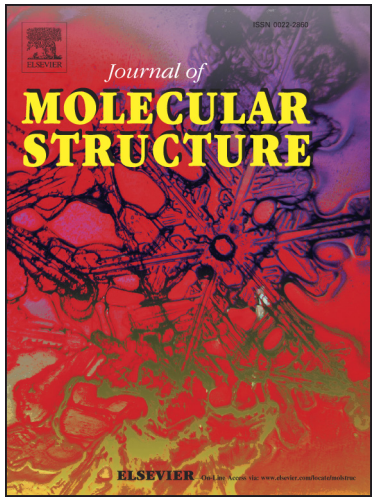

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Spectroscopic study of photo and thermal destruction of riboflavin

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Abstract

Influence of temperature and light irradiation on the spectroscopic properties of aqueous solutions of riboflavin was studied using linear dichroism method, absorption and fluorescence spectroscopy. It was established that in a wide temperature range 290-423 K there is a decline of absorbance and fluorescence ability, which is explained by thermodestruction of riboflavin. It is shown that the proportion of molecules, which have undergone degradation, are in the range of 4-28%, and depends on the concentration and quantity of temperature effects. Introduction of hydrochloric and sulfuric acids, as well as different metal ions leads to an increase in the photostability of riboflavin solutions by 2-2.5 times. The observed phenomena are explained by the formation protonation form of riboflavin and a complex between the metal ions and oxygen atoms of the carbonyl group of riboflavin, respectively.

Key words: riboflavin, absorption, luminescence, thermal destruction, the spectrum of the linear dichroism, photochemistry, ion metals.

1. Introduction

Riboflavin is one of the most important water-soluble vitamins involved in many biochemical processes [1,2]. However, solutions of organic compounds are mostly used in

practice; their molecules can be in a different molecular forms each characterized by own absorption and fluorescence spectra [3], as well as in multi-component mixtures where a variety of intermolecular interactions may affect the spectral-luminescence characteristics of organic compounds. Along with the spectral-luminescence characteristics of the solutions, an important parameter that determines the working resource is a photo and termostability. Photophysical and photochemical processes occurring in solutions of riboflavin fairly well studied [4-7]. During the production of various riboflavin containing drugs they can be thermally treated for sterilization purposes, as a result significant changes of riboflavin properties may occur as a side effect. [8]. Therefore, the study of spectral-luminescent properties, photochemical degradation mechanisms, the influence of temperature and the development of non-chemical methods of stabilization of riboflavin is of great practical importance. That's why the purpose of present work is to study the effect of the concentration of riboflavin and various metal ions on the intermolecular interactions in solutions of riboflavin, as well as on their thermal and photo-stability.

2. Methods and experiment

Riboflavin powder with the "chemically pure" grade was used (Fig.1). Distilled water was used as a solvent. Electronic absorption spectra were measured by a Specord 50 SA spectrophotometer (Analytikjena, Germany), with specified +/-0.003 D accuracy and 0.3 nm spectral resolution over the 190-1100 nm range.

Figure 1.

Measurement of the fluorescence spectra was carried out by fluorescence setup, assembled on the base of two MDR-76 monochromators (LOMO, Russia). Photomultiplier tube FEU-38 (Russia) was used as detector. PMT signal was amplified and then registered by KSP-4 plotter. The angle between the excitation light and fluorescence observation was about 45^0. To avoid reabsorption, fluorescence measurements were

carried out with thin layers of the solutions, in which the absorption of the excitation light did not exceed ~5%. Depending on the concentration of the solution, special quartz cells with thickness of the layer in the range of 0.1-50 mm were used. In the case of absorption measurements, the cell thickness D and solution concentrations C were chosen so that C×D remains constant. In this case, despite the difference in the cell and concentration, the amount of absorbing molecules remains the same, allowing the direct quantitative comparison of spectrums. During irradiation by unfiltered light of a mercury-quartz lamp PRK-2 the distance between the lamp and the object was 15 cm, the irradiated area of the cell (diaphragm) 15.2 sm^2, optical power 60 mW. Riboflavin solutions were subjected to accelerated aging by heat treatment: for solutions by boiling under normal conditions and by keeping solutions in a thermostat at 343K-423K in tightly sealed cuvettes. A special hear-resistant capsule was used during the thermal treatment of aqueous solutions of riboflavin. In order to reduce the pressure of water vapor a cooling unit was mounted to the capsule: water vapor condensates in this unit and flows back to the heat-resistant capsule. Dispersion of optical rotation and linear dichroism spectra were recorded on a circular dichrographs Jasko-20, applying a double Fresnel parallelepiped specifically designed and fabricated for the visible and UV spectral region.For ease of comparison, the presented absorption and fluorescence spectra are normalized to unity. Calculation of charge distribution on the atoms was held during the quantum chemical calculations of molecular structure of riboflavin (Fig.1) using the program package MOPAC 2009 [9] by semi-empirical method AM1 with a standard set of parameters [10]. All calculations were performed for isolated molecules in a vacuum. Prior geometry optimization of molecules was performed using a limited method Hartree-Fock with Polak-Ribiera algorithm up to 0.001 kcal/(Å×mol) and consideration of various options initial conformations.

