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Flavonols as metal-ion chelators: complex formation with Mg^{2+} and Ba^{2+} cations in the excited state

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Abstract

The excited state complexation of flavonol derivatives with alkali and alkali-earth metal ions were investigated.

It was found that in the excited state chelating magnesium complexes are more stable than in the ground state, and they are produced both from normal and from phototautomer forms of flavonols. The 'external' barium complexes with flavonols containing electron donor groups in 4′-position behave similarly in the excited state. The 'external' barium complexes of unsubstituted and 4′-methoxy substituted flavonols dissociate in the excited state. Their disruption occurs as a result of the excited state intramolecular proton transfer (ESIPT) in flavonol molecules.

Ejection of metal ion from the crown-cycle was found for the magnesium and barium complexes of azacrown-flavonol – M_2L. In the case of Ba_2L complex the process of cation ejection results in the formation of a complex which does not exist in the ground state.

The authors assumed that the short-wavelength band in the emission spectrum of unsubstituted flavonol in acetonitrile might be due to the fluorescence of flavonol molecules forming a hydrogen bond with the solvent.

Keywords: Flavonols; Chelators; Magnesium complexes; Barium complexes; Spectroscopy

1. Introduction

Alkali (Na^+, K^+) and alkaline-earth (Mg^{2+}, Ba^{2+}) metal cations play an important role in various biological phenomena. Presently, the biochemical functions in intracellular and extracellular media are being studied extensively. This requires the development of highly specific fluorescence indicators that would allow one to determine the concentration of the mentioned metal ions in different parts of the living cell, particularly in the cytoplasm [1].

Flavonols (3-hydroxyflavones) are promising candidates for the development of new fluorochromic indicators for both ion chelating and biomembrane structure studies [2–7]. Using flavonols as a basis, the spectral methods for quantitatively determining Sr^{3+}, Ga^{3+}, Th^{3+} [8], Al^{3+} [9], Sn^{2+} [10] cations in solution have been developed. Flavonols are also demonstrating promising results in the studies of proteins [11,12] and micelles [2,13].

Some representatives of this class of compounds possess all of the properties which are necessary for indicators and biosensors, i.e. the cation binding sites, high molar absorbances, substantial quantum yields, biocompatibilities and relative simplicity of synthesis.

Flavonols are also interesting because they exhibit high spectral sensitivity to solvent properties connected with excited state intramolecular proton transfer (ESIPT) reaction [14–24].

If ion chelating properties could be supplemented by ESIPT, then one may be able to develop several simple analytical methods based on ratiometric analysis of two fluorescence bands of fluorescent probe.

As we wished to focus on flavonol applications for alkali and alkaline-earth ion assay in biological systems, we conducted preliminary investigations of complex formation of several flavonols [25] in the ground state with Mg^{2+} and Ba^{2+} ions, which differ substantially by their ionic radii [26]. It was found that according to ion radius two types of

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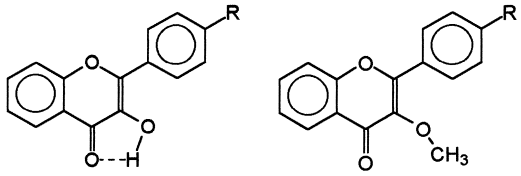


I - IV *V - VI*

I - R= H,
II - R = OCH_3,
III - R= $N(CH_3)_2$,
IV - R=monoaza-15-crown-5

V - R= $N(CH_3)_2$,
VI - R= H

Scheme 1.

complexes of different structure and spectroscopic parameters were formed.

As in our previous study [25], here we focus on the research of flavonols having substituents in the side phenyl fragment, which differ by their electron donor properties (Scheme 1). Our special interest is flavonol **IV**, for which the electronic properties of the substituent, monoaza-(15-crown-5)-cycle, change substantially with the coordination of the metal cation. In order to estimate the contribution of ESIPT into flavonols' fluorescent properties, we also studied several models – 3-methoxyflavones (**V**, **VI**) for which proton transfer was absent. Due to the presence of long-wavelength $n\pi^*$-transition the latter compound had no fluorescence (similar to the case of unsubstituted flavon [27]). These compounds were used for comparison of spectral characteristics of complexes formed in the ground state.

The present investigation is devoted to the study of fluorescent characteristics of flavonol complexes with Mg^{2+} and Ba^{2+} ions as well as to the establishment of the mechanism of complex formation in the excited state.

2. Experimental

3-hydroxyflavone derivatives were obtained according to the Algar–Flinn–Oyamada reaction [28,29]. Methylation of **I** and **III** was performed according to [29]. The obtained flavonols were recrystallized from ethanol and dried in a vacuum.The structure and purity of the studied flavonols were confirmed by element analysis, ^{1}H-NMR and IR-spectroscopy [25].

Acetonitrile, which was used as the main solvent in the present investigations, was dried under P_2O_5 and then rectified in dry atmosphere [30]. The concentration of water in the solvent was detected by the method of FTIR spectroscopy as peaks of OH-groups of water in acetonitrile (3636 and 3544 cm^{-1}). According to this method, the concentration of water in acetonitrile and in acetonitrile solutions of flavonols does not exceed 5×10^{-4} mol/l. Mg^{2+} and Ba^{2+} perchlorates were obtained by roasting the corresponding crystallohydrates at 215°C under low pressure −0.15 mm Hg [31]. The extent of drying was controlled gravimetrically.

2.1. Spectroscopic studies and measurement of equilibrium constants

Absorption spectra were measured with a 'Hitachi U3210' spectrophotometer, and fluorescence spectra with a 'Hitachi F4010' fluorescence spectrophotometer. Fluorescence quantum yields were determined with respect to quinine sulfate solution in 0.1 N H_2SO_4 ($\varphi = 0.56$) (for flavonols **I** and **II**) and with respect to fluorescein in carbonate buffer pH = 9.93 ($\varphi = 0.90$) [32] (for **III** and **V**). All the measurements were performed at isothermic conditions at a temperature of 20.0 ± 0.1°C.

Stability constants (K_S) and stoichiometric composition of the formed complexes were calculated by non-linear iteration least-squares method from the spectrophotometric titration data with the use of techniques described elsewhere [33–36].

The evaluation of dissociation constants of 3-hydroxy group in flavonols **I**–**IV** in the ground state (pK_d) were conducted in 30% aqueous ethanol solutions. For the estimation of the corresponding dissociation constants for the excited state (pK_d*), the method of Förster [37] was used.

2.2. Kinetic studies

The lifetimes of fluorescent forms and time resolved fluorescence spectra were measured with the spectrometer operating in the single photon counting mode [38,39]. Data analysis was performed by special techniques [38–40] which allowed for simultaneous determination of the excited state lifetimes of different fluorescent species in conditions of photochemical reactions between them and the rate constants of these reactions.

The rate constants of radiative (k_f^x) and radiationless (k_d^x) deactivation of excited states of flavonols and of their complexes being formed in the ground state were calculated by the following formulae:

$$k_f^x = \frac{\varphi_x}{\tau_x}, \qquad k_d^x = \frac{1-\varphi_x}{\tau_x}$$

here φ_x and τ_x are fluorescence quantum yield and the excited state lifetime of compound X, respectively.

