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ORIGINAL PAPER

The Antioxidative Activity of Riboflavin in the Presence of Antipyrin. Spectroscopic Studies

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Abstract The effect of Antipyrin upon the antioxidant activity of the riboflavin has been evidenced using chemiluminescent system luminol–hydrogen peroxide, in Tris–HCl buffer, pH 8.5. It was found that riboflavin antioxidant activity depends on the reaction time and the Antipyrin concentration. Using ESR spectroscopy the hydroxyl radical generation, in the mentioned chemiluminescent system, was evidenced. The interaction between reactants was also investigated by UV-VIS and fluorescence spectroscopy. The effect of Antipyrin concentration upon the riboflavin fluorescence has also been investigated. The fluorescence quenching by Antipyrin is not significant and subsequently the riboflavin fluorescence quenching doesn't indicate an electron transfer process through diffusion-controlled mechanism. The results are discussed with relevance to the redox processes of riboflavin.

Keywords Chemiluminescence · Fluorescence · Riboflavin · Antipyrin · Free radicals

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Introduction

Riboflavin (RF) is the precursor for essential flavocoenzymes [flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD)]. FAD and FMN are the prosthetic groups of numerous enzymes that catalyze the various electron transfer reactions that occur in energy-producing, biosynthetic, and detoxifying and electron-scavenging pathways [1]. 2,3-Dimetyl-phenyl-3-pyrazolin-5-one (Antipyrin) is an antipyretic drug and used frequently due to its analgesic properties. Antipyrin has been used in many research studies to determine the cyclooxygenase activity of the liver [2, 3], being used as a marker for hepatic metabolic activity. It is rapidly distributed over the total body water and the hepatic clearance can be approximated by the systemic clearance, since the drug is almost completely metabolized by the hepatic P450 enzymes [4]. The advantage of a low clearance drug to measure the hepatic enzymatic activity is the insensitivity of the metabolic breakdown to the hepatic blood flow. The low clearance of Antipyrin has the consequence that the metabolic breakdown is slow, so after the complete and fast absorption, the bioavailability will be high. This is necessary to compete with biomolecules for free radicals. Antipyrin reacts fast with free radicals having a reaction rate constant in the order of 10^{10}–10^{11} l mol^{-1} s^{-1}. It was observed that in vivo experiments with Antipyrin as a marker for oxidative stress are complicated since the concentration of phenolic derivatives of Antipyrin is low. In this regard a method was developed to determine Antipyrin and its phenolic derivatives in plasma by means of reverse phase high performance chromatography combined with a mass spectrometer [4, 5]. Recently, the putative inhibitory effect of the aminoantipyrin, isopropylantipyrin and Antipyrin against human neutrophil burst and their scavenging activity against reactive oxygen

species, have been investigated [6]. It was found out that aminoantipyrin prevent phorbol-12-myristate-13-acetate-induced neutrophil burst with high efficiency, while isopropylantipyrin had little effect and Antipyrin had no effect at all.

By means of chemiluminescence method, using the luminol-hydrogen peroxide system [7–10], the luminol properties have been evidenced. Our investigations concerning the riboflavin antioxidant activity, using luminescence and chemiluminescence (with luminol as chemiluminescent compound) methods, deals with its investigation in the presence of KI, KBr, thiourea [11], cyclodextrins [12, 13], amino acids [14]. It was found that the antioxidant activity of riboflavin is decreased by I^- or Br^-, and thiourea having an opposite effect. An increase of the riboflavin fluorescence polarization in the presence of these additives was observed as a consequence of the fluorescence lifetime decrease [11]. As regard cyclodextrins and aminoacids, the order of improving the antioxidant activity of riboflavin was found to be: γ-CD>α-CD>β-CD [13] and Cys, His, Lys increase while Arg decreases the riboflavin antioxidant activity [14].

This work followed the previous mentioned papers and deals with the investigation by luminescence methods of the effect of Antipyrin on the riboflavin antioxidant activity, in order to get more details about their interaction and on the redox processes of the riboflavin in this complex system.