3. Results and discussion

3.1. Influence of metal ions and the concentration.

The concentration dependence of the absorption and fluorescence spectra of aqueous riboflavin solutions were studied (Fig.2).

Figure 2a,b.

It can be seen from the Fig. 2 absorption and fluorescence spectra in the range 10^{-6}-10^{-5} M concentration remain constants. The absorption spectrum of riboflavin has four maximums with λ_{max}=220 nm, λ_{max}=265 nm, λ_{max}=370 nm and λ_{max}=440 nm. An aqueous solution of riboflavin has an intense fluorescence band with λ_{max}=527 nm. A further increase in concentration leads to a decrease of the intensity and broadens the absorption spectra. Concentration increase in fluorescence spectra is manifested by the drop in the intensity, in other words, the concentration quenching of fluorescence occurs. Observed phenomena in the absorption and fluorescence spectra in aqueous solutions can be explained by formation of non-luminescent aggregates of dye molecules with solvent molecules acting as a connecting bridge between dye molecules through hydrogen bonding. This phenomenon is widely spread among organic dyes [11-13]. It is known that, ability to form different aggregates is explained by the structure of the chromophore dyes, namely large dipole moments, alternation of opposite charges and flat structure of the chromophore, which results in high energy of intermolecular interactions [14].

Linear dichroism spectroscopy proved to be very informative and accurate method of determining the degree of aggregation of riboflavin in the aquatic environment. In [15-17] it was shown that a linear dichroism can be observed when a solution containing aggregates is pumped through a one millimeter flow cell with the speed of 2 mm/s.

Figure 3.

To measure the linear dichroism the velocity vector of the laminar hydrodynamic flow is oriented at an angle of about 45^0 in regard to the polarization plane of the linearly

polarized light impinging on the cell. In conditions of incomplete dissolution or its aggregation up to colloidal state in the storage process of solutions, riboflavin aggregates are represented as elongated microcrystals. In conditions of the laminar flow of these solutions through flow cell they become optically anisotropic due to partial orientation of riboflavin aggregates in hydrodynamic laminar flow. In such case the linear dichrograph registers nonzero spectral curve with bands maxima corresponding to the maxima of the absorption bands of aggregated riboflavin whereas band amplitudes are proportional to the degree of aggregation. Aging of aqueous solutions of riboflavin promotes its aggregation and that can be evidenced by the appearance and growth of the linear dichroism bands (similar to curve 2 in Fig.3) under conditions of laminar flow through flow cell.Addition of hydrochloric and sulfuric acids, various metal ions, such as manganese chloride, nickel acetate, copper and aluminum sulfates, to diluted riboflavin solutions ($c=10^{-5}$M) leads to the about double increase of the absorption bands with λ_{max}=220 nm and λ_{max}=265 nm, and to 1.3 times increase of λ_{max}=370 nm and λ_{max}= 440 nm bands. No significant changes in the fluorescence spectra were observed.

3.2. The study of thermal stability

Aqueous solutions of riboflavin were subjected to thermal effects 423K in a time interval from 20 to 60 minutes. Degradation of the riboflavin molecules determined optical density at a wavelength λ_{max}=445 nm before and after heating of aqueous solutions. As can be seen from Fig. 4a at temperature influence on aqueous solutions of riboflavin there is a significant deformation of its electronic absorption spectra.

Figure 4a,b.

In this case, along with the fall of absorbance bands with λ_{max}=445, 375 and 268 nm, an increase of absorption at λ_{max}=220 nm is observed. The hypochromic shift by 7 nm is observed at the band with λ_{max}=375 nm (Fig. 4a).

Hypochromic shift of absorption is accompanied by the decrease of fluorescence intensity without changes in shape (Fig. 4b). Many authors attribute such hypochromic shifts of electronic absorption and fluorescence spectra to the partial aggregation of molecules in solution. However, during the thermal influence on the riboflavin solution aggregation of the solute molecules is not observed. The absence of aggregates in solutions of riboflavin under the exposure to high temperatures can be explained by following considerations. Firstly, increase of the solution temperature leads to the dissociation of molecules [18,19]. Secondly, in the event of temperature influence symmetric reductions of absorption bands is not observed. It was experimentally determined that the temperature effect on aqueous solutions of riboflavin leads to a decrease of absorption bands in the range of 24-42%. Thirdly, the absence of aggregation in aqueous solutions of riboflavin is proved by the analysis of linear dichroism spectra. Use of similar method of pumping of aqueous solution of riboflavin after thermal treatment showed that they did not have optical activity. For such solutions, the linear dichroism is zero. Based on the experimental results, it follows that under thermal effect of riboflavin solutions its destruction is observed. Analysis of the fluorescence spectra, which are more sensitive to changes in environment, shows that at a temperature of 423K up to 75% of riboflavin undergo destruction. Such a change in the amount of riboflavin molecules which undergo degradation is observed at a constant temperature, depending on the exposure time. So, ~15% riboflavin molecules are destructed during treatment at 423K for 20 minutes, reaching ~28% and ~42% after 40 and 60 minutes of treatment, respectively. It is important to mention that the proportion of riboflavin molecules that undergo degradation depends on the temperature and time of heat exposure. During thermal exposure within 40 minutes at a temperature 373 K the fraction of molecules which undergo degradation of riboflavin is 4% at 393K - 7%, and more than 423K ~ 20%. The observed phenomena suggest that under thermal effect on the structure of riboflavin solutions formed products differ from photo-destruction products.