Evaluation of k_f^y and k_d^y for the component Y, which does not exist in the ground state, was performed using the value of the radiative rate constant of initial species X and the rate constant of transformation of X into Y ($k_{x\to y}$) by the formulae:

$$k_f^y = \frac{S_y}{S_x}\frac{k_f^x}{k_{x\to y}\tau_y}, \qquad k_d^y = \frac{1-\varphi_y}{\tau_y},$$

here φ_y and τ_y are the fluorescence quantum yield and excited state lifetime of the species Y, and S_x and S_y are the

area under the emission bands for X and Y in the steady state fluorescence spectra.

2.3. Quantum chemical calculations

Optimization of the geometries of flavonol molecules, their complexes and hydrogen bonded forms, as well as the calculation of electron density distribution in molecules and their thermodynamic characteristics were performed by PM3 method [41].

The estimation of enthalpies of hydrogen bond formation in flavonol **I** molecule was performed by the formulae:

$$\Delta H_f = H_f^h - H_f^b, \qquad \Delta H_f^* = H_f^{*h} - H_f^{*b}$$

for structures with intermolecular hydrogen bonding and

$$\Delta H_f = H_f^h - H_f^b - H_f^S, \qquad \Delta H_f^* = H_f^{*h} - H_f^{*b} - H_f^S$$

for structures with intermolecular hydrogen bonding.

In these formulae, H_f^h and H_f^{*h} are the enthalpies of formation of molecules with intramolecular hydrogen bond in the S_0 and S_1 states, respectively, H_f^b and H_f^{*b} are the enthalpies of formation of the same molecules without intramolecular hydrogen bond in the S_0 and S_1 states, and H_f^S is the enthalpy of formation of solvent molecules.

The comparison of enthalpies of hydrogen bond formation in the ground and in the excited states for any particular structure was performed for an unrelaxed Frank–Condon state, in which the geometry of flavonol **I** molecule and the relative positions of molecules forming intermolecular hydrogen bonds in the S_1-state does not change substantially compared to the ground state. The contribution of entropy factor was assumed to remain approximately the same for the S_0 and S_1 states.

This assumption can not be applied to the structures with hydrogen bonds of different type. In such cases we used the value of relative change of enthalpy of formation as a measure of the strengthening of intramolecular and intermolecular hydrogen bonds, which was calculated by the formula:

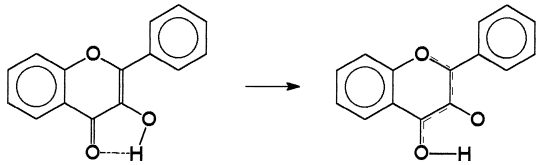
$$\Delta H_f^r = \frac{\Delta H_f^* - \Delta H_f}{\Delta H_f} 100\%$$

3. Results and discussion

3.1. Fluorescent properties of complex-free flavonol molecules

As we mentioned above the reaction of proton phototransfer (ESIPT) is characteristic to flavonols (Scheme 2). Fluorescent properties of normal (N) and phototautomer (T) forms of unsubstituted flavonol and kinetics of proton transfer were extensively studied [18,29,42–43].

The spectroscopic data obtained for various flavonols are presented in Tables 1 and 2. These data, together with our previously published results [14], demonstrate that in fluorescence spectra of flavonol **I** in acetonitrile solutions, a noticeable emission from normal form is observed in addition to an intensive phototautomer band. This fact is difficult to understand in view of the high rate of ESIPT process .



Scheme 2.

It is well known that fluorescence of normal form is often revealed in aprotic solvents containing impurities with proton donor and proton acceptor groups [14] (the traces of water, for example).

The logarithm of the stability constant of flavonol–water adduct determined in [25] was 0.17. At experimental conditions, the working concentration of flavonol in acetonitrile was approximately 1.0×10^{-5} mol/l, and the concentration of water was less than 5×10^{-4} mol/l. In this case the concentration of the flavonol–water adduct must be less then 7.4×10^{-9} mol/l. Even if the stability constant of the complex in excited state is slightly higher than in the ground state, it is impossible to detect the presence of the water–flavonol adduct at its given concentration (especially since its quantum yield is 1.64×10^{-3}).

In order to verify if the observed fluorescence was due to trace amounts of water which may have remained in acetonitrile even after special purification, we also performed special fluorescence studies in a water–acetonitrile mixture.

Molecules of water can form hydrogen bonds both with the carbonyl oxygen atom and with the 3-hydroxy group of studied flavonols (structure B, Scheme 3). Owing to the higher proton acceptor ability of water molecules, their hydrogen bonds with the flavonol hydroxy group must be stronger than those formed with acetonitrile molecules (structure C, Scheme 3) [18]. That is why we can expect different spectral characteristics of the hydrogen bonded complexes B and C.

Results of our experiments showed that along with the addition of water to acetonitrile a small increase of the quantum yield of normal form (φ_N) of flavonol **I** occurred. Its maximal value of 1.64×10^{-3} was observed at a water concentration of more than 2 mol/l. The lifetime of species N in acetonitrile, which was initially equal to 90 ps, increased to 330–360 ps upon the addition of even small amounts of water and did not change as the water concentration was increased further (up to 2.5 mol/l). Thus, the fluorescent properties of the flavonol and of the adduct flavonol–water in acetonitrile solutions are different. Consequently, we can conclude that the observed fluorescence of N form is not due to the presence of water in acetonitrile.

The comparison of radiationless deactivation rate constants of k_d^N(obtained from quantum yield and lifetime of

Table 1
Spectral characteristics of flavonols and of their complexes with Mg^{2+} and Ba^{2+} ions[a]

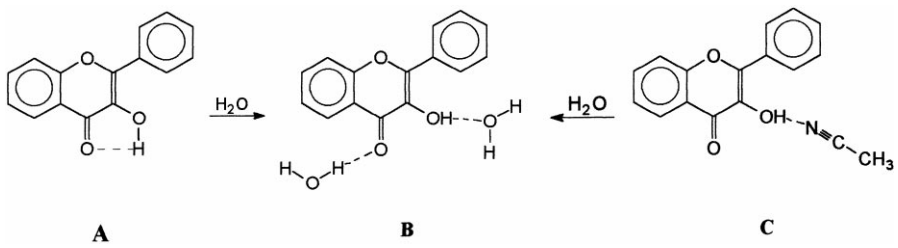
Flavonol	Normal form			Phototautomer		Complex with Mg^{2+} ion			Complex with Ba^{2+} ion		
	$\bar{\nu}_{abs}$	$\bar{\nu}_{fl}$	$\Delta\bar{\nu}_{St}$	$\bar{\nu}_{fl}$	$\Delta\bar{\nu}_{St}$	$\bar{\nu}_{abs}$	$\bar{\nu}_{fl}$	$\Delta\bar{\nu}_{St}$	$\bar{\nu}_{abs}$	$\bar{\nu}_{fl}$	$\Delta\bar{\nu}_{St}$
I	29 440	24 630	4810	18 860	10 580	23 840	20 480	3360	28 600	24 420	4180
II	28 740	23 220	5520	18 860	10 080	23 920	20 220	3700	27 620	22 860	4760
III	25 180	18 980	6200	17 300	7880	22 340	18 240	4100	23 340	18 220	5120
IIIa	29 340	24 780	4560	18 640	10 700	–	–	–	–	–	–
IV[b]	25 060	19 151	5900	17 370	7690	22 600	18 600	4000	28 080	18 680	9400[c]
						23 840	19 880	3980	26 180		7500
V	26 220	19 840	6380	–	–	23 680	18 620	5060	24 320	18 500	5060
VI	33 820	–	–	–	–	33 280	–	–	32 940	–	–

[a] $\bar{\nu}_{abs}$, $\bar{\nu}_{fl}$-positions of long-wavelength absorption and emission band maxima, cm^{-1}; $\Delta\bar{\nu}_{St}$- fluorescence Stokes shifts, cm^{-1};
[b] Characteristics of ML complex (top line) and of M_2L (bottom line).
[c] The Stokes shifts for emission band of BaL complex were calculated in respect to absorption bands of BaL and Ba_2L.

