the short molecular axis (y) passing through the O–N atoms and the peak with the highest energy (H_Z) was assigned to the z axis perpendicular to the molecular plane. Additional studies in polarised light showed [80] that in a concentrated solution the lowest triplet state of phenoxazine is populated via S_0-S_1 excitation, the transition moment of which is parallel to the y axis. However, since in solution the molecule can be bent, the magnetic y axis cannot coincide exactly with the direction of the N–O bond. This is also confirmed by studies of the hyperfine structure of the spectra of phenoxazine free radicals [52], since the hypothesis of the sp^3 hybridisation of the nitrogen atom gives a better explanation of the experimental results than the hypothesis of sp^2 hybridisation corresponding to a planar molecule.

7. Coumarin and its Derivatives

Coumarin and related compounds are known in biology as uncoupling agents for mitochondrial oxidative phosphorylation and as anticoagulants (dicoumarol). The triplet state of coumarin and its derivatives was observed for the first time by Graber et al. [82] The spectrum of coumarin in ethanol at 77°K is presented in Fig. 10 ($\Delta m = \pm 1$ transitions). The position of the H_{X_3} peak was calculated since its measurement is hindered by the superposition of the spectrum due to the ethanol radical. The narrow line at 3201 G corresponds to $H_{t.q.}$ The zero-field splitting energies and constants and the values of τ are listed in Table 13. Except for 6-substituted coumarins, the zero-field splitting energies are intermediate between the values for benzene and naphthalene [2]. This and also the high value of τ show that the triplet state of coumarin and its derivatives is of a $\pi-\pi^*$ type. Graber et al. [82] chose for coumarin the same designation of the axes (Fig. 10) as for naphthalene [1,2]. Qualitatively this is justified by the fact that the energy of the zero-field splitting of the triplet level for planar $\pi-\pi^*$ molecules, associated with the z axis, is negative [81]. Two other energies characterise the distribution of spin density in the molecular plane. Table 13 shows that the values of D^*/hc for coumarin and

its monosubstituted derivatives are higher than for polysubstituted derivatives. This is evidence of a greater delocalisation of spin density in the latter. On the other hand, chromanone and chromanol, which contain one unsaturated ring, have still higher values of D^*/hc, which are comparable with that of benzene (0.193 cm^{-1}).

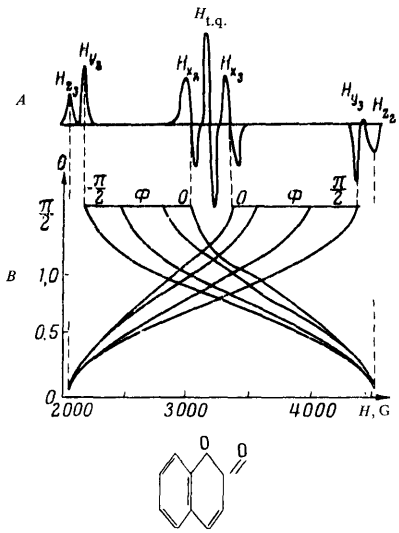


Figure 10. *A*) ESR spectrum of coumarin in the triplet state ($\Delta m = \pm 1$ transition) in ethanol at 77°K (ν = 9239 MHz); *B*) distribution of resonance fields.

Table 13. Parameters of the ESR spectra of the triplet state of coumarin and its derivatives.

Compound	X/hc	Y/hc	Z/hc	D^*/hc	τ, sec.
Coumarin	0.0094	0.0680	−0.0774	0.1244	0.54
4-Hydroxycoumarin	0.0137	0.0680	−0.0817	0.1301	1.5
7-Hydroxycoumarin	0.0147	0.0642	−0.0788	0.1177	1.1
6-Methylcoumarin	0.0026	0.0661	−0.0636	0.1124	0.6
Aesculine (6β-glucosido-7-hydroxycoumarin)	0.076	0.0600	−0.0524	0.0980	1.5
7-Nitro-3,4-benzocoumarin	—	—	—	0.0870	2.3
4-Chromanone	—	—	—	0.1584	1.8
4-Chromanol	—	—	—	0.1354	0.9
Dicoumarol	0.0172	0.0680	−0.0807	—	1.3

The excited triplet state of dicoumarol in low and high fields was observed by Gull et al.[83] in an ethanol–dimethylformamide mixture at 77°K. The $\Delta m = \pm 1$ ESR spectrum consists of six canonical peaks (molecular axes $x \neq y \neq z$). In the $\Delta m = \pm 2$ region a more intense peak with H_{min} = 1468 G (ν = 9239 MHz) was observed. The decay time τ of the ESR signal at H_{min} was 1.3 s after switching off the light (Table 10), which agrees with the phosphorescence decay time. The zero-field splitting energies calculated from the spectra are listed in Table 13.

