Energy Transfer from the Aminophthalate Dianion to Fluorescein

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Energy transfer from the excited aminophthalate dianion species to fluorescein at pH 8.32 (Tris–HCl buffer) was studied. The excited aminophthalate dianion species was obtained either by excitation with UV radiation (330 nm), with fluorescence emission, or by the well-known chemical reaction luminol–hydrogen peroxide in an alkaline medium, with chemiluminescent emission, both with λ_{max} at 425 nm. The influence of Co^{2+} and Mg^{2+} on fluorescence and chemiluminescence (CL) was studied. It was found that at low concentrations (10^{-7}–10^{-9} M), these ions do not modify the fluorescein fluorescence, however, the CL is strong affected. The effect of the concentration of these elements, which exert an influence on CL even at a high dilution (nanomolar concentration), was determined. In the case of Co^{2+} the prooxidant character is stronger than in the case of Mg^{2+}, and therefore the CL enhancer effect is higher. Compared to the system without catalyst, their presence ensures stronger, prolonged, and stable light emission. The emission spectra, in the presence of fluorescein, show two bands with maxima at 425 and 520 nm, the second one being specific to fluorescein emission. The intensity of aminophthalate dianion luminescence is lower and the duration shorter in the presence of fluorescein. The influence of Co^{2+} and Mg^{2+} catalyst and fluorescein concentration on the energy transfer process was studied. The efficiency of the energy transfer process for these two situations (fluorescence and CL) was compared. An attempt was made to replace hydrogen peroxide with superoxide anion (solubilized by means of crown ether) and its effect upon the energy transfer process was observed.

KEY WORDS: Energy transfer; aminophthalate dianion; fluorescein; chemiluminescence.

INTRODUCTION

The study of energy transfer is important owing to the numerous applications in biochemical research, especially because of the dependence of the transfer rates on the distance between the donor and the acceptor. Although the process of fluorescence energy transfer has been intensively studied, considerably less is known about chemiluminescence (CL) energy transfer, a physi-cal process with applications in analytical chemistry and biochemistry [1–3,6,7]. Immunology measurements were carried out using the energy transfer method [3,6].

One knows that, after the homogenizing of the reactants, the intensity of CL (I_{CL}) increases at first and then decreases exponentially in time, as the reactants are consumed. This characteristic is described by the following equation:

$$I_{CL}(\text{photons/s}) = \Phi_{CL} \text{ (photons molecules reacted)}$$
$$\times (dC/dt)$$

where Φ_{CL} is the efficiency of the CL process, which may be defined as the number (or rate) of photons emitted per number (or rate) of the molecules which react, and

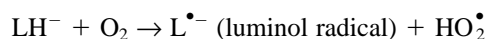[1] Institute of Physical Chemistry, Splaiul Independentei 202, 77208 Bucharest, Romania.
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dC/dt represents the number of reacted molecules per time unit [1,3–5,7–9].

The duration of CL may vary from fractions of seconds to 0.5 h; this time is long enough to be influenced by the various factors. Therefore the reaction products of a chemical process with CL emission can be determined by fluorometric analysis [10–13]. One of the biochemical applications of CL is offered by the possibility to determine the free radicals of oxygen [3,4].

The excited aminophthalate dianion species may be obtained either by excitation with UV radiation, with fluorescence emission, or by the well-known chemical reaction luminol–hydrogen peroxide in an alkaline medium, with chemiluminescent emission, both with λ_{max} at about 425 nm [8,14–17]. It is known that the mechanism of CL emission is based on the following equations:
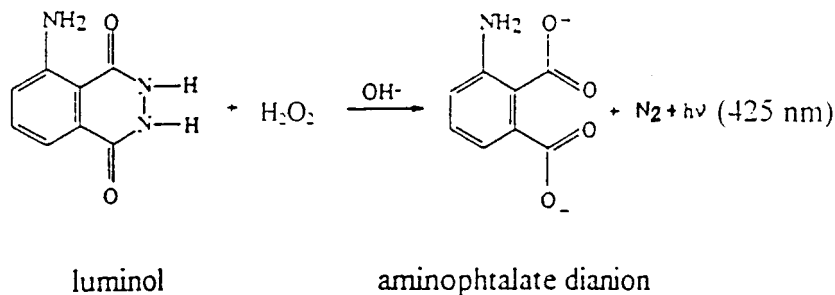
$$LH_2 \text{ (luminol)} \rightarrow LH^- \text{ (luminol anion)} + H^+$$

$$LH^- + O_2 \rightarrow L^{\bullet-} \text{ (luminol radical)} + HO_2^{\bullet}$$