Table 2
Lifetimes, quantum yields, radiationless deactivation rate constants of flavonol derivatives[a] and their complexes with alkaline-earth metals in acetonitrile

Flavonol	Normal form (N)				Phototautomer (T)				Complex with Mg^{2+} (Mg_iL)				Complex with Ba^{2+} (BaL)			
	$\varphi\times10^2$	τ	k_f	k_d	$\varphi\times10^2$	τ	k_f	k_d	$\varphi\times10^2$	τ	k_f	k_d	$\varphi\times10^2$	τ	k_f	k_d
I	0.09	0.09	1.0×10^7	1.2×10^{10}	4.17	0.70	4.0×10^7	1.4×10^9	10.50	1.79	5.9×10^7	5.0×10^8	4.23	0.28	1.5×10^8	3.4×10^9
II	0.11	0.11	1.0×10^7	9.0×10^9	3.63	0.29	1.3×10^8	3.5×10^9	28.70	3.92	7.1×10^7	1.8×10^8	23.91	0.92	2.6×10^8	8.3×10^8
III	6.48	0.51	1.3×10^8	1.8×10^9	2.34	0.10	2.4×10^8	9.7×10^9	73.32	3.68	2.0×10^8	7.3×10^7	56.34	2.38	2.4×10^8	1.8×10^8
IV[b]	4.58	0.56	8.7×10^7	1.7×10^9	3.72	0.14	2.2×10^8	6.9×10^9	79.30	3.48	2.2×10^8	6.0×10^7	46.8	1.81	2.7×10^8	2.8×10^8
									62.41	1.62	3.9×10^8	2.3×10^8				
V	68.60	1.95	3.5×10^8	1.6×10^8	–	–	–	–	4.54	1.11	4.1×10^7	8.6×10^8	8.4	0.40	2.1×10^8	2.3×10^9

[a] φ denotes the quantum yield of fluorescence; τ is the life time of fluorescing forms, ns; k_f and k_d are the constants of radiative and radiationless deactivation, s^{-1}.
[b] Characteristics of MgL complex (top line) and of Mg_2L (bottom line).



Scheme 3.

Table 3
Rate constants of photochemical processes observed in the excited state for flavonols and their complexes with Mg^{2+} and Ba^{2+} ions

Flavonol	N → T	N → MgL	T → MgL	Mg_2L → MgL	N → BaL	T → BaL	BaL → T
I	$\approx3.5\times10^{11}$	–	8.2×10^9	–	–	–	3.3×10^9
II	8.7×10^9	–	4.3×10^{10}	–	–	–	1.4×10^8
III	1.7×10^9	2.4×10^{11}	2.5×10^{11}	–	4.8×10^{10}	1.0×10^{11}	–
IV	1.8×10^9	8.8×10^{10}	4.2×10^{10}	3.5×10^9	1.8×10^{11}	5.1×10^{10}	–
V	–	1.7×10^{10}	–	–	7.2×10^9	–	–

the normal form of flavonol **I**) and the rate constant of proton transfer $k_{N\to T}$ (ESIPT) reaction for **I** (Table 3) shows that the latter is about 30 times (or using data [17] – about 15 times) higher. It is known that the total values k_d^N consist of three main contributions: first, the rate constant of radiationless decay in normal form due to the intersystem crossing, second, the rate constant of radiationless deactivation, induced by the process of proton phototransfer, and, third, the rate constant of proton transfer [44]. Since the k_d^N value cannot be smaller than $k_{N\to T}$, we suggest that decreased k_d^N is not related to the fluorescence of intramolecular hydrogen bond species (structure A, Scheme 3). If we assume

the opposite and assign the observed short-wavelength fluorescence to the structure with the intramolecular hydrogen bond, the lifetime of normal form evaluated from the k_d^N value (either obtained by us or reported elsewhere [17]) would not exceed a decimal fraction of picosecond. This value is much shorter than the τ_N obtained from experiment.

The presence of fluorescence of form N in acetonitrile may most likely be explained by the presence in solution of a definite quantity of flavonol molecules with the broken intramolecular hydrogen bond (Structure C, Scheme 3). The authors of [17] deny this possibility. They start from the assumption that if in the ground state the intermolecular hydrogen bonds with acetonitrile exist, they should be disrupted upon electronic excitation. In such a case, the next steps would be the restoration of intramolecular hydrogen bond and proton transfer reaction. According to this reasoning, the slow proton transfer might be observed. However, the authors of [17] found only the fast ESIPT process for flavonol in their picosecond emission spectroscopy experiments in acetonitrile. Based on these results, the conclusion was made [17] about the absence of intermolecular hydrogen bonding between flavonol and acetonitrile molecules in the excited state.

However, it was not taken into account that the acidity of the OH-group increases substantially upon excitation and therefore the intermolecular hydrogen bond of flavonol with acetonitrile molecules in S_1-state must become stronger in comparison with the ground state. The intramolecular proton transfer reaction in this case will hardly be probable. This conclusion is supported by model calculations of enthalpy of hydrogen bond formation (ΔH_f) for the structures A and C from Scheme 3 (in S_0 and S_1 states). In both cases, on excitation the decrease of ΔH_f is observed. This corresponds to strengthening of both intra and intermolecular hydrogen bonds. The relative changes of enthalpy ΔH_f^r obtained for the structures A and C are of the same order: 155 and 118%, respectively.

Thus we can suggest that in acetonitrile solution there exists some amount of flavonol **I** molecules which form hydrogen bond with the solvent, and these bonds are retained in the S_1-state. For such solvent-bonded molecules the proton phototransfer reaction does not occur, and they cause the presence of a low intensive short wavelength band in flavonol fluorescence spectrum.

Inclusion of electron donor groups to position 4′ of the side phenyl ring increases the electron density on the oxygen atom of the 3-hydroxy group (Table 4), which corresponds to the decreased proton mobility of this group, as well as to the decrease in its acidity. As shown in Fig. 1, the dissociation constants of the 3-hydroxy group in the S_1 state pK_d^* depend linearly ($r = 0.987$) on the calculated values of charges on oxygen atoms of OH group.

The decrease of 3-hydroxy group acidity upon inclusion of electron donor substituents also results in weakening of intra and intermolecular hydrogen bonds in the ground state. Thus, the relative amount of molecules with intermolecular hydrogen bonds must decrease. This leads to the decrease of intensity of the corresponding short wavelength fluorescence band.

Table 4
Charges on the oxygen atoms of carbonyl and hydroxyl groups, calculated by PM3 method. Dissociation constants of studied flavonols in the ground and the excited states

	S_0			S_1			ΔpK_d
	q_o (C=O)	q_o (O–H)	pK_d	q_o (C=O)	q_o (O–H)	pK_d^*	
I	−0.342	−0.213	+9.12	−0.360	−0.139	−1.21	10.33
II	−0.345	−0.219	+9.59	−0.363	−0.164	+4.45	5.14
III	−0.345	−0.220	+10.18	−0.370	−0.179	+6.74	3.44
IV	−0.345	−0.219	+10.23	−0.366	−0.176	+6.75	3.48

Comparison of radiationless rate constants of normal forms k_d^N (Table 2) and rate constants of ESIPT $k_{N\to T}$ of compounds **II–IV** (Table 3) shows that they are close in value. Therefore we may conclude that the observed emission of normal forms are determined mainly by the rate of proton phototransfer. In this case the short wavelength fluorescence band of flavonols **II–IV** may be attributed mainly to the emission of species with the intramolecular hydrogen bond.