Thus we have attempted to give as far as possible complete information about the characteristics of various classes of biologically active compounds in a triplet excited state. Summarising, we emphasise yet again that the ESR method yields the greatest amount of information, particularly in studies on large biological molecules, such as those of DNA and proteins, where the triplet states have been identified, and also for compounds which show weak phosphorescence. Next we shall discuss the role of the excited triplet state in the photochemistry of these molecules.

III. THE TRIPLET STATE IN THE PHOTOCHEMISTRY OF BIOLOGICALLY IMPORTANT MOLECULES. TWO-QUANTUM REACTIONS

1. Photochemistry of Porphyrins in Solid Solutions

When ethanol solutions of porphyrins are illuminated at 77°K with light corresponding to the long-wavelength absorption maxima of the pigments, a triplet state ESR signal arises, whose intensity persists during the period of irradiation. The formation of free-radical products is not observed. However, when solutions of porphyrins are irradiated with the unfiltered light of a mercury lamp, the intensity of the ESR signal of the triplet state falls and at the same time free-radical products accumulate[8,9,32]; they have been identified[11] as the radical products of the decomposition of ethanol ($CH_3\dot{C}HOH$, $\dot{C}H_3$, and $\dot{C}HO$) and the porphyrin radical. With increased concentration of the pigment, the yield of radicals rises. The use of calibrated photometric grids showed that the concentration of ethyl[8,32,42] and pigment[48,50] radicals varies with the square of the intensity of the incident light. Therefore it was suggested[32] that the process has a two-quantum mechanism and that free radicals are formed via a triplet state, the second quantum for porphyrins being greater than the exciting quantum.

Simultaneously with the accumulation of free radicals, the absorption spectrum of the pigment undergoes significant changes. In particular, in the spectrum of Mg-aetioporphyrin[8,42,84,85] the intensities of the initial absorption band at 542 and 578 nm and of the Soret band fall (virtually to zero in a dilute solution after prolonged illumination) and a new absorption arises at 450, 605, and 686 nm. When the temperature of an irradiated specimen is gradually raised, the 686 nm absorption vanishes, the 450 nm peak diminishes, and new bands arise between −150° and −125°C with maxima at 660 and 750 nm, the intensity of which also falls to zero on further heating and the spectrum is regenerated. In order to demonstrate that the photochemical reaction proceeds via a triplet state, the authors of the review investigated the effect of intermittent illumination (with light pulses of a duration shorter than that required for the establishment of phosphorescence) on the yield of free radicals in an ethanol solution of Mg-aetioporphyrin, Mg-mesoporphyrin, and Zn-tetraphenylporphyrin[19]. The dark intervals lasted 1–2 s, which, for an exponential phosphorescence decay law, corresponds to virtually complete decay of the glow. It was shown that, under interrupted illumination, the yield of radicals (the areas under the integral curves were compared) is lower by a factor of 15–25 (depending on the duration of the light pulse) then under continuous illumination of the same integral intensity (Fig. 11). This finding is satisfactorily consistent with calculated values[19].

In order to demonstrate directly that the process has a two-quantum mechanism, a method involving the illumination of the solution by two independent light sources was employed[7]. As already stated, the illumination of the solutions with yellow light did not result in the accumulation

of radical products at 77°K. Irradiation of the specimens with ultraviolet light (between 365 and 248 nm) also does not lead to the formation of free radicals in appreciable concentrations[16]. Under simultaneous illuminations by two independent sources, one of which excites the molecules to the triplet state and the light of the other ($\lambda \leqslant$ 320 nm) is absorbed by the pigment molecule in the excited triplet state, free radicals of the porphyrins and the matrix accumulate (Fig. 12A). The yield of radicals increases with the energy of the second quantum. It was also shown that in this case the yield of radicals increases in direct proportion to the intensities of both the exciting (578 nm and the additional) light (302 and 248 nm). Analogous evidence for the two-quantum process mechanism was obtained in a study of the variation of the absorption spectrum of Mg-aetioporphyrin[86]. Initially the authors showed that, when an ethanol solution of Mg-aetioporphyrin is illuminated at 77°K with light having a wavelength of 578 nm, its absorption spectrum does not change. Also the authors did not observe measurable changes in the absorption spectrum of Mg-aetioporphyrin on illumination with ultraviolet light alone (λ_2 = 365, 320, and 313 nm).