Experimental

Materials The system luminol (LH_2)-hydrogen peroxide (H_2O_2) at concentrations of $LH_2=2.5\times10^{-5}$ M and $H_2O_2=$ 30 mM in 0.2 M Tris–HCl buffer, pH 8.5 was considered the Reference System (RS). LH_2 and H_2O_2 are from Merck and the Tris buffer from Sigma. Antipyrin (AP) is from Aldrich. Riboflavin (RF) is from Fluka, vitamin C and β-cyclodextrin (β-CD) are from Merck. Thiourea, (5,5-dimethyl-1-pyrolin)-*N*-oxide (DMPO) and α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (PBN) are from Sigma. Solutions were prepared in distilled water.

Methods and apparatus The chemiluminescence (CL) measurements were undertaken with a chemiluminescence-measuring device TD 20/20 Turner Design, USA. The points on the plot were obtained by integrating the light signal on periods of 4 s. Five measurements were made and an average value calculated, obtaining a maximum 10% relative scattering of the results from the mean value. The working volume was 1,000 μl.

The extinction of the CL emission, S, was calculated according to the equation:

$$S(\%) = \frac{(I_0 - I)}{I_0} \times 100$$

Where I_0 and I represent CL intensity measured for the reference system and for the reference system in the presence of Antipyrin; both values were measured 5 s after the beginning of the reaction.

$I_{CL}=f(t)$ variation enables the determination of the rate constant of the reaction, for the upward part of the plot, $-k_2$, (attributed to the consumption of free radicals) as well as for the downward part of the curve, k_1, (attributed to the reaction of forming free radicals), considering that the CL reaction is of the first order. k_1 and k_2 values were determined using the following equation:

$$k = \frac{1}{\Delta t} \times \ln\frac{I_i}{I_0}, \Delta t = t_i - t_0$$

in which t_i represents the time at the i moment, t_0 represents the initial time, I_i is the intensity of CL signal at the moment i and I_0 is the intensity of the CL signal at the initial moment. The values of the rate constants were calculated on different time ranges, in this way:

- 5–300 s ($-k_2$) for the RS; RS/1 μM RF; RS/1:1, RF:AP systems;
- 5–60 s ($-k_2$) and 65-300 s (k_1) for the RS/1 μM RF/2.5 and 5 μM AP system;
- 5–75 s ($-k_2$) and 80-300 s (k_1) for the RS/1 μM RF/10 μM AP system;
- 5–70 s ($-k_2$) and 75-300 s (k_1) for the RS/1:1, RF: vitamin C and RS/1:1, RF: β-CD systems;
- 5–45 s ($-k_2$) and 50 -300 s (k_1) for the RS/1 μM RF—1:1, AP–βCD and RS/1 μM RF—1:1, AP–vitamin C systems;
- 5–80 s ($-k_2$) and 85-300 s (k_1) for the RS/1 μM RF—1:1, AP–thiourea system.

The fluorescence spectra (emission and excitation) were recorded with Perkin Elmer 204 spectrofluorimeter, interfaced to a computer, permitting a prestabilized reading time of the data. Usually the time range between two measurements is 550 ms.

The ESR spectra were recorded with a JEOL, JES-FA 100 spectrometer (X band frequencies), at room temperature (approximately 20 °C). The instrument setting: microwave frequency 9.455 GHz; microwave power 1 mW; center field 335.7 mT; sweep width 7.5 mT; gain 1,000; modulation amplitude 0.1 mT; modulation frequency, 100 kHz; sweep time 30 s; time constant 30 ms. The simulations were performed with Winsim-program standard.

The absorption measurements were recorded with Perkin Elmer Lambda 35 UV-VIS Spectrometer.

Molecular dynamics method was achieved with the Hyperchem (trial version) program [15] choosing total energy as property.

Results and discussion

The effect of Antipyrin on oxidation process

Chemiluminescence measurements

It is well known that LH_2–H_2O_2 in alkaline solution yields aminophtalate dianion excited species and leads to the appearance of oxygen free radicals such as: superoxide anion, singlet oxygen, hydroxyl radical [16, 17]. The result of the multiple interactions is the light emission, as luminol is consumed. In the presence of an antioxidant compound (which consumes free radicals), CL intensity will decrease, while the effect of a prooxidant compound (which increases the concentration of free radicals) will be an increase in CL intensity.

