Triplet States in DOPA-Melanin and in Its Complexes with Kanamycin and Copper Cu(II) Ions

L. Najder-Kozdrowska[a,*], B. Pilawa[b], E. Buszman[c], A.B. Więckowski[a], L. Świątkowska[d], D. Wrześniok[c] and W. Wojtowicz[a]

[a]Division of Spectroscopy of Functional Materials, Institute of Physics, Faculty of Physics and Astronomy University of Zielona Góra, Szafrana 4a, 65-516 Zielona Góra, Poland

[b]Department of Biophysics, [c]Department of Pharmaceutical Chemistry, [d]Department of Instrumental Analysis School of Pharmacy, Medical University of Silesia, Sosnowiec, Poland

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The mono- and biradical forms of DOPA-melanin (DOPA-eumelanin) were studied by EPR spectroscopy in 100–300 K temperature range. The existence of triplet states in DOPA-melanin was proved. The analysis of EPR spectra has shown that in DOPA-melanin and its complexes with kanamycin and Cu^{2+} ions, two kinds of paramagnetic centres exist. The first of them are in doublet ground state with spin $S = 1/2$ and they obey the Curie law. The paramagnetic centres of the second group are in thermally excited triplet state with spin $S = 1$ and in this case the Curie law is not fulfilled.

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1. Introduction

Melanin is a very widespread polymer existing in living organisms. The most popular is eumelanin. DOPA--melanin (DOPA-eumelanin) is a synthetic melanin, which is very similar to natural eumelanin. Melanins reveal the ability of binding to drugs [1–4], and metal ions [5, 6]. Binding the drug with melanin has the character of electrostatic interactions between positive charged drug molecules and carboxylic or *orto*-semiquinone groups of melanin which have a negative charge at normal pH = 7. The complex of drug with melanin can also arise in charge transfer reaction or with participation of van der Waals forces [4, 7–9]. Drugs complexed with DOPA-melanin can be responsible for toxic effects in human organism. Radicals formed during (DOPA-melanin)–drug interactions can result in negative effects in pigmented tissues, e.g. toxic retinopathy, hyperpigmentation of the skin, hair bleaching, irreversible extrapyramidal disorders and some ocular and inner ear lesions. Mechanism of drug binding to DOPA-melanin and generation of paramagnetic centres in DOPA-melanin as the result of binding of aminoglycoside antibiotics has not been well recognized.

As the possible bonding places for metal ions (including copper Cu(II) ions) in melanin are given the next structures: carboxylate groups, amine groups, hydroxyl groups and also quinone or semiquinone structures [9, 10]. According to Hong and Simon [6] the metal binding sites are analogous to other biological systems.

Chemical structure of melanins is not clearly elucidated. It is however commonly accepted that eumelanin is a heterogeneous macromolecule composed of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2--carboxylic acid (DHCIA) units [11]. One of the most unusual properties of melanin is its persistent electron paramagnetic resonance (EPR) signal which indicates that radical centres are present in this biopolymer [11, 12]. At X-band the EPR signal of eumelanin is a single slightly asymmetric line 0.4–0.6 mT wide with a g-factor close to 2.004. Melanin radicals are stable but their EPR signal intensity can be easily modified by a number of different agents, including metal ions and drugs [4, 5, 11].

The effect of pH on the concentration of paramagnetic centres in melanin was studied in the temperature range of 100–300 K [13–14]. The obtained results indicate to the existence of equilibrium between dia- and paramagnetic forms of melanin involving quinone, semiquinone and hydroquinone however, the mentioned studies provided no information on biradical forms in melanin.

We have used samples of DOPA-melanin and its complexes with aminoglycoside antibiotic — kanamycin and Cu(II) ions and have studied the EPR signal from DOPA--melanin radicals, as well as the influence of drug and metal ions on DOPA-melanin EPR signal. We have performed temperature measurements of EPR signal of DOPA-melanin in order to verify the hypothesis that paramagnetic centres in thermally excited triplet state with spin $S = 1$ can exist in DOPA-melanin.

* corresponding author; e-mail: lkozdrowska@wp.pl

2. Materials and methods

2.1. Materials

Samples of synthetic DOPA-melanin were prepared by oxidative polymerisation of 3,4-dihydroxyphenylalanine (L-DOPA) in 1/15 M phosphate buffer at pH = 8 according to the Binns method [15]. The reaction products contain both indolo-5,6-quinone and 5,6--dihydroxyindole forms. DOPA-melanin was complexed with kanamycin, concentration of which was 1×10^{-3} M. For the preparation of Cu(II) complexes, 1×10^{-3} M copper(II) chloride solution was used. Complexes of (DOPA-melanin)–kanamycin, (DOPA-melanin)–Cu(II), [(DOPA-melanin)–kanamycin]–Cu(II) and [(DOPA--melanin)–Cu(II)]–kanamycin were prepared.