These assumptions are confirmed by the fact that the ability of fluorescent decomposition products is absent, and the absorption spectra of the degradation products are in the ultraviolet region of the spectrum. To assess the absorption spectrum of the destruction products spectrum of adsorption of pure riboflavin were subtracted from the curve 3. The resulting spectrum is shown in Fig. 5 curve 4.

Figure 5.

As seen in Figure 5 the absorption spectrum of products thermodestruction has a maximum λ_{max}=210, 260 nm and a broad band in the range wavelength of 290-360 nm. From the analysis of the absorption spectra of the degradation products we assumed that the main cause of deformation and discoloration of the absorption spectra of an aqueous solution of riboflavin is splitting drug molecules urea and 1, 2–dihydro–6,7–dimethyl–2–keto–1–ribitol–3–quinoxaline carboxylic acid. Similar assumption put forward by the author in [20] where it is shown that the products of thermal degradation of riboflavin are quinoxaline derivatives.

3.3. The study of photostability

It was found that, as water solution was exposed to the irradiation by unfiltered light of PRK-2 lamp, absorption of λ_{max}=265 nm, λ_{max}=370 nm, λ_{max}=440 nm bands falls accompanied by hypsochromic shift of about 8-12 nm. Decrease in the intensity of band with λ_{max}=527 nm is observed in fluorescence spectra (Fig. 6).

Figure 6a,b.

Comparison of optical density of the concentrated and dilute solutions at λ_{max}=447 nm for the same irradiation duration showed that the photostability of riboflavin solution doesn't depend on solution concentration. It should be noted that the photobleaching is irreversible, absorption and fluorescence spectra of irradiated solutions of riboflavin are not restored with time. This means that during photolysis the destruction of riboflavin occurs. It

is known that under the same conditions of irradiation by light organic solvents destruct differently [21], leading correspondingly to different bleaching of the same compound in various solvents. Therefore, the photodestruction of riboflavin solution, apparently, can be explained by the fact that at first water molecules are photodissociated by photon: $H_2O + h\nu \longrightarrow H + \dot{O}H$ [21]. It can be seen from the scheme for water photolysis, that the OH group is formed and the free hydrogen atom. Next, the formed OH radicals (may act as the alkali) react with an active part of riboflavin molecule and side chain of molecule coupling is destructed which leads to destruction of the molecule of riboflavin. Similar assumption is described in [22] where it is shown chemical changes are typical for riboflavin molecules when exposed to light. Thus in an alkaline solution of riboflavin 4 atoms are segregated from the side chain of riboflavin and enters lumiflavin (6,7,9-trimethyl-isoalloxazine). Photochemical reaction in neutral and acidic medium proceeds differently: side chain is segregated as a whole and lumichrom is formed (6,7-dimethylalloxazin). It should be mentioned that in aqueous solutions in the presence of hydrochloric acid and sulfuric acid alongside with irradiation in the absorption spectrum band λ_{max}=265 nm bathochromic shift at 15-18 nm and a new band with λ_{max}=402 nm is observed. Furthermore, during irradiation of the solution being composed from hydrochloric acid or sulfuric acid there is a drop of riboflavin absorption ability at a wavelength λ_{max}=445 nm in relation to a neutral environment. For example, under irradiation in a neutral aqueous environment within 80 minutes, the optical density value decreases from 1.22 to 0.39. At the same time irradiation of the solution having the composition in its normal 0.025M and 0.05M hydrochloric acid, absorbance value decreased from 1.22 to 0.80 units and from 1.22 to 0.85 respectively. Further increase of HCl in solution does not significantly change the optical density of the solution. These results indicate that the quantum yield of photo-transformation of riboflavin molecules in acidic media in relation to a neutral environment is reduced by approximately in 2 times.

Decrease in the quantum yield of photo-transformation can be explained by the formation of the protonated form of riboflavin. In riboflavin there are 4 nitrogen atoms (N) and 6 oxygen atoms (O) which can form a hydrogen bond with the proton acid and interfere the degradation of the side chain of riboflavin. At this certain photostability of solution is reached. This assumption is confirmed by the fact that the nitrogen atoms have two free electron pairs that can create steric hindrances in the formation of hydrogen bonds between the oxygen atoms. Moreover, steric hindrances may also be created by a nitrogen atom N (3). Number of hydrogen atoms that can lead to the neutralization of electron pairs on the nitrogen atoms is increased by increasing the amount of acid in an aqueous solution of riboflavin. Thus favorable conditions are occurred for the formation of hydrogen bonds between the oxygen atoms with the nitrogen atom a group NH.