The fact that proton phototransfer is a main channel for non-radiative decay of flavonol normal forms is supported by the investigation of fluorescent characteristics of compound **V**. In this molecule ESIPT is impossible, and thus the observed k_d^N value is nearly one order of magnitude lower and the quantum yield φ_N is one order of magnitude higher compared to the case of flavonols, which exhibited proton transfer.

In our experiments we found a good linear correlation between logarithms of ESIPT rate constants calculated by PM3 method and excited state negative charges on 3-hydroxy group oxygen atoms. In view of the similar linear correlation for pK_d^* values, we may conclude that the ESIPT rate in this series of compounds is determined mainly by the change in acidity of the 3-hydroxy group in the excited state (Fig. 1). The fluorescence quantum yields and the positions of short-wavelength emission bands for flavonols **III** and **IV** differ significantly from those for flavonols **I** and **II**. This may be due to substantial shift of electron density in the excited state from nitrogen containing electron donor substituents of **III** and **IV** to benzopyrone fragment. Upon decrease of intramolecular charge transfer (ICT), the acidity of the 3-hydroxy group in the S1 state increases substantially and results in an increase of ESIPT rate and, correspondingly, in a decrease of the quantum yield of normal form. Thus, in acetonitrile in the presence of sulfuric acid, the concentration of which is sufficient for protonation of the nitrogen atom and complete suppression of ICT (**IIIa**), fluorescence characteristics of the N and T forms of **III** (Table 1) are similar to those of flavonols **I** and **II**. This fact allows us to conclude that the observed fluorescent properties of amino substituted flavonols are determined by superposi-

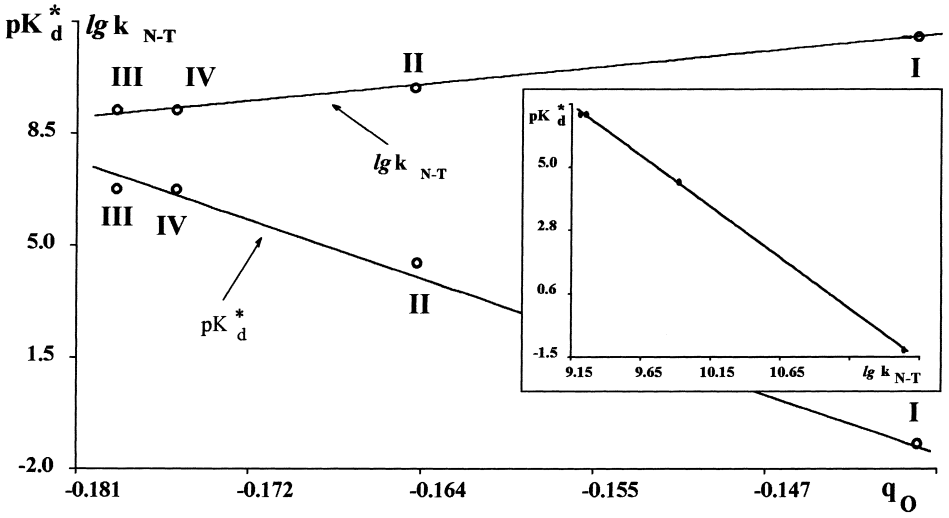


Fig. 1. The correlation of S_1 state hydroxy group dissociation constant indecies (pK_d^*) and logarithms of ESIPT rate constant ($pk_{N\rightarrow T}$) from the values of negative charge on the oxygen atom of carbonyl group. The analogous correlation of ($pk_{N\rightarrow T}$) and pK_d^* is presented in the included figure.

tion of the ICT and ESIPT processes, but not by switching between them as was suggested in [29].

3.1.1. Spectral properties of chelating complexes of flavonols with Mg^{2+} ions and kinetics of their formation in the exited state

Recently we have estimated that in the ground state flavonols **I–IV** form strong magnesium complexes. The metal ion replaces the hydrogen atom of the 3-hydroxy group and forms a donor–acceptor bond with the oxygen atom of the carbonyl fragment. Thus, a five membered chelate cycle is formed. The supposed structure of such chelating complexes ML is presented in Fig. 2a.

The absence of proton phototransfer, which is the main way that radiationless deactivation occurs for complex-free flavonols, gives chelating complexes the significant lifetime in the S_1 state and higher values of fluorescence quantum yields. An additional factor, which reduces the efficiency of radiationless deactivation, is a rigidity of chelating complexes in comparison with complex-free molecules. The last fact is also confirmed by fluorescence Stokes shifts values of magnesium complexes (Table 1), which are as small as those observed for complex-free flavonols.

As we have mentioned above, intramolecular excited state charge transfer (ICT) from the lateral phenyl ring to benzopyrone fragment is typical for flavonols **III** and **IV** with dimethylamino- and monoaza-5-crown-15 substituents. Formation of a chelating complex intensifies the electron acceptor properties of the carbonyl group, which increases the efficiency of ICT, and additionally stabilizes the highly polar S1 state. The result of this is a significant decrease (almost of the order of 1.5) in the radiationless deactivation constants of these complexes, $k_d^{C_{Mg}}$, which provides a high intensity for them.

The results of our investigation of time resolved fluorescence spectra of flavonol chelates correspond well to the data obtained from quantum chemical calculations. According to these calculations, complexes with Mg^{2+} ion must be stronger in the excited state than in the ground state. The time-resolved fluorescence spectra of compounds **I** and **III** in the presence of magnesium perchlorate, recorded at a 35 ps time interval (and normalized on the area under the spectral curve) are presented on Fig. 3a,b. In all cases the emission bands of magnesium complexes were observed. Calculations of kinetic constants showed that the formation of chelating complex of flavonols **III** and **IV** in the excited state occurs not only from the normal form, but also from the phototautomer (Fig. 2a). The values of rate constants of both mentioned processes for compound **III** were practically identical, while for crown-flavonol **IV** the chelating complex formed slightly faster from the normal form (Table 3). As flavonols **I** and **II** had high ESIPT process rates, their excited state complexation with magnesium ions was registered only from the phototautomer (Fig. 2a).

The schemes of mutual transformations of flavonol normal forms, of their phototautomers and of magnesium chelate complexes are presented in Fig. 2.