(DOPA-melanin)–kanamycin complex

250 mg synthetic DOPA-melanin were added to 250 cm^3 of 1×10^{-3} M kanamycin solution.

(DOPA-melanin)–Cu(II) complex

120 mg synthetic DOPA-melanin were added to 120 cm^3 of 1×10^{-3} M solution of copper(II) chloride.

[(DOPA-melanin)–kanamycin]–Cu(II) complex

50 mg of (DOPA-melanin)–kanamycin complex were added to 50 cm^3 of 1×10^{-3} M solution of copper(II) chloride.

[(DOPA-melanin)–Cu(II)]–kanamycin complex

50 mg of (DOPA-melanin)–Cu(II) complex were added to 50 cm^3 of 1×10^{-3} M kanamycin solution.

All samples were stirred for 24 h, filtered and dried.

The amount of kanamycin and Cu(II) ions bonded with DOPA-melanin is presented in Table I.

TABLE I

Amount of kanamycin and copper(II) ions bonded with DOPA-melanin.

Sample	Amount of kanamycin and Cu(II) ions bonded with DOPA-melanin [μmol/mg]	
	kanamycin	Cu(II)
(DOPA-melanin)–kanamycin	0.4278	–
(DOPA-melanin)–Cu(II)	–	0.5181
[(DOPA-melanin)–Cu(II)]–kanamycin	0.0520	0.5175
[(DOPA-melanin)–kanamycin]–Cu(II)	0.3907	0.0227

2.2. Methods

2.2.1. EPR spectroscopy

EPR measurements were performed using an X-band spectrometer S/EX, RADIOPAN, Poznań. The microwave radiation frequency and the magnetic field modulation frequency were 9.3 GHz and 100 kHz, respectively. The amplitude of modulation frequency was $\Delta B_{\mathrm{mod}} = 0.05$ mT. The EPR spectra were recorded as the first derivative of absorption in the temperature range 100–300 K and at the attenuation 20 dB that correspond to microwave power equal to 0.7 mW. The g factor of melanin radicals was determined from the equation

$$g = h\nu/\beta B_r\,, \tag{1}$$

where h — Planck's constant, β — Bohr's magneton, ν — microwave frequency, B_r — magnetic induction of resonance field. The value of $g-$ factor for melanin radicals in studied samples is within the range 2.0044–2.0045.

The experimental intensity I (which is proportional to the concentrations of paramagnetic centers) for EPR signal of melanin radical was calculated from the formula

$$I = (A_{\mathrm{s}}/A_{\mathrm{r}} m_{\mathrm{s}})(\Delta B_{\mathrm{pp}})^2, \tag{2}$$

where A_{s} — amplitude of EPR signal of the melanin sample, A_{r} — amplitude of EPR signal of ruby crystal used as the inner reference, m_{s} — mass of sample, ΔB_{pp} — linewidth (peak-to-peak) of the first derivative of absorption of EPR signal for melanin radicals.

The intensity I calculated in this way was used for numerical analysis of experimental data.

2.2.2. The influence of measurement temperature on EPR spectra

Paramagnetic substances can behave in different ways during measurements of the effect of temperature on EPR spectra, namely the EPR signal intensity can be temperature dependent or not. In paramagnetic substances with more than one kind of paramagnetic centres, each of them can show a different behaviour.

The exchange interaction between two paramagnetic centres with spin $S = 1/2$ results in the formation of a singlet state with $S = 0$ or a triplet state with $S = 1$ [16]. The difference of energy between singlet and triplet states is expressed by exchange energy J. For negative values of exchange energy J (antiferromagnetic coupling), an increase in EPR line intensity is observed when measurement temperature is on the rise, whereas for positive

values of J (ferromagnetic coupling), EPR line intensity decreases with increasing temperature.

For determination of the ratio of centers in singlet and triplet states in the whole amount of centers in doublet, singlet and triplet states, we have used the procedure given by Yen and Young [17] during their investigations of bitumens.