Distribution of charges on the atoms of the molecules of riboflavin (Fig. 1) shows that the highest negative charges are nitrogen atoms N (2) and N (3). However, the nitrogen atom N (3) is located at a great distance from the oxygen atoms O (3)-O (6). Therefore, neutralization of electron pairs on the nitrogen atom N (3) contributes to the formation hydrogen bonds. Attachment of hydrogen to the nitrogen atom N (3) may be as follows:

Possibility of such reaction is confirmed experimentally. As an example, Figure 7 shows the absorption spectra of aqueous riboflavin solution with the addition of hydrochloric acid in the normal range of 0,025-0,075, which are subjected to light radiation. At this time, the irradiation to all solutions with different amounts of HCl remained constant at 80 minutes.

Figure 7

As seen in Figure 7 the addition of hydrochloric acid, leads to an increase in optical density, in relation to a neutral solution irradiated and a significant stabilization of the

solution is observed. This confirms that in acid media the riboflavin molecules exhibit some degree of protonation, which increases the photostability of the solution by 2 times with respect to a neutral shape.

A similar pattern under irradiation by light is observed in the absorption and fluorescence spectra of aqueous riboflavin solutions in the presence of metal ions. Presence of metal ions in solution decreases the photodestruction rate of riboflavin by 1.5-2.5 times (Table 1).

Table 1.

In our opinion, stabilization of riboflavin is caused by the formation of a complex between the metal ions and oxygen atoms in the hydroxygroup of riboflavin molecule. Theoretical estimates of acidity of hydroxygroup of flavonoid derivatives by the partial and total charge on the oxygen atoms in hydroxygroups show that the acidity has no significant effect on the structure of the formed complexes [23,24], as observed in our experiment. Furthermore, it is noted that the complexation of metal ions with flavonoids is caused by pronounced electron donor properties of latter and by low redox potential, which for most flavonoids is in the range of 0.25-0.75 [25]. It is assumed that the complexation reaction of flavone derivatives with metal ions is characterized by the binding of ligand with metal ions due to electron transfer from d-orbital of metal to π*-orbital of flavone. By analogy with flavone derivatives riboflavin is also characterized by the bond of ligand with a metal ion. The structure of the formed complex is largely dependent on the electron density distribution in ligand molecule [24], particularly on the charge on the oxygen atoms, where the electron density is maximized. Indeed, our calculations of molecular structure showed that the negative charge is maximized on the oxygen atom of carbonyl group (-0.33 and -0.307), while charges on the other oxygen atoms are in the range -0,291:-0,329 (Fig.1). So it can be assumed that riboflavin forms a complex with metal ions via the oxygen atom in carbonyl group. This in turn leads to substantial stabilization of its solutions. In the case of

aqueous solutions with metal ions riboflavin destruction of side chain conjugation is impossible due to complex formation with metal ions. This in turn leads to substantial stabilization of its solutions. In this case, during initial irradiation radicals interact with the active part of the complex, and lead to its destruction, and then to degradation of riboflavin. This explains the increase in the exposure time necessary for destruction of an aqueous solution of riboflavin in the presence of metal ions.

Conclusion

It is shown that the proportion of molecules have undergone degradation in the range 4-28%, depending on the concentration and quantity of temperature effects.

It is shown that the products of thermal degradation of riboflavin are urea and quinoxaline carboxylic acid absorption spectrum, which is located in the ultraviolet region of the spectrum and is in satisfactory agreement with the absorption band of urea.

Introduction of hydrochloric and sulfuric acids, various metal ions in aqueous solutions of riboflavin leads to an increase in the photostability of the last 2-2.5 times due to protonation forming a complex form of riboflavin and between metal ions and oxygen atoms of the carbonyl group of riboflavin, respectively.