The $HO_2^{\bullet}$ radical is the hydrate form of the superoxide radical ($O_2^{\bullet-}$). It is possible that, under specific conditions, equilibrium $HO_2^{\bullet} \Leftrightarrow O_2^{\bullet-}$ could be attained. $O_2^{\bullet-}$ reacts spontaneously, yielding oxygen in the singlet excited state:

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + {}^1O_2$$
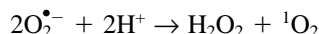
The singlet oxygen transfers energy to aminophthalate dianion.

The general reaction for aminophthalate dianion production with CL emission is shown in Scheme I.

Studies were carried out on the singlet–singlet energy transfer making use of the luminol–hydrogen peroxide system in alcaline buffer in the presence of fluorescein [2,18,19], and different mechanisms were advanced for the interpretations of the results for the respective systems and medium conditions. It was found that fluorescein is a weak enhancer of CL, increasing the conversion of luminol to luminol radical. The enhanced CL of luminol is transferred to fluorescein, with a new emission maximum appearing around 525 nm.

The equations which describe the energy transfer process in the two cases, CL and fluorescence, are similar and are based on Förster's theory [20,21]. The rate constant of fluorescence energy transfer from donor to acceptor is

$$k_{TF} = r^{-6}\chi^2 \, J \, n^{-4}\phi_d/\tau_d \times 8.71 \times 10^{23} \quad (s^{-1}) \quad (1)$$

and the rate constant of CL energy transfer is

$$k_{TCL} = r^{-6}\chi^2 \, J \, n^{-4} \, K_{CL} \times 8.71 \times 10^{23} \quad (s^{-1}) \quad (2)$$

where r is the distance between donor and acceptor; χ^2, the relative orientation factor of the donor and acceptor transition dipoles; n, the refractive index of the medium; and K_{CL}, the emissive rate of the donor.

$$J = \int [F_d(\nu)\varepsilon_a(\nu)]/\nu^4 d\nu \quad (3)$$

where J is the spectral overlap integral; $F_d(\nu)$, the emission spectrum of the donor, with the total intensity normalized to unity; $\varepsilon_a(\nu)$, the extinction coefficient of the acceptor; ϕ_d/τ_d, the the emissive rate of the donor; and R_0, the characteristic distance (Förster distance) at which the efficiency of energy transfer is 50%. At this distance one-half of the donor molecules decay by energy transfer and one-half by radiative and nonradiative processes:

$$R_0 = 9.79 \times 10^3(\chi^2 \, J \, n^{-4}\phi_d)^{1/6} \quad (\text{Å}) \quad (4)$$

$$R_0 = 9.79 \times 10^3(\chi^2 \, J \, n^{-4}Q)^{1/6} \quad (\text{Å}) \quad (5)$$

where Q is the quantum coefficient of the donor CL in absence of acceptor.

This contribution aims at a qualitative study on the chemiluminescence energy transfer from the aminophthalate dianion to fluorescein in buffered (Tris–HCl, pH 8.32) solution. The influence of Co^{2+} and Mg^{2+} addition and of the fluorescein concentration on the energy transfer process is determined. The R_0 values are calculated in the two cases of CL and fluorescence energy transfer.

Scheme I. Left: Luminol. Right: Aminophthalate dianion.
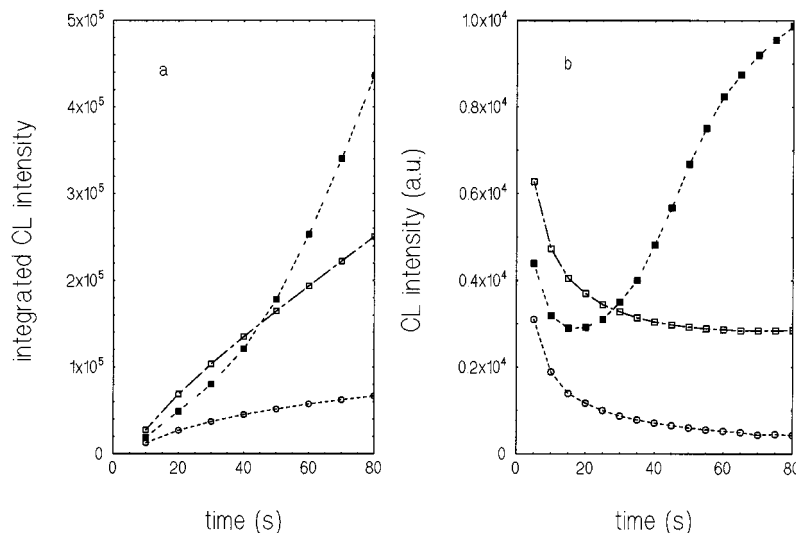
Fig. 1. Co^{2+} (■) and Mg^{2+} (□) effects on time variation of the chemiluminescence. Reference system: LH_2 (2 μM)–H_2O_2 (25 μM) in Tris–HCl buffer, pH 8.32 (○).