The EPR line intensity connected with the radicals in doublet state ($S = 1/2$) changes with temperature according to the Curie law

$$I_{\mathrm{d}} = (1/2)aN_{\mathrm{d}}/kT\,, \tag{3}$$

where I_{d} — line intensity of paramagnetic centers in doublet state ($S = 1/2$), N_{d} — number of paramagnetic centers in doublet state ($S = 1/2$), k — Boltzmann's constant, T — temperature of measurement, a — constant.

In the case when two electrons are strongly coupled, we can have the ground state being a singlet ($S = 0$) and the excited state being a triplet ($S = 1$). We can observe the triplet states in the EPR spectrum when the excitation energy singlet–triplet J is comparable with the thermal energy kT of lattice vibrations.

The EPR line intensity connected with the biradicals in triplet state ($S = 1$) changes with temperature according to the formula given by Bleaney and Bowers [18]:

$$I_{\mathrm{t}} = 2aN_{\mathrm{s\text{–}t}}/\left\{kT\left[\exp(J/kT) + 3\right]\right\}, \tag{4}$$

where I_{t} — line intensity of paramagnetic centers in triplet state ($S = 1$), $N_{\mathrm{s\text{–}t}}$ — sum of the number of centers in singlet ($S = 0$) and triplet states ($S = 1$), J — excitation energy singlet–triplet.

The g-factor of paramagnetic centers where the unpaired electron is localized on carbon C, nitrogen N, or oxygen O is nearly equal to the free electron g-factor, therefore the g-factors for paramagnetic centers (radicals) in doublet and triplet states are nearly the same and the two EPR lines overlap.

Putting $C = (1/2)aN_{\mathrm{d}}/k$, and $B = 2aN_{\mathrm{s\text{–}t}}/\{k[\exp(J/kT) + 3]\}$, we can present the total intensity of the EPR line in the form

$$I = I_{\mathrm{d}} + I_{\mathrm{t}} = C/T + B/\left\{T\left[\exp(J/kT) + 3\right]\right\}, \tag{5}$$

or

$$IT = C + B/\left[\exp(J/kT) + 3\right]. \tag{6}$$

Knowing the ratios B/C, it is possible to calculate the fraction of the centers in singlet and triplet states in the whole amount of centers in doublet, singlet and triplet states. The fraction is given by the formula

$$N_{\mathrm{s\text{–}t}}/(N_{\mathrm{d}} + N_{\mathrm{s\text{–}t}}) = (B/C)/(4 + B/C)\,. \tag{7}$$

The occurrence of a deviation from the formula $IT = C$ for a measured sample points to the existence of thermally excited paramagnetic centres in triplet state with spin $S = 1$, besides paramagnetic centres with spin $S = 1/2$.

The EPR signal of melanin radicals undergoes microwave saturation [19–21], therefore the linewidth ΔB_{pp} of EPR signal is determined by spin–lattice relaxation time T_1 and by spin–spin relaxation time T_2 [22, 23]. The relaxation time T_1 is dependent on temperature of measurement, however, the relaxation time T_2 changes only slightly with temperature. The relaxation time T_2 is dependent on concentration of paramagnetic centres. The study of temperature dependence of linewidth ΔB_{pp} of EPR spectrum permits to evaluate which kind of interactions predominates in investigated samples: spin–lattice or spin–spin interactions. At low temperatures, the linewidth ΔB_{pp} of EPR signal is determined by spin–spin interaction, because low temperature causes a decrease of spin–lattice interactions.

3. Results and discussion

3.1. Temperature dependence of the product IT of EPR signals of DOPA-melanin and its complexes with kanamycin and copper Cu(II) ions

Examples of EPR spectra of DOPA-melanin and its complexes with kanamycin and copper Cu(II) are shown in Fig. 1. EPR spectra of melanin radicals for all samples look similar but they possess different intensity I and linewidth ΔB_{pp}. Copper(II) ions cause the decrease of melanin radicals in samples, so we have obtained the EPR spectrum with the lower intensity. Copper Cu(II) ions are bonding with melanin radicals [21, 24].

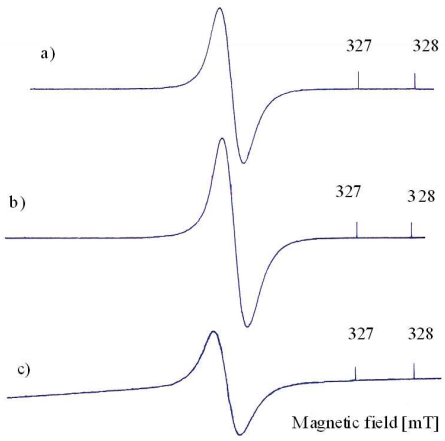

Fig. 1. EPR spectra of melanin radicals recorded at 300 K: (a) DOPA-melanin (1×10^4), (b) (DOPA--melanin)–kanamycin complex (1×10^4), (c) (DOPA--melanin)–copper(II) complex (2.5×10^4). The values of receiver gain are given in parentheses.