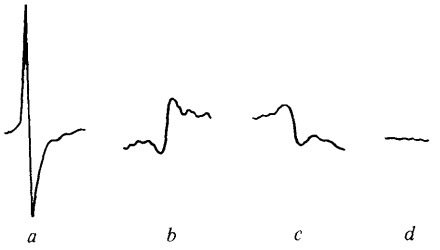


Figure 11. Yield of free radicals in alcoholic solution of Mg-aetioporphyrin as a function of illumination: *a*) with continuous light under steady-state excitation conditions for 30 s; *b*)–*d*) with intermittent illumination for 55 min with periods of exposure to light of 1.8×10^{-2} s, 9×10^{-3} s, and 6×10^{-3} s respectively.

Under simultaneous illumination by the total quanta ($h\nu_1 + h\nu_2$), the initial absorption bands of Mg-aetioporphyrins at 542 and 578 nm become less intense and a new absorption arises at 686 nm (Fig. 12*B*). The latter confirms that both radical products and the new 686 nm absorption arise by a two-quantum mechanism via the triplet state and that the product absorbing at 686 nm is a radical. Similar results were obtained for the dications of haematoporphyrin and protoporphyrin. When the temperature is raised to −165°C and above, yellow light is sufficient for the formation of Mg-aetioporphyrin radicals. The free radicals recorded give rise to only one chromophore line[11,42], the yield of radicals being again proportional to the square of the incident light intensity.

The kinetics of the formation of ethyl and pigment radicals have been investigated[48–50,93]. The logarithmic form of the kinetic curves for the destruction of triplet molecules and the accumulation of ethyl and porphyrin radicals can be fitted fairly satisfactorily by two intersecting lines. The observed photochemical process can be treated kinetically as the sum of two first-order reactions having rate constants k_1 and k_2 respectively, with $k_1 = 10k_2$. It was shown that the formation of one ethoxy-radical corresponds to one porphyrin radical[49,50] at a low light intensity. In order to demonstrate the reductive nature of the reaction, a study was made of the effect of electron-accepting additives (O_2, riboflavin) on the kinetics. It was found that electron-accepting impurities diminish the rate of destruction of molecules in the excited triplet state and the formation of radical products, the constant k_1 showing a much stronger dependence on O_2 and riboflavin than k_2.

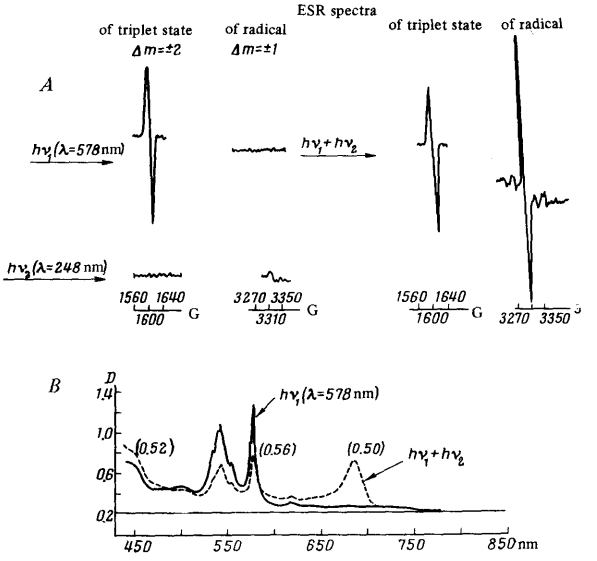


Figure 12. Effect of the two-quantum mechanism. Formation of free radicals in ethanol solution of Mg-aetioporphyrin on illumination by one ($h\nu_1$) and two ($h\nu_1 + h\nu_2$) light sources. *B*) formation of the 686 nm product under the same conditions.

It is noteworthy that, on illumination with visible light at 77°K, an appreciable change in the yield n_{st} of triplet molecules as a function of the oxygen content of the specimen was not observed. However, τ is also independent of the oxygen content of the specimen at 77°K[47]. Consequently the mechanism of the action of oxygen does not include direct quenching of the triplet state of porphyrins at 77°K. The observed inhibition of the formation of ethanol and porphyrin radicals in the presence of oxygen is consistent with the available data concerning the inhibition by oxygen of the photochemical reduction of tetrapyrrole pigments[59]. On the whole, the data permit the conclusion that the mechanism of the photochemical reaction of porphyrins in solid solutions at 77°K involves electron transfer from the alcohol to the porphyrin molecule in the excited triplet state. This results in the formation of a porphyrin radical-anion, which can then combine with a proton, forming a porphyrin radical—the semireduced form. The "686 nm" form of Mg-aetioporphyrin is a porphyrin radical-ion.