The reactions for complex formation in the excited state are bimolecular, however, their rate constants $k_{N\rightarrow ML}$ and $k_{T\rightarrow ML}$ exceed (for flavonols **III** and **IV**, more than an order of magnitude) the rate constant for diffusion controlled reaction in acetonitrile. It is known, however, that the diffusion rate increases in the case of electrostatic interaction between the particles in solution. Approximate calculations of rate constants for diffusion controlled bimolecular reactions of charged particles – Mg^{2+} ions and bipolar flavonol molecules **III** and **IV** in acetonitrile – allowed us to estimate limiting values of $k_{N\rightarrow ML}$ and $k_{T\rightarrow ML}$. They were estimated as approximately $2–3\times10^{11}\ s^{-1}/l\times mol$ for compound **III** and $6–8\times10^{10}$ for compound **IV**. These data were similar to rate constants obtained from experiment, presented in Table 3. This fact permitted us to conclude that the reactions

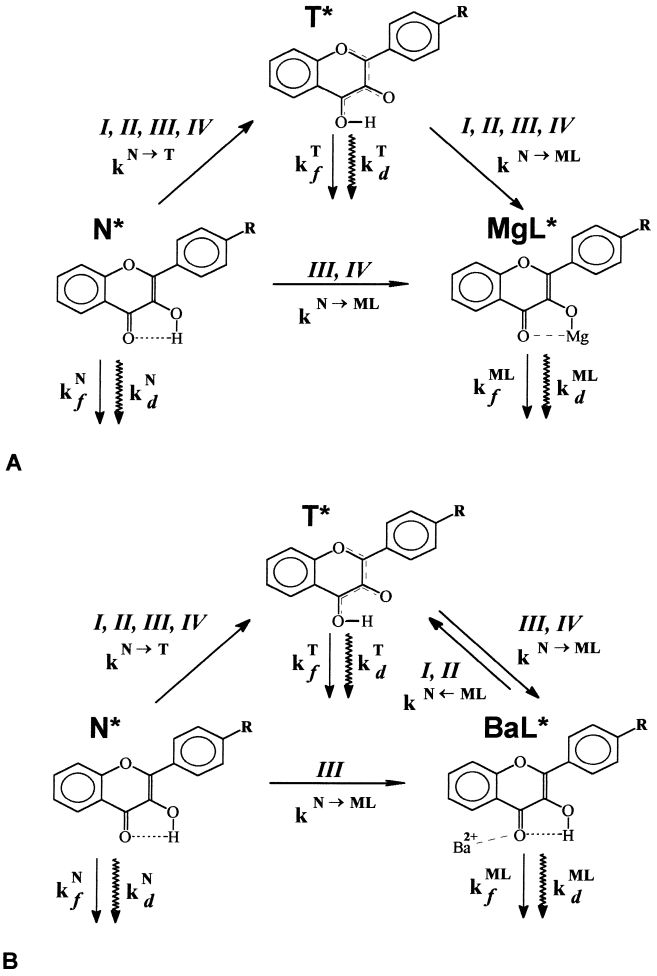


Fig. 2. Mutual transformations of neutral and tautomer forms of studied flavonols, and their complexes with Mg^{2+} and Ba^{2+} ions.

of chelate **III** and **IV** formation were determined mainly by a diffusion process.

The behavior of crown-flavonol **IV**, having two complex formation centers, is of special interest to us. Recently we found that complexation with Mg^{2+} ions in the ground state primarily proceeds through the stage of initial formation of chelating complex. The crowned complex of M_2L type with metal ion coordination by the crown-cycle is formed on the next stage of interaction. An increase of Mg^{2+} ion concentration leads to the same changes both in the absorption and in the fluorescence spectra of flavonol **IV**. In the fluorescence spectra, the emission of N and T, as well as ML and M_2L complex emission were observed. In the complex formed by crown-cycle, the crown-bonded Mg^{2+} ion prevents charge transfer from the crown-nitrogen atom to the benzopyrone fragment. That is why the absorption and emission bands of M_2L complex are shifted towards shorter wavelengths compared to analogous bands in spectra of chelate ML (Table 1).

A decrease of charge transfer from the nitrogen atom to the benzopyrone fragment caused by the presence of Mg^{2+} ion in crown-cycle of M_2L complex prevents delocalization of positive charge of another Mg^{2+} ion, forming a five membered chelate cycle. This fact leads to lower stability and greater efficiency of radiationless processes in M_2L complexes in S_1-state. As a result, the $k_d^{M_2L}$ value is as large as the analogous rate constant for unsubstituted flavonol **I**.

Our analysis of time resolved fluorescence spectra (Fig. 3c) shows that the M_2L complex is less stable in the excited state than in the ground state. M_2L complex decomposes in the S_1-state with formation of product having the same fluorescence characteristics as the ML complex. This fact allowed us to assume that the observed process of the M_2L complex dissociation occurs by ejection of metal ion from the crown-cycle (Scheme 4).

Similar results were described in [45–47] for calcium and lithium complexes of azacrown-containing coumarins and merocyanins. However, in some cases the dissociation of the complex occurred in a very short time of approximately 4 ps. This dissociation has been interpreted by several authors as cleavage of the donor–acceptor bond between metal ion and the nitrogen atom of crown-cycle [45], and by other authors as the ejection of calcium ion [46,47] from the crown-cycle.

In our case the destruction of crown-complex of **IV** with ion Mg^{2+} proceeds noticeably slowly ($k = 3.3 \times 10^9\ c^{-1}$). This fact may be explained by greater 'durability' of the Mg–N bond, in comparison with similar bonds of other alkaline and alkaline-earth metals [48].

3.1.2. 'External' flavonol complexes with Mg^{2+} and Ba^{2+} ion

The barium ions do not form chelating complexes with flavonols, since in this case the ion radius exceeds the dimensions of the cavity between the hydroxyl and carbonyl groups. Nevertheless, addition of $Ba(ClO_4)_2$ to acetonitrile solution of compounds **I–III** and **V** leads to changes in their electronic absorption spectra. This fact permits us to make a conclusion about complex formation of flavonols with Ba^{2+} cations.

The possibility of replacement of the hydrogen atom of the flavonol hydroxy group by Ba^{2+} ion (Scheme 5a) seems to be improbable. Unlike Mg^{2+} ions, whose bonding with oxygen atom has partial covalent character, the Ba^{2+} ions form only ionic bonds. Hence, in such case, an anionic form of flavonols **I–IV** must appear in acetonitrile solution containing Ba^{2+} ion. According to the data presented in Table 4, the investigated flavonols are too weakly acidic to form even a detectable concentration of anion under the conditions of our experiments. This fact was confirmed by our spectral studies: even in the concentrated solutions of $Ba(ClO_4)_2$ in acetonitrile neither absorption nor emission of an anionic form were observed.

In our opinion, the formation of a donor–acceptor bond between Ba^{2+} ion and the oxygen atom of the 3-hydroxy group (Scheme 5b) is also improbable. According to the data in Table 4, negative charges on this atom are much less than on the oxygen atom of the carbonyl group, and tend to essential reduction in excited state. Hence, it is reasonable to