The dependence of the product IT versus temperature T showed that the Curie law is not fulfilled (Fig. 2). As a fitting formula for this dependence, the equation having a physical sense was chosen. Two aspects of the choice were important, namely to obtain a good fitting of experimental data to the proposed theoretical model and to perform a sound interpretation of results. The temperature dependence of the product IT, obtained from EPR experiments, was fitted by Eq. (6).

In Fig. 2a–e, the dependence of the product IT on temperature T for investigated samples, which is the sum

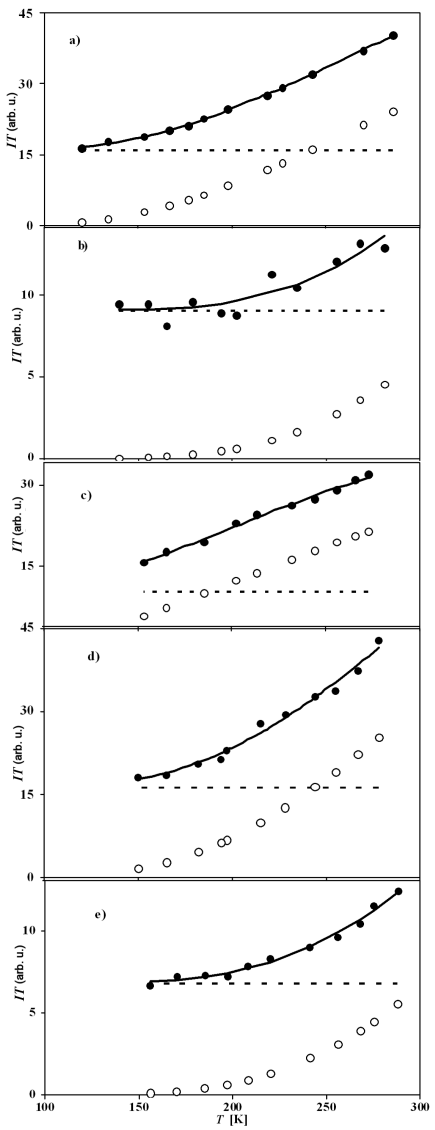

Fig. 2. Dependence of intensity I and temperature T product IT versus temperature T for paramagnetic centres in doublet states with spin $S = 1/2$ (dots) and for paramagnetic centres in thermally excited triplet states with $S = 1$ (empty circles) for (a) DOPA-melanin, (b) (DOPA-melanin)–Cu(II), (c) (DOPA-melanin)–kanamycin, (d) [(DOPA-melanin)–kanamycin]–Cu(II), (e) [(DOPA-melanin)–Cu(II)]–kanamycin. Filled circles represent the experimentally measured values IT and the solid line is a fitting of these points by Eq. (6).

of IT for paramagnetic centres with spin $S = 1/2$ and $S = 1$, is shown. The dotted curves concern paramagnetic centres in doublet ground state with spin $S = 1/2$ and the Curie law is fulfilled in this case. The curves marked with roundels reflect the behaviour of paramagnetic centres in thermally excited triplet state with spin $S = 1$ which do not obey the Curie law. In this case, a raise in temperature of measurements is accompanied by an increase of product IT of EPR line originating from spin $S = 1$.

The addition of Cu(II) ions to DOPA-melanin causes a reduction in intensity of the EPR line coming from melanin radicals in the case of samples (DOPA-melanin)–Cu(II) and [(DOPA-melanin)–Cu(II)]–kanamycin (Fig. 2b,e) as well as it results in a clear predomination of paramagnetic centres in doublet ground state ($S = 1/2$) over paramagnetic centres in thermally excited triplet state ($S = 1$). Such a result was not observed for other samples (Fig. 2a,c,d). Paramagnetic centres in thermally excited triplet state with spin $S = 1$ outnumber those with spin $S = 1/2$ at a certain temperature that is equal to about 250 K for DOPA-melanin and 170 K for (DOPA-melanin)–kanamycin complex. On this ground, we can say that kanamycin added to DOPA-melanin polymer increases the number of paramagnetic centres with spin $S = 1$. Because the mechanism describing how drugs are bound to melanin is not well recognised yet [19, 20, 25], it is difficult to explain fully this effect. It is known that intensity of EPR line of melanin increases after the addition of drugs [19, 20, 26]. This phenomenon is clearly seen at room temperature. The effect of temperature manifests itself in the occurrence of the charge transfer reaction and the latter results in the change of electron configuration in melanin.