Acknowledgements

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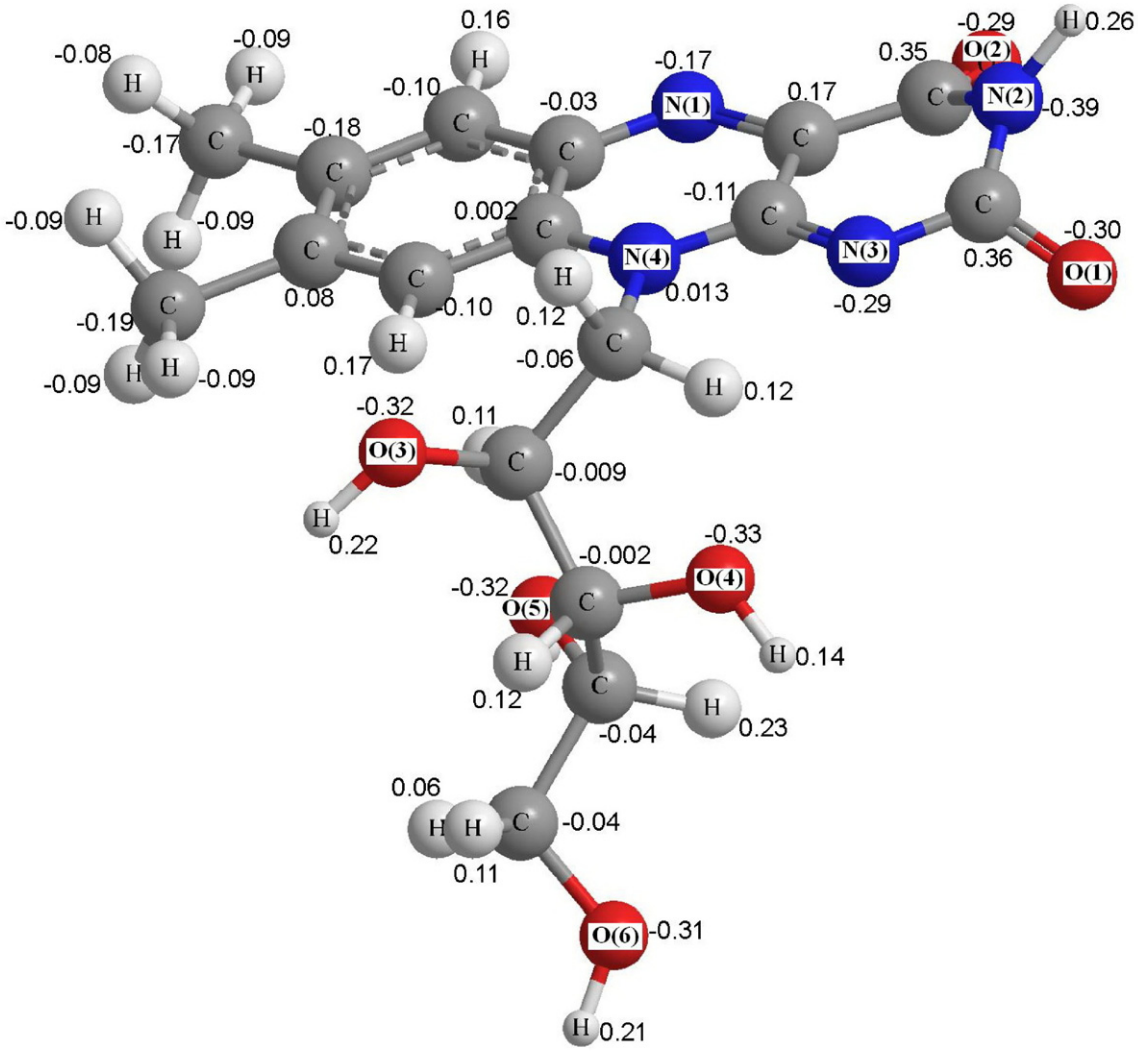
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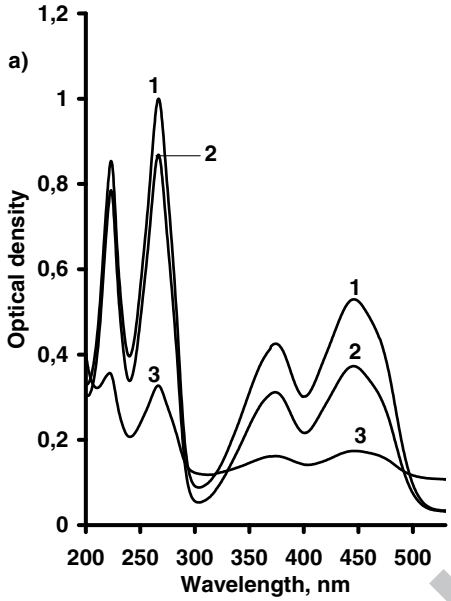

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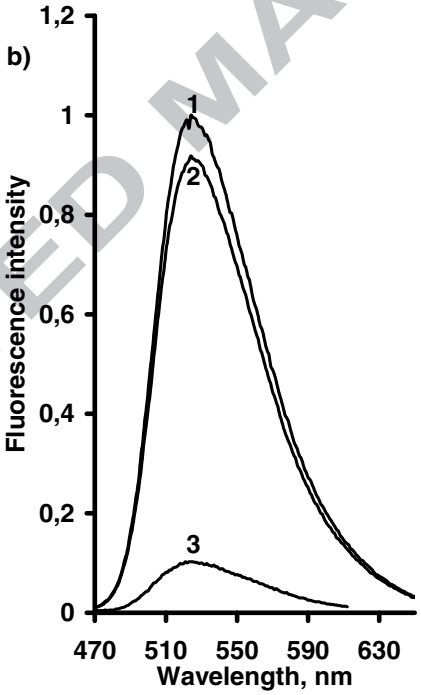