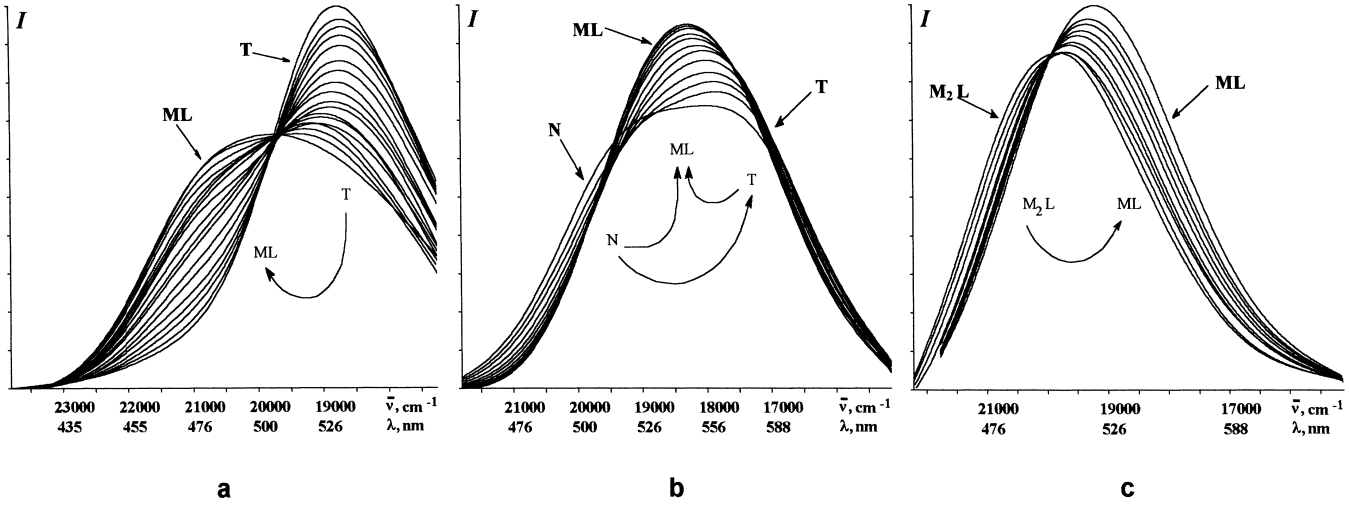
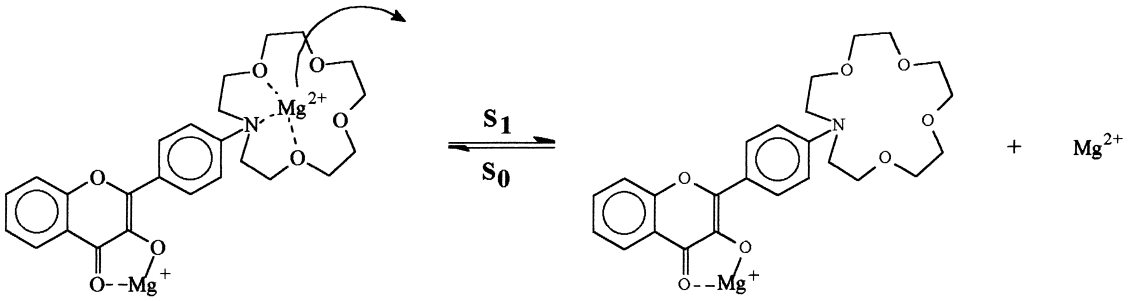
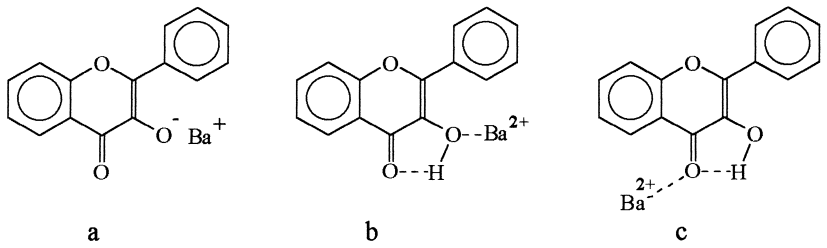


Fig. 3. Time resolved fluorescence spectra of chelating flavonol complexes with Mg^{2+} ion: a – **I**, b – **II**, c – **III** (recorded at a 35 ps time interval and normalized by the area under the spectral curve).



Scheme 4.



Scheme 5.

expect decreased stability and even destruction of complex (Scheme 5b) in the excited state.

Furthermore, the linkage of hydroxy group and metal ion, which has significant positive charge, should lead to an increase in proton mobility and acceleration of the ESIPT-process. Our experimental results contradict this expected behavior. Actually, upon increase of metal ion concentration the proton transfer in the excited state slows down or even stops completely.

Based on the changes that were observed in absorption spectra on formation of barium complex, as well as on the comparison of charges on oxygen atoms of hydroxyl and carbonyl groups, we made an assumption that the Ba^{2+} ion forms complexes with the external electron pair of the carbonyl oxygen atom (Scheme 5c). It is probable that the non-chelating magnesium complexes with 3-methoxyflavones **V** and **VI** are of similar nature.

It is clear that the stability of 'external' complexes depends on the negative charge value on the oxygen atom of the carbonyl group. In the ground state these charges are practically identical for all of the flavonols studied (Table 4). The measured pK_S values for barium complexes of flavonols **I–IV** in the S_0-state are also very close to one another (0.72–0.89). The magnesium complexes of flavonols **V** and **VI** are less stable than analogous barium complexes: for flavonol **V**, $pK_S = 0.43$, while for **VI** $pK_S = 0.32$.

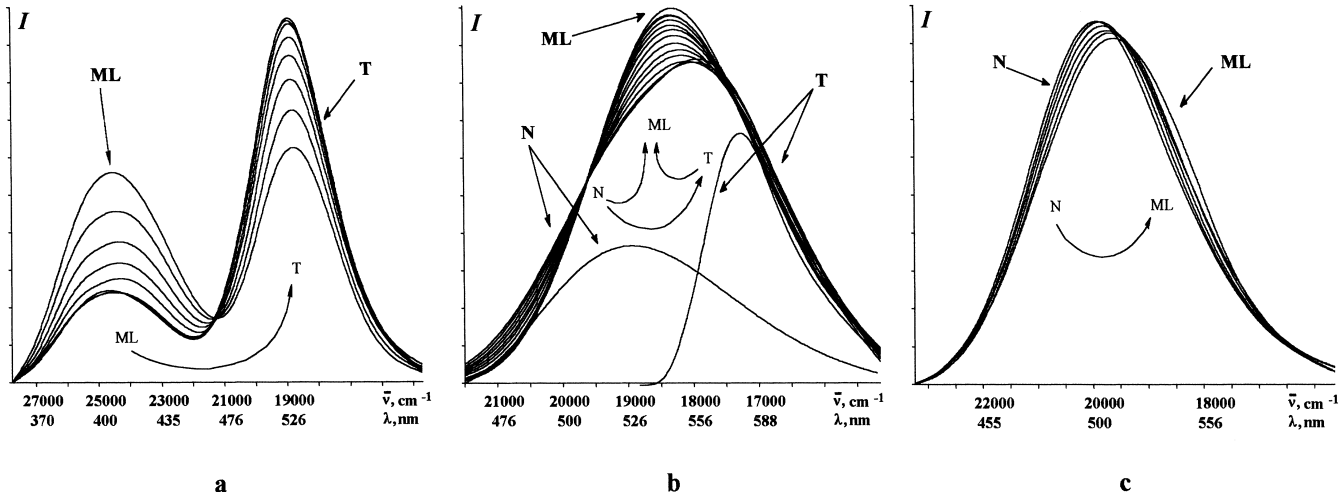


Fig. 4. Time resolved fluorescent spectra of 'external' flavonol complexes: a – **I** with Ba^{2+}, b – **III** with Ba^{2+}, c – **V** with Mg^{2+}. In (b) the emission bands of normal and tautomer forms are represented by dotted lines.

The increase of electron density upon excitation is typical for the oxygen atom of the carbonyl group, so the increase in stability of the 'external' complex in the S_1-state was expected.

Despite the similarity of absorption spectra and close stability constants, the 'external' complexes of flavonols with Ba^{2+} ions differ in their fluorescent properties.