Thus, CL measurements were recorded in order to evaluate the antioxidative ability of RF in various systems. The variation of CL intensity as a function of time allows the determination of the quenching of CL reaction for the systems which mainly contain RF and AP.

The CL emission kinetics produced by LH_2 (2.5×10^{-5} M)–H_2O_2 (30 mM) reaction, in Tris–HCl buffer 0.2 M, pH 8.5 in the presence of Antipyrin, has been studied. As can be seen in the Fig. 1, at low concentration of Antipyrin, 2.5 and 5 μM, respectively, the CL intensity increases and depends on the reaction time. At higher concentrations of Antipyrin, 15 μM respectively, the CL intensity decreases comparatively with the reference system, fact which could be due to the decrease of oxygen free radicals produced in the CL reference system; the Antipyrin has in this case an antioxidant effect. The CL changes in the Reference System, in the presence of AP and different compounds are presented

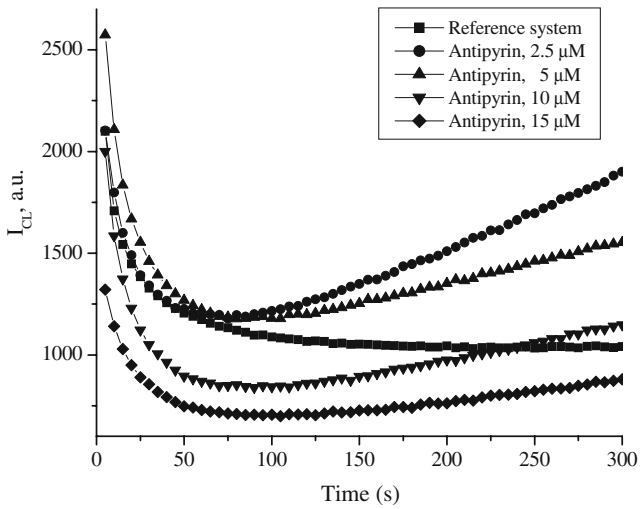

Fig. 1 The effect of Antipyrin on the CL Intensity of the LH_2 (2.5×10^{-5}M)–H_2O_2 (30 mM), in Tris–HCl buffer pH 8.5 (Reference System)

Table 1 The chemiluminescence changes in the RS, in the presence of AP and different compounds, 5 s from the beginning of the CL reaction

System	I_{CL} (a.u.)	S (%)
RS	2,099	–
RS/1 μM RF	1,153	45.06
RS/1 μM RF/15 μM AP	1,321	37.06
RS/RF-vitamin C, 1:1	239	88.61
RS/1 μM RF/1:1, AP–vitamin C	1,046	50.16
RS/RF–β-CD, 1:1	1,931	7.95
RS/1 μM RF/1:1, AP–β-CD	1,723	17.91
RS/RF+AP, 1:1	557	73.45
RS/1 μM RF/1:1, AP–thiourea	100	95.21

in Table 1. From CL curves in the time range t_1–t_2, kinetic rate constants (Table 2), have been determined.

For the comparison, in the same systems, the effect of vitamin C (instead of AP), using the same molar ratios (as RF/AP), was also investigated. It is well known that vitamin C is a very good antioxidant [18].

Figure 2 shows the variation of CL intensity in the presence of RF and mixtures RF/vitamin C, RF/β-CD and respectively, RF/AP. It was found (Fig. 2) that the presence of vitamin C in the LH_2–H_2O_2–RF system leads, in the first moments of the reaction (0–50 s), to a decrease of the CL intensity. After 50 s from the reaction start, the CL intensity increases due to the consumption of vitamin C in the system. As can be seen in the Fig. 2, in the first moments of the reaction the CL intensity is lower in the presence of vitamin C compared to Antipyrin, fact due to the stronger antioxidant effect of vitamin C.