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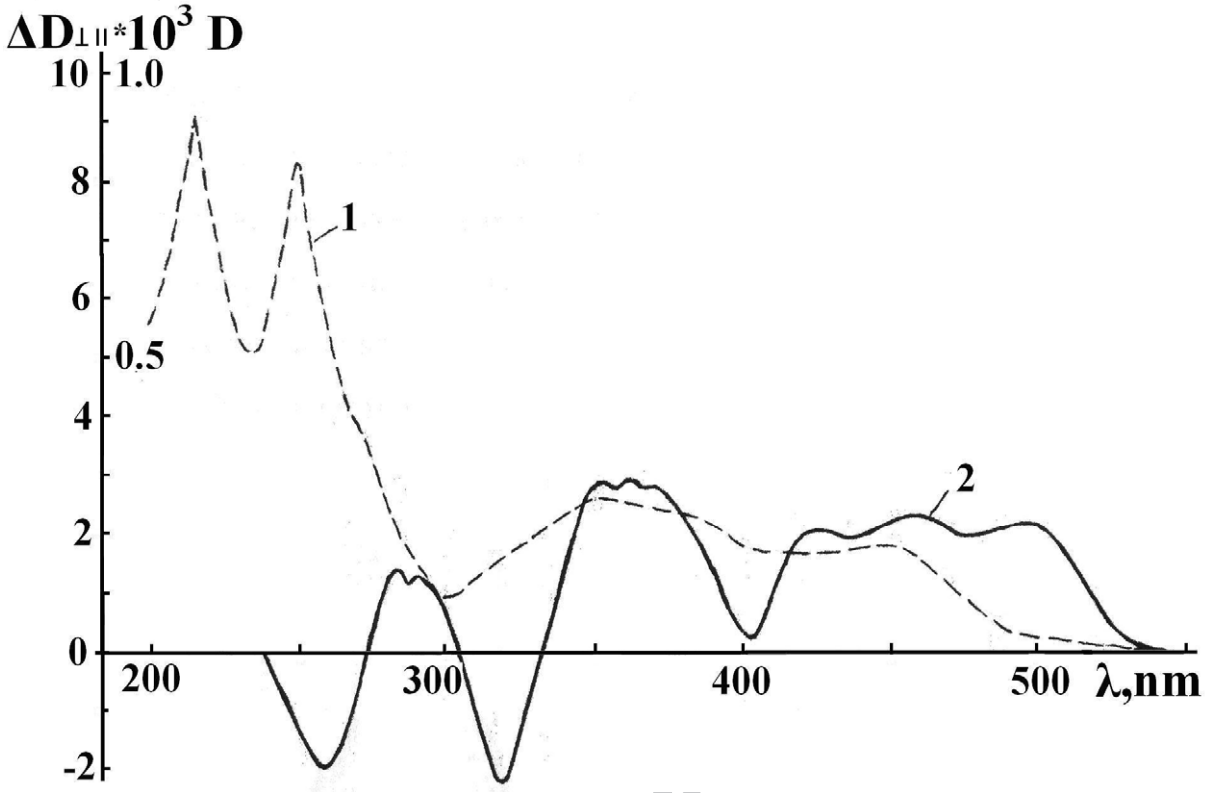

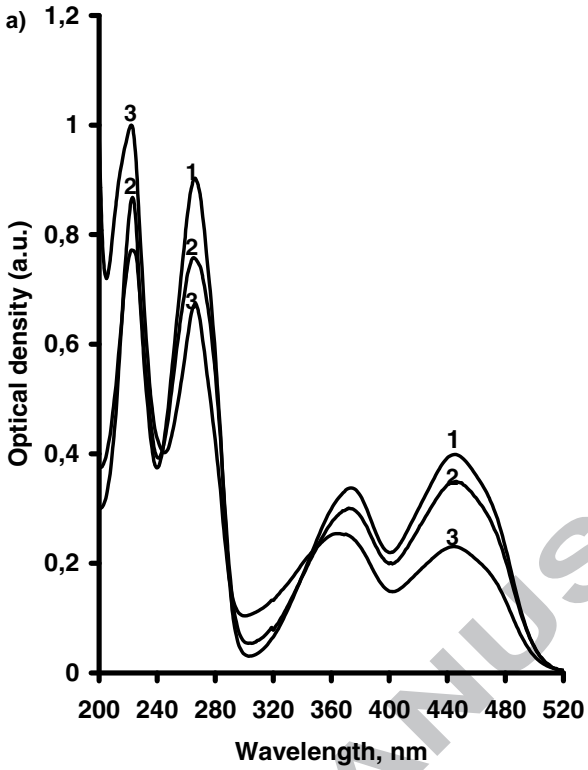

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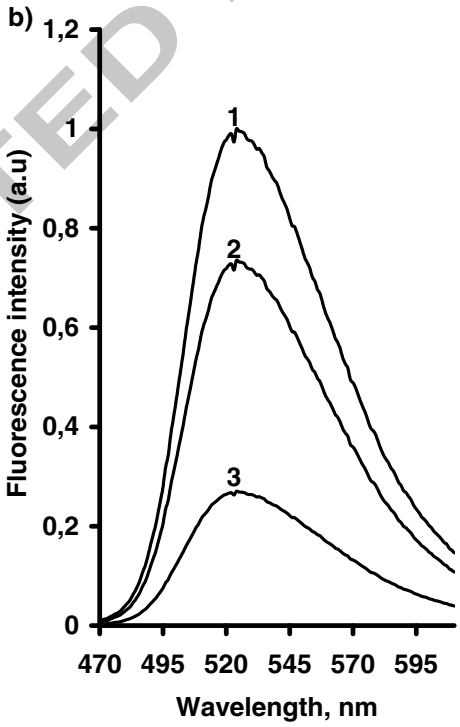