The emission bands of barium complexes of flavonols **I** and **II** have maxima at $24\,400\,cm^{-1}$ (410 nm) and $22\,820\,cm^{-1}$ (438 nm), respectively. The positions of these bands are close to those of emission bands of the normal forms of appropriate flavonols. So, one can assume that the electronic structure of complexed and complex-free forms **I** and **II** is similar. The weakening or absence of the intramolecular hydrogen bond, leading to downturn of the efficiency or even complete termination of proton transfer, decreases the probability of radiationless deactivation in barium complexes in the S_1 state. As a result, their fluorescence is more intensive than those of normal forms **I** and **II**.

The π-electronic structure of flavonols **III** and **V**, their 'external' barium complexes and magnesium chelates in the excited state are dependent on ICT. Though the metal ion in barium complexes does not directly influence the π-electronic system of flavonol molecules (in chelates this influence is transmitted mainly through the oxygen atom of the hydroxyl group), the presence of a donor–acceptor bond between the positively charged ion and the oxygen atom of the carbonyl group of benzopyrone fragment leads to an increase of ICT and to the additional stabilization of the complex with transferred charge in the excited state as well.

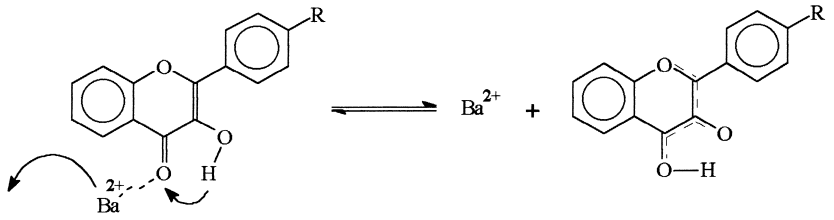
The nature of the excited state of 'external' complexes with essential interfragmental charge transfer appears to be similar to the nature of chelated complexes. Therefore the complexes of these two types have similar spectral characteristics (Table 3), irrespective of metal ion position.

Due to smaller stability and smaller rigidity of 'external' complexes, the probability of radiationless deactivation in them is higher than in chelate complexes of the corresponding flavonols. That is why fluorescence quantum yields of 'external' complexes are lower. Particularly, this statement confirms the example by the flavonol **V** complex. Replacement of the hydrogen atom of the 3-hydroxy group by a more bulky methyl fragment leads to an increase in steric hindrance. As a result, the methoxy-group of **V** is turned out at an angle of 60–80° from the plane of the molecule. The torsion angle between the benzopyrone fragment and the side phenyl cycle is also increased [49,50]. This leads to a decrease in carbonyl group basicity and, consequently, reduces the stability of 'external' complex with metal ions in both the ground and excited states. Thus, barium and magnesium complexes of flavonol **V** have high k_d^Cvalues, small fluorescence lifetimes and low fluorescence quantum yields.

The 'external' complexes differ also by their behavior in the exited state. The emission band of phototautomer in fluorescence spectra of flavonols **I** and **II** in solution does not completely disappear even at significant concentrations of Ba^{2+} ions. The analysis of time-resolved spectra of compounds **I** and **II** showed that in this case the phototautomer fluorescence is observed not only due to the ESIPT process. The significant portion of phototautomer is formed as a result of dissociation of the 'external' barium complexes occurring in the exited state (Fig. 4a). This process appears to take place through the replacement of metal ion by hydrogen atom, transferred to carbonyl group during ESIPT (Scheme 6).

The fact of phototautomer formation at the barium complex excitation confirms the structure proposed by us for 'external' complexes.

We have assumed that the formation of 'external' complexes should result in significant weakening or even in cleavage of the intramolecular hydrogen bond and, as a consequence, must lead to decreased efficiency of proton transfer.



Scheme 6.

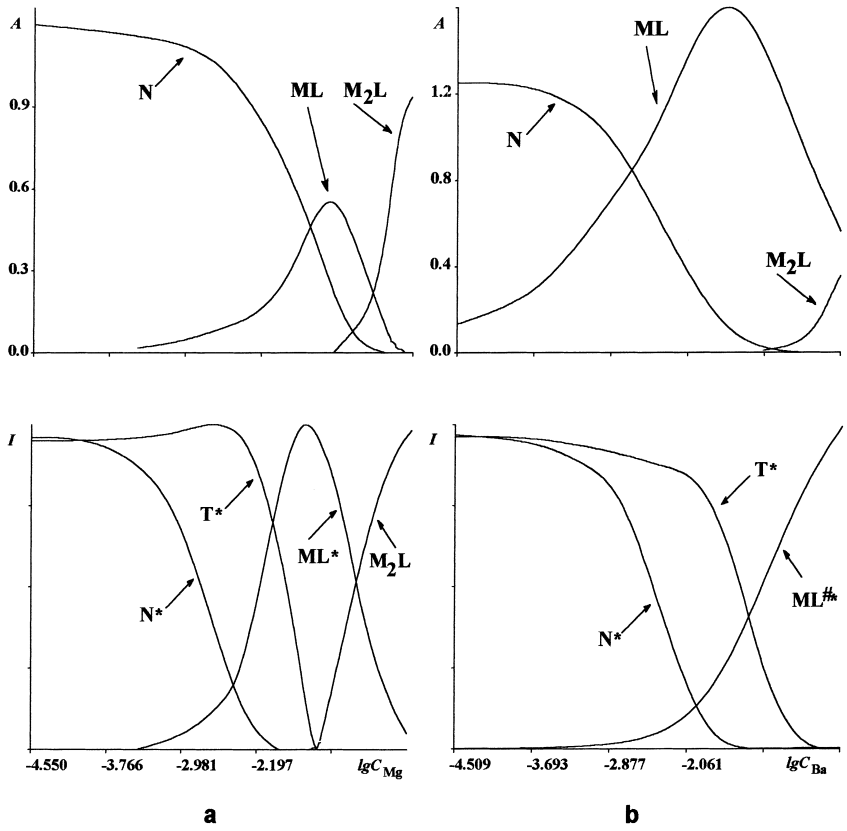


Fig. 5. Changes of relative content of different forms and complexes of crown-flavonol in respect to concentration of Mg^{2+} (a) and Ba^{2+} (b) ions. Luminescence spectra data (below) are normalized on intensity.

Comparison of ESIPT rate constants ($k_{N\rightarrow T}$) with constants of dissociation of 'external' barium complex under influence of ESIPT ($k_{ML\rightarrow T}$) for flavonols **I** and **II**, shows that the $k_{ML\rightarrow T}$ constants are by two orders of magnitude lower than the $k_{N\rightarrow T}$

As the 3-hydroxy group acidity in the excited state significantly affects the rate of proton phototransfer, the inclusion of electron donor substituents which reduce proton mobility in the lateral phenyl ring leads to the less effective dissociation of barium complex of flavonol **II**. Moreover, the dissociation of 'external' complexes of flavonol **III** was not observed at all.

In the last case, the backward process – an 'external' complex formation from N form of flavonols in S_1-state(Fig. 4 b) was observed. The same process took place also for flavonol **V** in its interaction with Mg^{2+} and Ba^{2+} ions (Fig. 4c). In addition, it was found that the barium complex of flavonol **III** was formed not only from the N form, but also from the T one. The total scheme of interactions in the exited state for various forms of compounds **I–III**, **V** and their complexes is presented in Fig. 2b.