Thus the photochemical reduction reactions of porphyrins at 77°K in solid solutions proceed via the triplet state and involve a two-quantum mechanism. Although

the results have been obtained for model systems and low temperatures (the two-quantum reaction in PMT takes place at room temperature[48,49]), processes of this kind must be taken into account in a consideration of the mechanism of real photobiological processes, in particular photosynthesis.

2. Photochemical Properties of the Triplet State of Flavins

When ethanol solutions of flavins (riboflavin, lumichrome, lumazine) and pteridines are irradiated with ultraviolet light ($\lambda > 310$ nm) at 77°K, a complex ESR signal due to free radicals is observed, increasing with the duration of illumination[16]. Analysis of the contour of the signal formed in the ethanol flavin solution showed that it is due to ethyl radicals, $CH_3\dot{C}HOH$, and a species giving rise to a single line with a mixed g-factor. The shape of the single line and its g-factor resemble those of the ESR spectrum of the radical formed in aqueous solution of flavin mononucleotide. The relative intensities of the ESR lines of the ethyl and flavin radicals remain constant regardless of the duration of irradiation and light intensity. The rates of formation of the radicals are the same.

In order to elucidate the reaction mechanism, a study was made of the effect of light intensity on the kinetics of the accumulation of free radicals and the destruction of flavin molecules in an excited triplet state. It was found that the concentration of free radicals at any instant is proportional to the concentration of molecules in the excited triplet state for various light intensities and it was shown that the reaction has a two-quantum mechanism and proceeds via the higher triplet state of flavins and pteridines.

Similar behaviour of riboflavin was investigated by Azizova et al.[87] in the sensitised photochemical decomposition of ethanol and aliphatic aminoacids at 77°K. On illumination with visible light exciting riboflavin to a triplet state, sensitised deamination of aliphatic aminoacids, the spectra of which lie in the range 180–220 nm, was observed. It was demonstrated by the method of intermittent illumination that the reaction proceeds via the triplet state of riboflavin.

3. The Excited Triplet State in the Photochemistry of Nucleic Acids and Their Bases

In 1964 Pershan et al.[88] investigated the mechanism of the formation of thymine free radicals under the influence of ultraviolet light. In particular, it was shown that the concentration of free radicals formed in DNA on ultraviolet irradiation falls when cobalt(II) ions are added. On the other hand, Bersohn and Esenberg[89] showed that the ions of bivalent paramagnetic metals cause marked quenching of the phosphorescence of nucleotides and nucleosides. Later Rahn et al.[75] showed that bivalent metals quench vigorously the triplet state of DNA. In particular, at a fairly high concentration of manganese ions, the ESR signal of the triplet state falls almost to zero. Under these conditions, the DNA fluorescence is not quenched. It was also shown that the number of DNA free radicals formed is comparable to the concentration of the molecules in the excited triplet state. Since the quenching of the DNA triplet state entails a decrease of the yield of thymine radicals in the same proportion, one may assume that the thymine triplet state precedes the formation of free radicals. Thus the formation of thymine free-radical products in DNA proceeds via the excited triplet state.

In a study of quenching of the triplet state of thymine and of uracil in acetonitrile solution, Lamola and Mittel[90] showed that the photodimerisation of thymine takes place wholely via its triplet state. The photodimerisation of uracil proceeds via two mechanisms, one of which also involves the triplet state.

Finally, we shall consider in somewhat greater detail the two-photon reactions of nucleic acid derivatives: purine, adenosine, and guanosine in alkaline aqueous and ethanol solutions at 77°K. These reactions were investigated for the first time by Hélène and coworkers[15,79], who showed that, when the compounds are irradiated with ultraviolet light ($\lambda > 250$ nm) in alcoholic and aqueous solutions, the intensity of the phosphorescence and the ESR signal of the triplet state falls, but the fluorescence intensity remains unchanged. The rate of quenching of phosphorescence is proportional to the square of the incident light intensity. This relation remains unchanged within the concentration range 10^{-4}–10^{-3} M. After unfreezing and subsequently refreezing the specimen, the intensity of its phosphorescence is restored to the extent of 90%. When the phosphorescence is almost completely quenched, the optical absorption spectra remain unchanged. Very large irradiation doses result in the appearance of a new absorption in the range 300–350 nm, which is particularly distinct for purine.

At the same time, when aqueous solutions of purine bases are irradiated with ultraviolet light, a single ESR signal is observed with a g-factor close to 2. On subsequent irradiation with visible light, the signal amplitude falls by 15–20%. In 2% ethanol solution, the ESR spectrum of ethyl radicals is observed together with the singlet spectrum. The rate of formation of the radicals is higher by a factor of 30 than in pure ethanol. Subsequent illumination with visible light leads to a decrease of the singlet ESR signal. The authors showed that the rate of formation of ethyl radicals at any time after the start of illumination is proportional to the instantaneous concentration of the excited triplet molecules and to the square of the incident light intensity.