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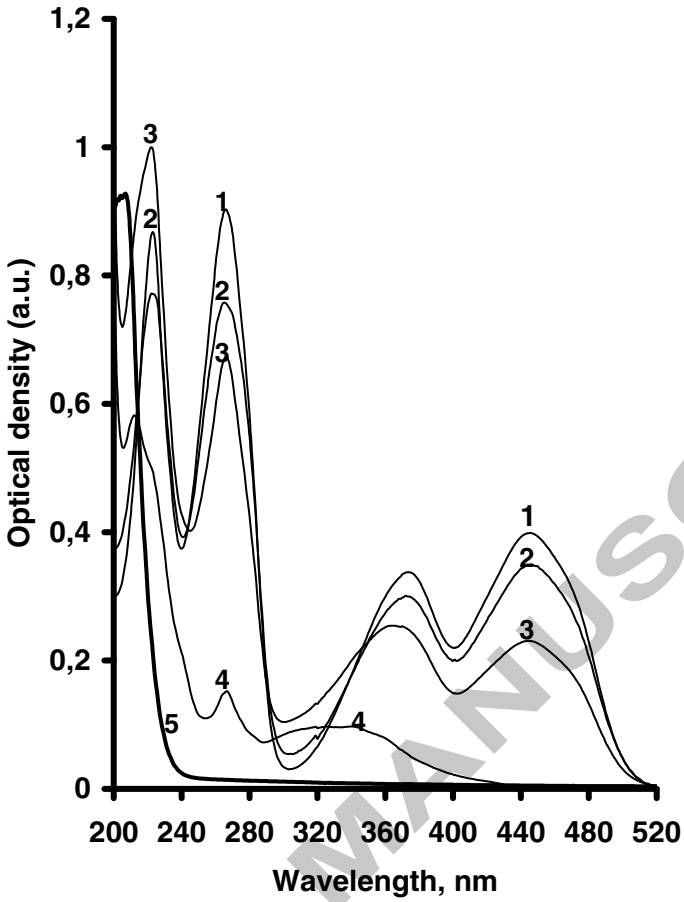


Figure 5

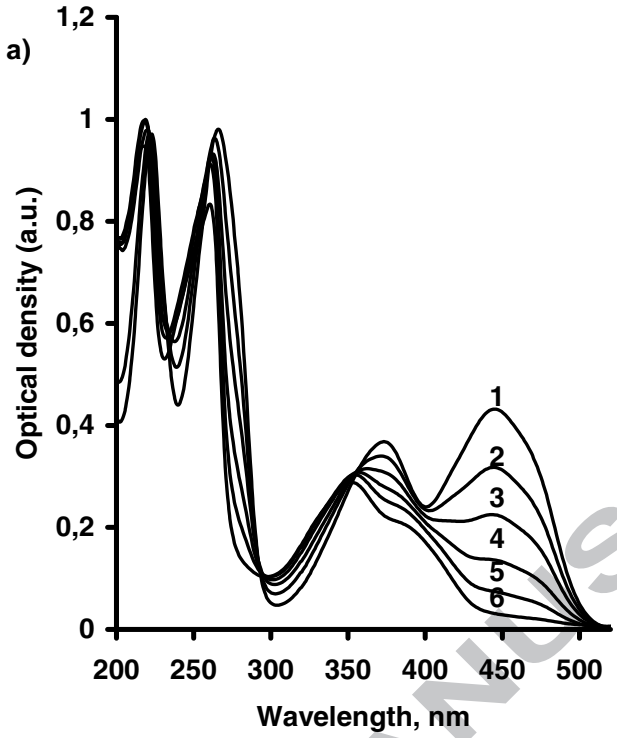
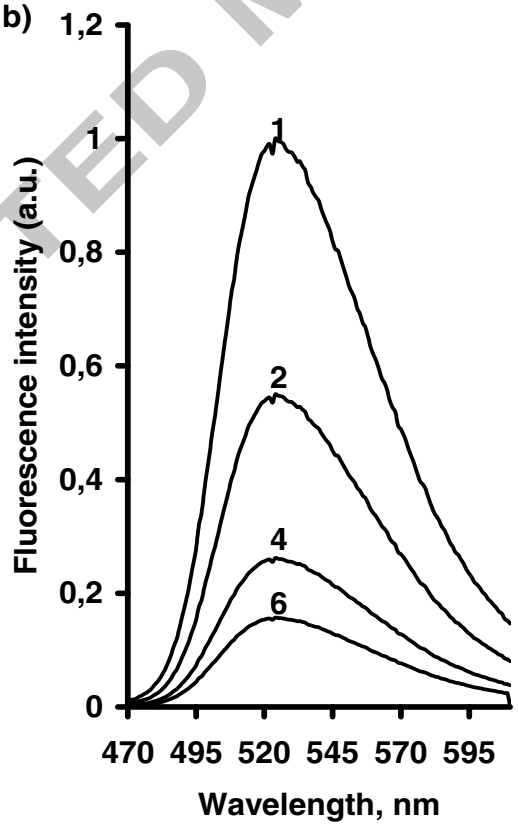