In the excited state the crown-flavonol **IV** forms barium complexes of two types – ML and M_2L. The structure of these complexes differ substantially from stoichiometrically similar complexes with Mg^{2+} ions. This difference arises for two main reasons. The first one is that monoaza-15-crown-5 ether forms stronger complexes with Ba^{2+} ion than with Mg^{2+} ion (for *N*-phenyl-15-crown-5, $pK_S = 3.57$ and 1.37, respectively) [25]. The second reason is that the Mg^{2+} and Ba^{2+} ions form complexes of a different nature with the benzopyrone moiety of flavonols. Thus, magnesium chelates are much stronger than 'external' barium complexes (for ex-

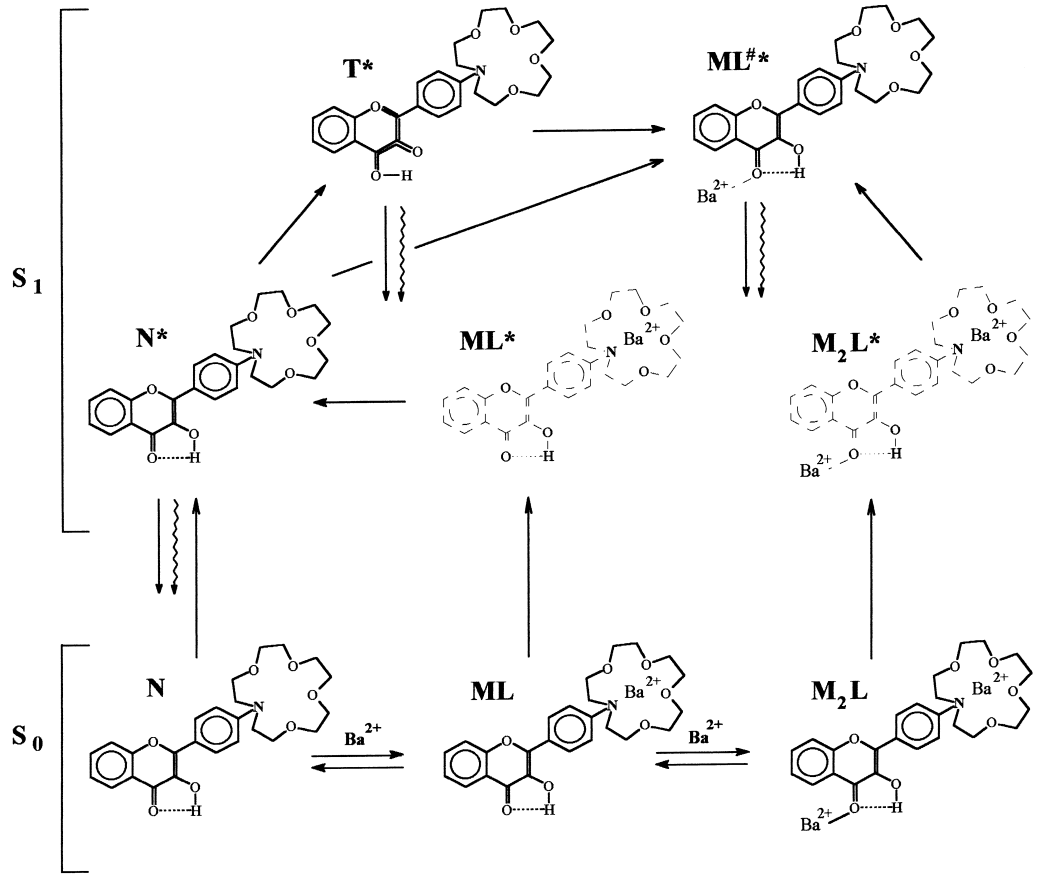


Fig. 6. The scheme of mutual transformations of barium complexes of crown-flavonol **IV** in the ground and the excited states.

ample, for flavonol **I** pK_S = 1.71 and 0.76, respectively). As a result, the increase of Ba^{2+} ion concentration in flavonol **IV** solutions in the ground state leads to the formation of a crown-complex ML at the first stage of interaction. Then, after binding of the second metal ion, an 'external' crown-complex M_2L forms.

The data presented in Fig. 5b show that changes in the fluorescence spectra, which were observed as the concentration of Ba^{2+} increased, did not correspond to changes in the absorption spectra. In the last case the band of flavonol **IV** as well as two bands of complexes ML and M_2L were present. However, the emission bands of normal (N*) and phototautomer (T*) forms of the flavonol, and only one band which might correspond to complexes, were distinguished in fluorescence spectra. The band of the complex has a high Stokes shift value relative to the absorption bands of complexes ML and M_2L (9400 and 7500 cm^{-1}, respectively). This fact does not permit us to attribute the observed emission band to any of the complexes existing in the ground state.

Taking into account that in the excited state Mg^{2+} and Ca^{2+} ions are often ejected from the crown-cycle [46,47], it is reasonable to assume that the same process was observed for barium complexes of flavonol **IV**. Hence, the excited ML* complex loses the Ba^{2+} ion from crown-cycle and gives the free ligand (N*). At the same conditions, the M_2L complex transforms to another complex $ML^{\#}$*, the spectral properties of which are similar to those of the barium complex of flavonol **III** (Fig. 6). Thus, it is possible to assume that the emission band in the fluorescence spectra (previously attributed to a complex of undefined nature) can be referred to as the emission of 'external' complex of **IV** with free crown-cycle – $ML^{\#}$*.

The analysis of spectral characteristic of the $ML^{\#}$* emission band confirms our assumption: its position is close to that of the emission band of magnesium chelate **IV**-ML with free crown-cycle, and also is close to positions of fluorescent bands of chelating and 'external' complexes of flavonol **III**. Estimated k_f and k_d values for the complex $ML^{\#}$* are also similar to the corresponding rate constants of 'external' complex of flavonol **III** (Tables 2 and 3).

The similarity of complexes $ML^{\#}$* (**IV**) and ML (**III**) was confirmed also by the analysis of time resolved fluorescence spectra. Excited state formation of barium complex of $ML^{\#}$* type from normal and phototautomer forms of **IV** was observed. However, since $ML^{\#}$* appears not only from the specified forms, but also as a result of destruction of M_2L complex, the dependence of conversion rates $T \rightarrow ML^{\#}$* and $N \rightarrow ML^{\#}$* on the Ba^{2+} ion concentration displays deflects from linearity.

4. Conclusions

1. The short-wavelength emission band in fluorescence spectra of unsubstituted flavonol **I** in acetonitrile can be attributed to the emission of molecules having intermolecular hydrogen bonds with solvent. However, we found that the analogous band in the spectra of flavonols with electron–donor substituents in 4′ position corresponds mainly to the emission of molecules with an intramolecular hydrogen bond.
2. It was found that chelating magnesium complexes of flavonols are more stable in the excited state than in the ground state. As a result, at excitation these complexes are produced with high efficiency both from normal and from phototautomer forms of the studied flavonols.
3. Competition of processes of complex formation in the S_1-state and proton phototransfer was observed for 'external' barium complexes. In the case of flavonols **I** and **II**, the hydroxy groups of which were characterized by higher acidity in the excited state, the ESIPT process and the photodissociation of the complexes prevail. The stability of barium complexes, obtained from flavonols **III** and **IV** having low pK_d^*, increases in the excited state. Formation of additional amounts of these complexes from free ligands was observed at excitation.
4. Ejection of metal ion from the crown-cycle was found for the magnesium and barium complexes of azacrown-flavonol-M_2L. In the case of the Ba_2L complex the process of cation ejection results in the formation of another complex which does not exist in the ground state.

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