In the presence of AP, CL intensity increases up to 50 s from the beginning of the CL reaction, fact that leads to a decreasing in the RF antioxidant activity. After 50 s, CL intensity strongly decreases; therefore, depending of the

Table 2 The kinetic parameters of CL process (the rate constants) in the presence of different compounds

System	$k_1\times10^2$, s^{-1}	$-k_2\times10^2$, s^{-1}
RS		0.28
RS/2.5 μM AP	0.20	1.04
RS/5 μM AP	0.10	1.34
RS/10 μM AP	0.13	1.23
RS/15 μM AP	0.10	0.78
RS/1 μM RF	–	0.24
RS/ 1: 1, RF - vitamin C	0.33	0.06
RS/ 1: 1, RF - β-CD	0.14	0.84
RS/ 1: 1, RF – AP	–	0.31
RS/1 μM RF/1:1, AP–β-CD	0.11	1.15
RS/1 μM RF/1:1, AP - vitamin C	0.25	0.71
RS/1 μM RF/1:1, AP - thiourea	0.65	3.07

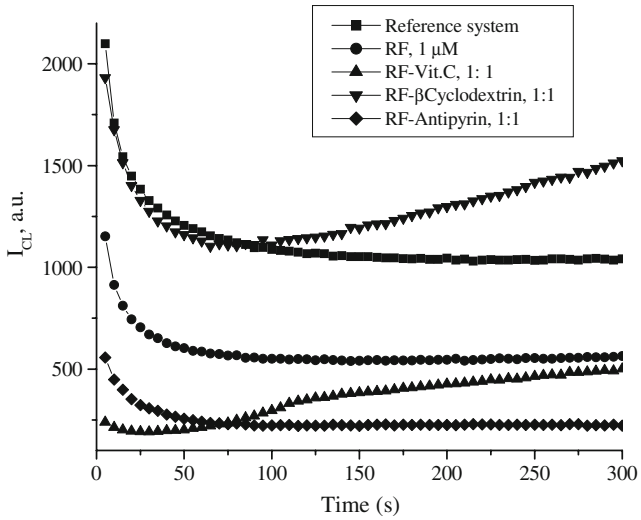

Fig. 2 The CL emission kinetics produced by LH_2 (2.5×10^{-5} M)–H_2O_2 (30 mM) reaction, in Tris–HCl buffer 0.2 M, pH 8.5 in the presence of riboflavin (RF) and RF–vitamin C, RF–β cyclodextrine and RF–Antipyrin mixture

reaction time, RF antioxidant activity is strongly influenced by the presence of AP.

The presence of β-CD in LH_2–H_2O_2 system in alkaline medium as well as in the LH_2–H_2O_2–RF system has as effect an increase in CL intensity suggesting that concentration of oxygen free radicals also increases in time. It is know that β-cyclodextrin can form inclusion complexes in aqueous solution with various organic compounds, one of the consequences of this process being the effect on the reactivity of included compound; cyclodextrin can protect it or can act as a catalyst on the reaction of included compound [19]. In case of the systems discussed in this paper seems that β-cyclodextrin acts as a catalyst for producing more free radicals.

It was found that the CL intensity in the LH_2–H_2O_2–RF system, in the presence of Antipyrin, depends on AP concentrations; CL intensity decreases when the AP concentration in the system is high. The quenching of the CL intensity in the presence of 15 μM AP is approximate 37%, that means that AP, at this concentration, has a protective effect into oxidation process. At lower AP concentrations (2.5 μM), in the LH_2/Tris–HCl pH 8.5/H_2O_2/RF system, the quenching of CL intensity is slow, because in this case riboflavin acts as catalyst of the reaction that generates hydroxyl radicals.

The effects attributed to the presence of Antipyrin, vitamin C, thiourea or β-cyclodextrin on the process taken in discussion and the consequence of the additives on riboflavin antioxidant activity in the Reference System were considered and compared. Thiourea, as vitamin C, have been chosen because are the most antioxidant biomolecules [18, 20]. Moreover, it was found that thiourea is the most active adjuvant for enhancing the absorption of quinine, whereas N,N′-diethylthiourea was the only significant adjuvant for quinidine. The urea and urea derivatives have little or no effect on the absorption of the drugs [20].