Thus in aqueous solutions purine bases are ionised with formation of a purine cation and an electron. Under the influence of visible light, the solvated electrons are bleached, liberated from traps, and undergo secondary interactions with cations and neutral purine and solvent molecules. In ethanol solutions two competing reactions develop—photosensitation and light-induced removal of an electron, the first predominating. According to the authors, photosensitation takes place at a high excited triplet level and does not involve the decomposition of the purine, since the absorption and fluorescence spectra remain unchanged. The base is ionised by a two-quantum mechanism. Since the sum of the energies of the triplet level (3.4 eV) (purine, adenosine, guanosine) and of the second quantum ($\lambda = 250$ nm; 5 eV) corresponds to an energy of 8.4 eV, it is sufficient to ionise the molecule in the solid phase (the ionisation potential in the gas phase is known to be about 9 eV).[79] The behaviour of pyrimidine bases and their nucleosides (cytosine, cytidine, thymine) is analogous, but the rate of accumulation of radicals is lower by a factor of 5–6 than for purine bases. Visible light does not affect the ESR signal. In the presence of ethanol, only the radicals of pyrimidine bases are formed and even after prolonged illumination ethyl radicals are

absent. Evidently pyrimidine bases are less sensitive to short-wave radiation and, when account is taken of energy transfer from purine to pyrimidine bases, this fact may help our understanding of the characteristics of the photochemistry of nucleic acids and their metabolism.

4. Two-Quantum Reactions of Aromatic Aminoacids

The first communication about the decomposition of aliphatic compounds photosensitised by aromatic aminoacids appeared in 1963.[32] The mechanism of the photochemical reaction was then investigated in detail by the same workers[11,42,85]. When ethanol solutions of aromatic aminoacids were illuminated at 77°K with the unfiltered light from a mercury lamp, the ESR signal of the excited triplet state fell and rapid accumulation of free-radical products was observed. The rate of accumulation was proportional to the concentration of aminoacids in solution, the square of the incident light intensity, and the intensity of the ESR triplet absorption. Ptak, Douzou, and coworkers[14,15] recorded the formation of free-radical products under analogous conditions.

Analysis of the ESR spectra showed[11,42] that the radicals formed are decomposition products of ethanol (ĊHO, ĊH$_3$, CH$_3$ĊHOH) and an aromatic aminoacid (a single line with a g-factor close to that of the free electron). The characteristics of the latter product (the radical is stable up to −143°C) are analogous to those of the radicals formed on irradiation of aqueous solutions of aromatic acids with ultraviolet light[91]. Since a correlation was observed between the kinetic curves for the destruction of the aminoacid molecules in an excited triplet state and the formation of CH$_3$ĊHOH and the aminoacid radicals (the slopes of the curves on a logarithmic scale are the same, but after prolonged irradiation the concentration of ethyl radicals increases faster owing to secondary processes), Gribova[32] suggested that the triplet state of the aminoacid is involved in the photochemical reaction (a similar process occurs in methanol and in EPA). This was demonstrated unambiguously by the method of interrupted illumination[8,9]. Santus and coworkers[15,79] observed two-quantum ionisation of aromatic aminoacids in boric acid. The two-quantum ionisation and two-quantum photosensitised decomposition of aliphatic aminoacids in the presence of aromatic aminoacids was demonstrated in a number of studies by Fesenko using the method of flash photolysis[12]. Here it is important to emphasise that, in the presence of aromatic aminoacids or dyes, which are excited by an energy much lower than the ionisation energy of aliphatic aminoacids, the latter are decomposed when the quanta are added together at the triplet level of the sensitiser.

Thus it is evident from the foregoing that the ESR method has been successfully employed in the study of complex biologically important molecules in an excited triplet state. It is also clear that the triplet state is important in chemical reactions and in the accumulation and transfer of energy in model systems *in vitro*. It is difficult to arrive at a direct conclusion concerning the involvement of the excited triplet state in photochemical processes *in vivo* on the basis of data obtained for model systems, owing to the extreme complexity of real biological structures and processes. However, the structural organisation of molecules, resembling the DNA double spiral, the close packing of protein molecules, or the structure of chloroplasts, is similar to the molecular arrangement in solid solutions. Therefore we believe that the study of the mechanism of reactions at a molecular level in simplified model systems (solutions of biologically important compounds) will permit a deeper understanding of the physical and chemical processes occurring in real biological systems.

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