Doping of DOPA-melanin with paramagnetic ions causes a decrease of EPR line intensity. This drop is the greater the higher the concentration of paramagnetic ions added [5, 11, 19, 21, 24, 27].

The sequence of the addition of components that form multicomponent complexes is of importance to results obtained. When metal ions are introduced to melanin as the first component, drug-binding sites became blocked [19]. In such a case, the dependence of the product IT on temperature for paramagnetic centres in thermally excited triplet state is weaker. When the drug is added first, the opposite situation is observed, namely the drug blocks binding sites for metal ions.

The temperature, beginning from which, paramagnetic centres with spin $S = 1$ predominate in [(DOPA--melanin)–kanamycin]–Cu(II) complex is the same as the temperature for pure DOPA-melanin (Fig. 2a,d). That means it is higher than that for DOPA-melanin complex with drug only (Fig. 2c). In the case of DOPA-melanin complex with Cu(II) ions and in that of complex, where metal ions were added before the addition of drug, no characteristic temperature was observed at which the product IT for paramagnetic centres with spin $S = 1$ was higher than that for paramagnetic centres with spin $S = 1/2$ (Fig. 2b,e). Metal ions block a considerable amount of DOPA-melanin binding sites and only a small amount of drug is bound to the polymer.

The data listed in Table II show the values of singlet–triplet excitation energy J/k, the constants C, B, and the ratio $N_{\mathrm{s-t}}/(N_{\mathrm{d}} + N_{\mathrm{s-t}})$, which is independent of temperature (see Eq. (7)) for samples studied. The lowest excitation energy was found for DOPA-melanin complex with drug and the highest one was typical of DOPA-melanin

complexes with metal ions (Table II). After the addition of Cu(II) ions to DOPA-melanin, a higher value of singlet–triplet excitation energy J/k is necessary for the existence of paramagnetic centres with spin $S = 1$. The increase of energy J/k depends also on the sequence of introducing kanamycin and Cu(II) ions to DOPA-melanin. The lowest value of J/k was found for DOPA-melanin and its complex with the drug. The introduction of kanamycin leads to a decrease of excitation energy J/k. This result confirms the hypothesis that the drug enables a better observation of paramagnetic centres in thermally excited state with spin $S = 1$ (a considerable predomination of the state with $S = 1$ over the state with $S = 1/2$) (Fig. 2c,d, Table II). The substantial increase in energy J/k and a considerable predomination of paramagnetic centres with spin $S = 1/2$ were found for complexes, where metal ions were introduced to DOPA-melanin as the first compound (Fig. 2b,e, Table II).

TABLE II

Values of parameters J/k, B, C and the ratio $N_{\mathrm{s-t}}/(N_{\mathrm{d}} + N_{\mathrm{s-t}})$ calculated for simultaneous existence of triplet state ($S = 1$) and doublet state ($S = 1/2$) in DOPA-melanin and its complexes with kanamycin and copper(II) ions (Eq. (7)).

Sample	Function	Model of simultaneous existence of triplet state ($S = 1$) and doublet state ($S = 1/2$)			
		J/k [K]	B	C	$N_{\mathrm{s-t}}/(N_{\mathrm{d}} + N_{\mathrm{s-t}})$
(DOPA-melanin)	$I = f(T)$	76.1	419.1	15.9	0.89
	$IT = f(T)$	76.8	427.6	16.0	0.87
(DOPA-melanin)–kanamycin	$I = f(T)$	55.0	224.2	10.1	0.86
	$IT = f(T)$	55.8	226.9	10.4	0.85
(DOPA-melanin)–Cu(II)	$I = f(T)$	145.4	821.5	9.1	0.96
	$IT = f(T)$	125.6	415.4	8.9	0.92
[(DOPA-melanin)–kanamycin–Cu(II)	$I = f(T)$	94.2	824.4	16.2	0.93
	$IT = f(T)$	94.2	822.9	16.2	0.93
[(DOPA-melanin)–Cu(II)]–kanamycin	$I = f(T)$	136.5	647.8	6.8	0.96
	$IT = f(T)$	144.7	855.2	6.9	0.97

Numerical analysis of EPR measurement results was performed with STATISTICA PL 5.1 of SYSTAT program using the quasi-Newton method.