EXPERIMENTAL

The luminol (LH_2)–hydrogen peroxide (H_2O_2) system with concentrations of LH_2 = 2 μM and H_2O_2 = 25 mM, in 0.2 M Tris–HCl buffer, pH 8.32, was considered the reference system. Luminol and hydrogen peroxide were obtained from Merck, while Tris–HCl was from Sigma. A concentrated luminol solution in DMSO was used, from which working dilutions were made with buffer. The concentration for fluorescein was of the micromolar order, while the Co^{2+} and Mg^{2+} ion concentrations were in the micromolar to nanomolar domain.

We worked with a volume of 1000 μl in the case of measurements carried out with a TD 20/20 chemiluminometer (Turner Designs). The data (Figs. 1 and 2) were obtained by integration of the signal on 4 s. Measurements were carried out in five replicas and averaged, obtaining a relative scattering of the results of up to 10% of the average value.

We worked on a 2500-μl volume in the case of measurements carried out with a Perkin–Elmer spectrofluorometer, provided with a 150-W Xe excitation lamp (in CL measurements the lamp was obturated). The device was interfaced to the computer, allowing preestablishment of the reading time (usually the interval between two measurements is 550 ms) and processing of the data (Figs. 3–8).

LH_2-anion superoxide, solubilized by means of ether corona 18C6 in DMSO, was used. KO_2 and 18C6 were obtained from Merck–Schuchardt, and DMSO was from Merck. The molar ratio of 18C6:KO_2 was 2:1 and the

measurements were effected with a Perkin–Elmer spectrofluorometer.

RESULTS AND DISCUSSION

Selection of the buffer is very important because it ensures the conditions, which evidence the modifications clearly, which prevail in the system. From this point of view it would be ideal for the reaction of the generator system to have no very rapid variation, to be able to
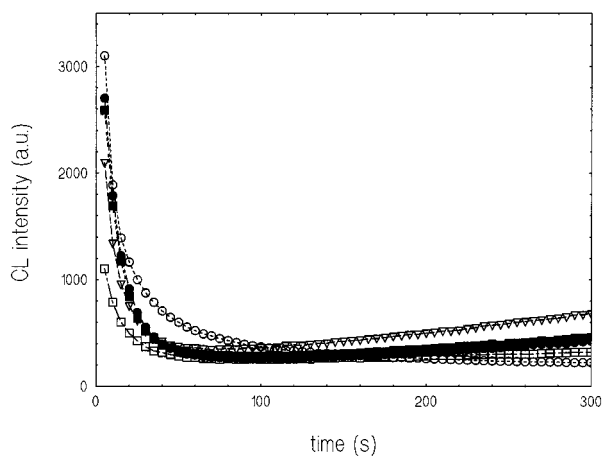
Fig. 2. Dependence of the total CL intensity on the various concentrations of fluorescein: 20 μM (□); 2 μM (▽); 0.4 μM (■);0.2 μM (●). Reference system as in Fig. 1 (○).
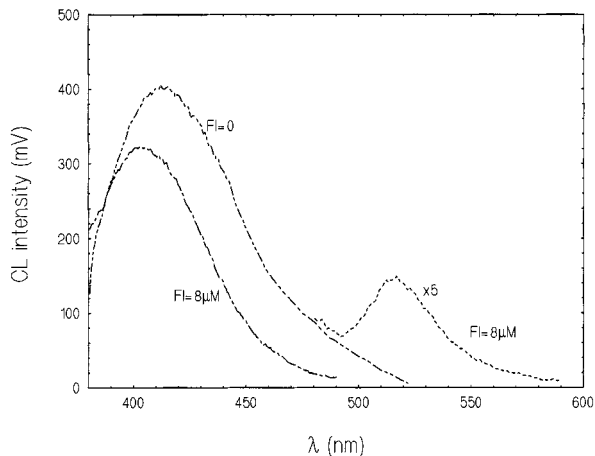
Fig. 3. CL spectra of aminophthalate dianion (a) and aminophthalate dianion with fluorescein (b).