As can be seen in Fig. 3, the CL intensity is strongly influenced by the presence of thiourea in the system, due to its strong antioxidant activity, that has an efficient participation in auto-oxidation process. Thus the antioxidant activity of riboflavin is improved. In what concern the interaction AP–vitamin C, it can be noticed that the CL intensity increases 25 s after the reaction start. The interaction AP–vitamin C, in the Reference System-RF, induces a rapid consumption of vitamin C and the antioxidant activity of riboflavin decreases. The interaction AP–β-CD leads to an attenuation of riboflavin antioxidant activity which is more pronounced than in case of AP–vitamin C interaction. Thus, the kinetics of the CL process is more rapid in the case of thiourea compared to vitamin C, for the same 1:1 molar ratio AP–thiourea and AP–vitamin C. Moreover, the inhibition percentage when the system contents thiourea, represent about 90%, this value supporting (suggesting) the strong antioxidant activity of thiourea in the mentioned system.

In case of vitamin C and β-cyclodextrin, the process takes place according to first order kinetique, in two steps: in the first step, the free radicals produced in the system are scavenge (approximately 125 s after the reaction start), and in the second step new structures with prooxidant character are formed, in which case free radicals concentration in the system increases. In case of thiourea one can see that free radicals are scavenged, RF preserving its antioxidant activity in time, due to the strong antioxidant effect of thiourea.

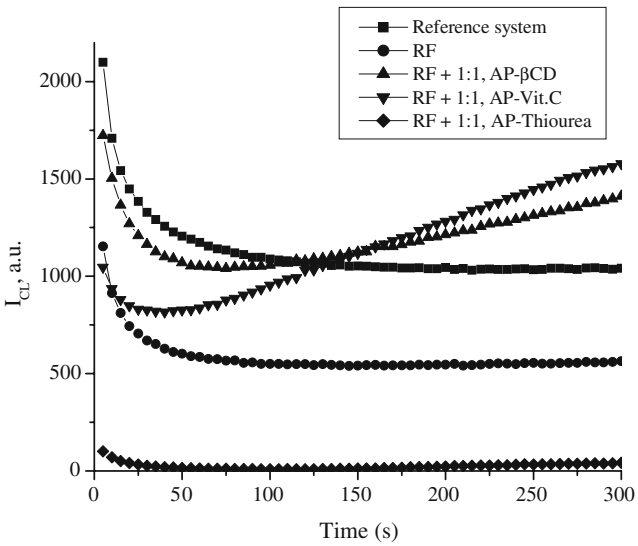

Fig. 3 The Antipyrine influence on riboflavin antioxidative activity, when others antioxidants are present in the system

These aspects could be observed also from Table 2, where the rates constant of the reaction attributed to the reaction of forming free radicals (k_1) as well as the rates constant of the reaction attributed to the consumption of free radicals ($-k_2$), are presented considering that the CL reaction is of first order. How we can observe, AP presents an antioxidant effect in the luminol–hydrogen peroxide CL system, the rate constant value attributed to the reaction attributed to the consumption of free radicals being higher than the rate constant value attributed to the reaction of forming free radicals. Moreover, one can observe that in the system containing AP–thiourea, the rate constant value of the reaction attributed to the consumption of free radicals is approximately five order of magnitude higher than of the rate constant value of the reaction corresponding to the free radicals forming.

ESR measurements

To evidence $HO^{\cdot}$ free radical generating in LH_2/Tris–HCl pH 8.5/H_2O_2/RF/AP system, some spin trapping experiments were done, using as spin trap compounds DMPO or PBN. The ESR (electron spin resonance) spectra of spin trap adducts were recorded for three systems: LH_2/H_2O_2/AP (I); LH_2/H_2O_2/RF/AP (II) and $LH_2/H_2O_2/Fe^{2+}$/RF/AP (III), immediately after preparation of reaction mixture and after few hours. In case of PBN, an ESR signal corresponding to a spin adduct was not observed for the system I; the same aspect was noticed 7 h from the reaction start. In case of system II, although no ESR signal was detected initially, 7 h after the reaction start, a weak ESR signal was registered. In case of system III, the ESR signal was instantly registered as well as 12 h after the reaction start.