Figure 6a



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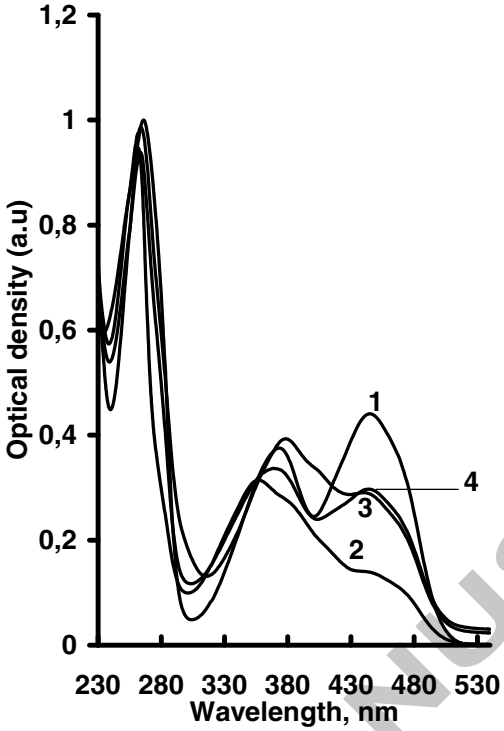


Figure 7

Figure 1. Structural formula and the distribution of charges in the riboflavin.

Figure 2. Concentration dependence of the absorption spectra (a) and fluorescence (b) riboflavin in water: 1-10-5, 2-2.5×10-4, 3-2×10-3 M.

Figure 3. The spectra of absorption (1) and linear dichroism (2) aggregated aqueous solution riboflavin in under laminar hydrodynamic flow.

Figure 4. The absorption spectra of (a) and fluorescence (b) of riboflavin in water ($c=2\times10^{-5}$ M) after thermal treatment at a temperature of about 423 K during: 1-0, 2-20, 3-60 minutes.

Figure 5. The dependence of the absorption spectrum of an aqueous solution of riboflavin at a temperature of 423K time 20-60 minutes (1-3), the absorption spectra of degradation products (4) and urea (5) ($c=2\times10^{-1}$M).

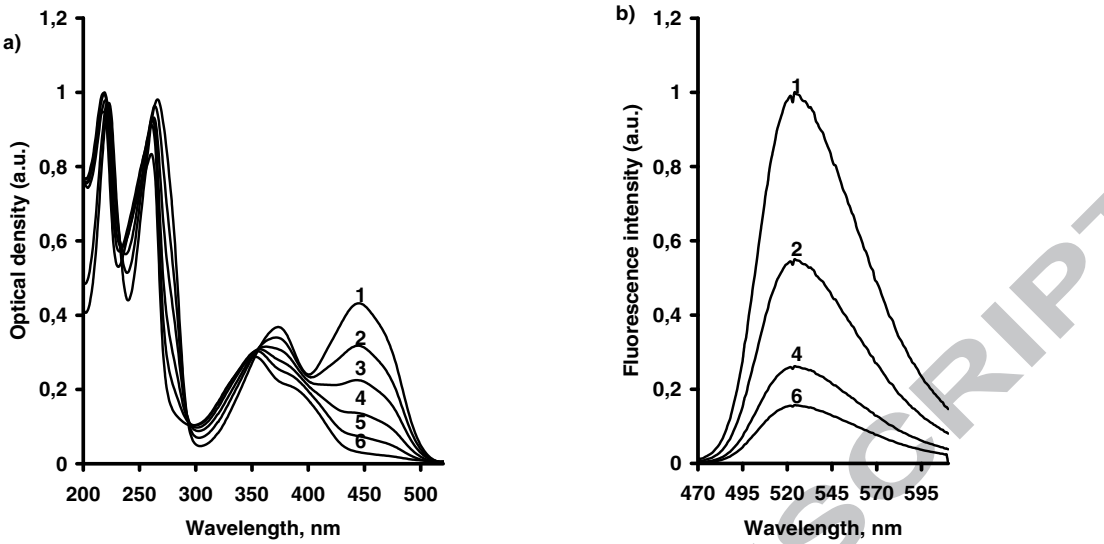
Figure 6. The absorption (a) and fluorescence (b) spectra of aqueous solutions of riboflavin exposed to irradiation by light: 1-0, 2-22, 3-47, 4-74, 5-100, 6-160 minutes.

Figure 7. Absorption spectra of aqueous solution of riboflavin ($c=10^{-5}$ M) as the unfiltered light irradiation: 1-unirradiated solution, 2-irradiated solution, 3-irradiated solution of HCl (0.025 M), 4-irradiated solution of HCl (0. 05 M).

Table 1. Duration of irradiation by light required for destruction of riboflavin in aqueous solutions (in minutes).

Solution	water	water+$Al_2(SO_4)_3$	water+$CuSO_4$	water+$MnCl_2$	water+NaCl	water+Ni(CH_3			
COO)$_2$	water+HCl	water+KBr	water+H_2SO_4						
Time	160	135	355	255	235	235	305	305	155

Graphical abstract



The absorption (a) and fluorescence (b) spectra of aqueous solutions of riboflavin exposed to irradiation by light: 1-0, 2-22, 3-47, 4-74, 5-100, 6-160 minutes.

Highlights

- The influence of temperature and light irradiation of aqueous solutions riboflavin was studied.
- The riboflavin has undergone destruction depending on the concentration and magnitude of temperature effects.
- The presence of various acids and metal ions in the solution leads to increased photostability.