3.2. Chemical structure of DOPA-melanin paramagnetic centres with spin $S = 1/2$ and $S = 1$

Our study of temperature dependence of EPR line intensity I shows that in DOPA-melanin and its complexes with kanamycin and Cu(II) ions, two kinds of paramagnetic centres with $S = 1/2$ and $S = 1$ exist. The obtained result reveals that the Curie law is not fulfilled and this indicates to the existence of paramagnetic centres with spin $S > 1/2$. Indolo-5,6-quinone is the main chemical unit of DOPA-melanin. The tautomeric forms of indolo-5,6-quinone are shown in Fig. 3c.

The existence of paramagnetic centres in thermally excited triplet state ($S = 1$) gives rise to the next task, namely the indication of localization site of two unpaired electrons which are responsible for the spin $S = 1$. In DOPA-melanin, three forms of indoloquinone chemical units can occur (Fig. 3): (a) 5,6-dihydroxyindole ($S = 0$) — fully reduced unit, and (b) monoradical canonic forms: 5,6-dihydroxyindol-5-yl ($S = 1/2$) and 5,6-dihydroxyindol-6-yl ($S = 1/2$) — semi-reduced (semi-oxidized) units, (c) tautomeric forms of indolo-5,6-quinone ($S = 0$) — fully oxidized unit [28]. The structure units of melanin polymer may or may not possess carboxylic groups –COOH [29]. This fact is shown in Fig. 3a.

Hydroquinone cannot have a thermally excited triplet state with electron spin $S = 1$, i.e. cannot be in a biradical form, because the thermal energy is too small for exciting. In hydroquinone the two hydrogen atoms in –OH groups are compensating the unpaired electrons in the quinone structure.

X-ray diffraction studies have shown that melanin consists of chain-like layers formed by indoloquinone units [30]. These layers are ordered parallel forming stacks. When in two neighbouring layers paramagnetic centres (radicals) with spin $S = 1/2$ exist, they can be coupled giving centres in singlet state ($S = 0$) and in thermally excited triplet state ($S = 1$). Therefore, paramagnetic centres in DOPA-melanin include monoradical forms with spin $S = 1/2$ and triplet states with spin $S = 1$. After providing thermal energy kT, the quinone

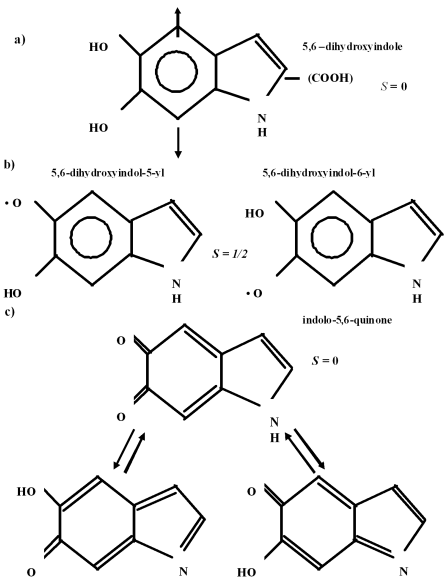

Fig. 3. Forms of indoloquinone structures in DOPA--melanin: (a) 5,6-dihydroxyindole ($S = 0$), (b) canonic forms of monoradicals: 5,6-dihydroxyindol-5-yl ($S = 1/2$) and 5,6-dihydroxyindol-6-yl ($S = 1/2$), (c) tautomeric forms of indolo-5,6-quinone ($S = 0$). The direction lines presented in Fig. 3a show that the monomer is a fragment of the macromolecular structure of melanin.

form of indolo-5,6-quinone with spin $S = 0$ can be excited to the triplet state structure with spin $S = 1$.

4. Conclusions

The study of temperature dependence of intensity I of EPR signal for DOPA-melanin and its complexes have shown the presence of two components in EPR spectrum. One of them obeys the Curie law and is associated with paramagnetic centres with spin $S = 1/2$. The other one does not fulfil the Curie law and originates from paramagnetic centres in thermally excited triplet state with $S = 1$. The existence of thermally excited triplet states ($S = 1$), besides doublet states ($S = 1/2$), in DOPA-melanin and its complexes with kanamycin and copper Cu(II) ions was proved. The singlet–triplet excitation energy J/k for analysed samples is within the range 55.0–145.4 K.

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