In case of DMPO, for system I no ESR signal neither immediately nor after the reaction start was observed. For system II, an ESR signal characteristic for DMPO-$HO^{\cdot}$ adduct was detected 7 h after the reaction start. In case of system III a quick decomposition was observed, and after 7 h from the reaction start only the spin adduct decomposition product was evidenced.

Figure 4 presents ESR spectra corresponding to the three systems previously mentioned. In Fig. 4a is presented the ESR spectrum corresponding to the spin adduct in case of system II, 7 h after the reaction start; DMPO was used as spin trapper. ESR parameters obtained from spectra

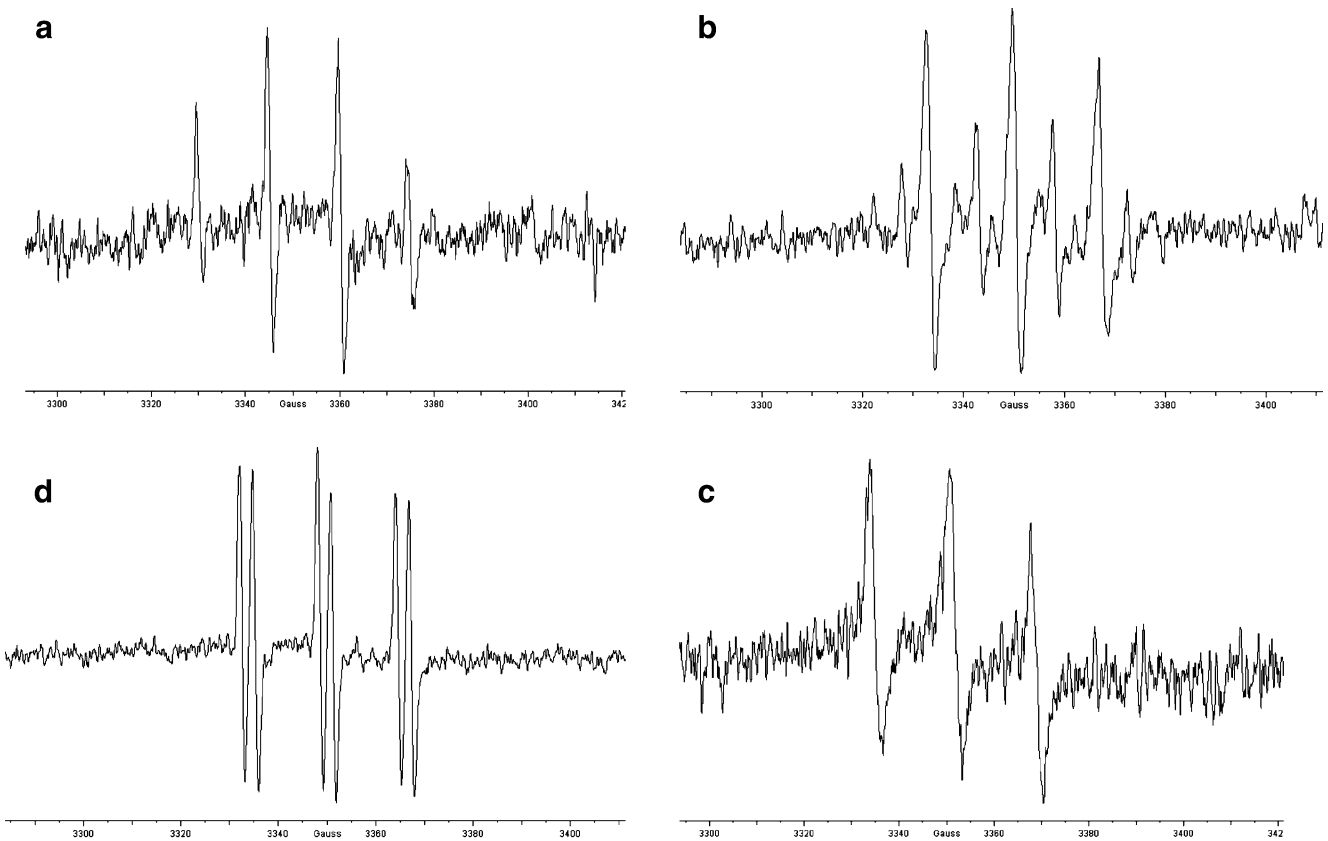

Fig. 4 ESR spectra corresponding to the LH_2–H_2O_2, in alkaline solution, -riboflavin–Antipyirin: **a** system II) LH_2–H_2O_2/riboflavin/Antipyrine, 7 h after the reaction start; DMPO as spin trapper; **b** system III) $LH_2/H_2O_2/Fe^{2+}$/riboflavin/Antipyrin, DMPO as spin trapper; **c** system III) $LH_2/H_2O_2/Fe^{2+}$/riboflavin/Antipyrin, 7 h after the reaction start, DMPO as spin trapper; **d** system III) $LH_2/H_2O_2/Fe^{2+}$/riboflavin/Antipyrin, PBN as spin trapper

simulation for this spin adduct are: a_N=14.96 G and a_H= 14.85 G. Figure 4b presents the ESR spectrum with two components for the system III. Parameters found from simulations are: a_N=14.96 G, a_H=14.85 (for the spin trap adduct) and a_N=17.03 G (a three line spectrum, corresponding to the DMPO-OH adduct degradation). After 7 h from the reaction start in the system III, ESR spectra of reaction mixture corresponds only to the degradation product of spin trap adduct (Fig. 4c) In Fig. 4d the ESR spectrum corresponding to the system III is presented; the spin trapper used was PBN. Parameters found in this case are: a_N=16.02 G and a_H=2.69 G.

These results do not evidence a spin trap adduct corresponding to a free radical which could derive from Antipyrin. If the riboflavin is used as a good scavenger, more radicals will react with the scavenger, caused by the competition between Antipyrin and riboflavin, leading to a lower conversion of Antipyrin.

The effect of Antipyrin on the riboflavin fluorescence

Absorption and Fluorescence measurements

By addition of lower AP concentrations (4.42×10^{-5}M÷ 11.06×10^{-5}M), the fluorescence intensity of RF increases, and was found to decrease continuously by gradual addition of higher Antipyrin concentration (Fig. 5), without any appreciable change in the position of maximum emission band. Figure 5 presents the fluorescence emission spectra of riboflavin in the presence of Antipyrin. One observed that the florescence excitation spectra agrees well with the absorption spectra.

The absorption spectrum of RF (c=2.83×10^{-5} M) is not affected in the presence of AP (c=11.06×10^{-5} M); thus, one can observe two RF absorption bands λ_{abs}: 373.50 and 445.62 nm with absorbances A=0.44 and 0.52 in presence of AP, and λ_{abs}: 373.14 and 445.26 nm with A=0.43 and 0.50 for RF without AP, therefore the RF–AP complex formation in the ground state doesn't take place.

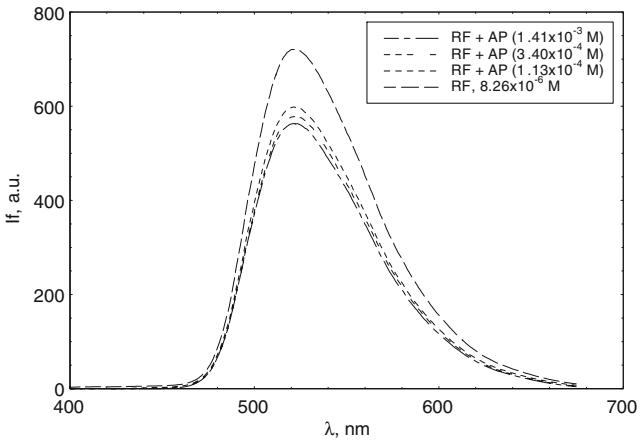

Fig. 5 The fluorescence emission spectra of RF in the presence of Antipyrin. The Antipyrin concentration increases downwards; λ_{ex}= 365 nm

One can conclude that the weak RF fluorescence quenching observed (Fig. 5) is not a fluorescence static quenching process by a nonfluorescent complex.

Table 3 Calculate values of potential energy of riboflavin (RF) and Antipyrin (AP) by Hyperchem (trial version) program [15]

Temperature (K)	$Energy_{RF}$ (kcal/mol)	$Energy_{AP}$ (kcal/mol)
225.92	−8987.22	−2692.35
256.67	−8984.05	−2692.78
272.46	−8986.33	−2692.28

Molecular dynamics on the riboflavin–Antipyrin interaction

To complete the investigation of the riboflavin–Antipyrin interaction, a Molecular Dynamics study was performed. It is well known that Molecular dynamics method allows the study of the structure and key properties like stability, diffusion, binding between molecules, and vibration or to study the dynamics of large macromolecules, including biological systems such as proteins, nucleic acids (DNA, RNA), membranes. Recently, it is widely used for drug design which is very common today in the pharmaceutical industry for the testing of a molecule's properties at the computer without the need to synthesize it (which is far more expensive). Dynamic events may play a key role in controlling processes which affect functional properties of the biomolecule [21].

In what concern computer simulation of the dynamics of riboflavin and Antipyrin biomolecules by the Molecular dynamics, we tried to found the possibility of describing and understanding of the structure–dynamics–function relationships of molecular processes in terms of interactions at the atomic level between these kinds of biomolecules.

To get information on potential energy of riboflavin ($Energy_{RF}$) and Antipyrin ($Energy_{AP}$) the temperature was scanning up to 300 K. From energetic point of view three random temperatures were chosen and the potential energy values obtained for these two biomolecule are presented in Table 3. Representing $Energy_{AP}$ (kcal/mol) vs. $Energy_{RF}$ (kcal/mol) for riboflavin, statistical parameters were found: Y=− 0.152X−4059.9; N=3; R^2=0.851; SD=0.150; F= 5.432; R^2 (CV)=0.812, where: SE is standard deviation of estimation, R is coefficient of multiple correlation, F is Fischer factor and R^2(CV) is cross validated correlation coefficient (R) [22]. Therefore, function of the potential energy value, $Energy_{RF}$ lower than $Energy_{AP}$, one can note that RF molecule is more stable to its interaction with Antipyrin.

Conclusions

Some aspects regarding the antioxidant activity of riboflavin and the influence of Antipyrin addition were studied by means of different methods, in principal chemiluminescence. Using of the chemiluminescent reference system LH_2/H_2O_2, Tris–HCl pH 8.5, and the influence of others additives which present biochemical interest such vitamin C, thiourea, β-cyclodextrine, were evidenced. The systems described in this paper can be considered as models for redox reactions which are taken part in biological organisms.

Thus, it was found out that Antipyrin, at concentrations in the 10–15 μM range decreases the chemiluminescence intensity of reference system that means that AP, at these concentrations, has a protective action against oxidative destruction caused by free radicals. In presence of riboflavin in the reference system, the decreasing of intensity is more pronounced, thus AP enhances its antioxidant activity. At lower concentrations of Antipyrin, the hydroxyl free radical is generated in the LH_2/H_2O_2, Tris–HCl pH 8.5/ riboflavin/Antipyrin system. Therefore, the action of antypirin on antioxidant property of riboflavin depends on the reaction time and on the concentration of Antipyrin.

By comparison, thiourea, vitamin C the increase of the riboflavin antioxidant activity is higher, the effect being dependent on the reaction time. β-cyclodextrine doesn't improve the riboflavin antioxidant activity because this molecule, by its cavity dimension, is a medium for free radicals generation in time.

The potential energy value of riboflavin is lower than the potential energy value of Antipyrin; the riboflavin molecule is more stable to its interaction with Antipyrin.

The effect of Antipyrin on the riboflavin UV-VIS absorption and fluorescence intensity is not significant, therefore antypirin does not react with riboflavin. These experimental data could be quite predictable for the new investigations as regard the future investigations on the effect of a drug like Antipyrin on the activity of a compounds such a riboflavin derivatives; hence the importance and the interest of these fluorescence studies.

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