determine the modifications of reaction rate assigned to different molecules in the system. It was found that Tris–HCl shows considerable stability over time, and it is frequently employed in biological studies. In a previous investigation [22] the influence of pH on the CL reaction of LH_2–H_2O_2 in Tris–HCl buffer, at pH values higher than 7.60 was studied. We found an exponential decrease in the CL intensity and a pH dependence on CL. For the present determinations, pH 8.32 was chosen because in this case the maximum CL intensity was evidenced (in this buffer the CL decreases at higher pH values), the decay curve being characteristic of the time evolution of an intermediary, in the excited state, with CL emission.

The effect of reactant concentrations on CL was determined, and we observed that at a luminol concentration within the range of 1–2 μM and an oxidizer concentration in the range of 25–55 mM, an increase in CL intensity occurs. In time, the CL intensity decreases exponentially, followed by a plateau, whose value depends on the substrate concentration.

The Chemiluminescence Energy Transfer Process in the Case of APhD–Fluorescein

Attempts to Optimize CL Emission. To obtain a higher CL intensity and slower kinetics (to maintain I_{CL} at a maximum constant value for a longer time), the effect of Co^{2+} and Mg^{2+} (ions with catalytic action) addition was studied. Figure 1a shows the variation of integrated CL intensity as a function of the integration time value (from the beginning of the reaction), while Fig. 1b shows the variation of CL intensity versus reaction time (in this case, the integration time is constant, 4 s). One can note the CL enhance activity of the ions even at very low concentrations (namolar in the case of Co^{2+}), the prooxidant character of these ions being evident. At the same time, only Co^{2+} changes the kinetics of the reaction. The different behavior of the two ions in the CL system may be accounted for by their chemical nature. Thus, Co^{2+} can change its valence according to the reactions

$$Co^{2+} + O_2 \rightarrow Co^{3+} + O_2^{\bullet-}$$

$$Co^{2+} + O_2 + 2H^+ \rightarrow Co^{3+} + H_2O_2$$
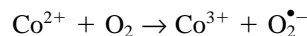
And when the Fenton system is involved,

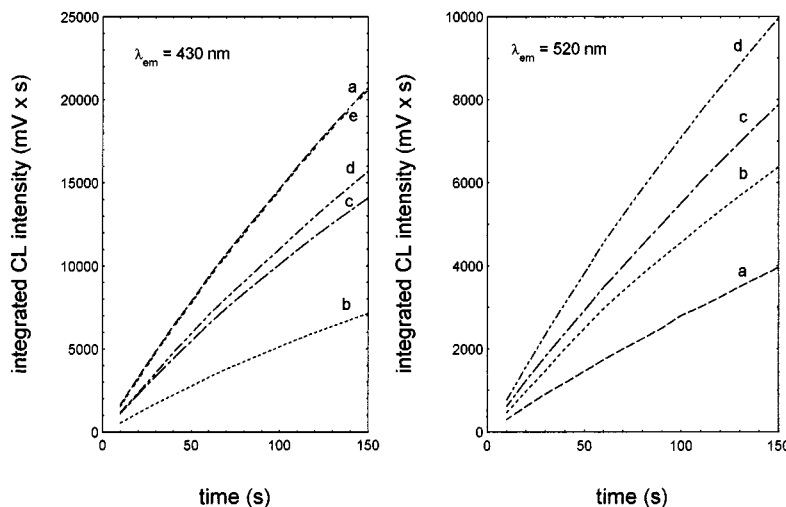**Fig. 4.** Time variation of integrated CL intensity for the system LH_2–H_2O_2–Mg^{2+} (1) with various concentrations of fluorescein: 2.4 μM (2), 8 μM (3), 16 μM (4), and 24 μM (5) at λ_{em} = 430 nm (A) and 2.4 μM (2), 8 μM (3), 16 μM (4), and 24 μM (5) at λ_{em} = 520 nm (B). $[Mg^{2+}]$ = 1.12 μM.
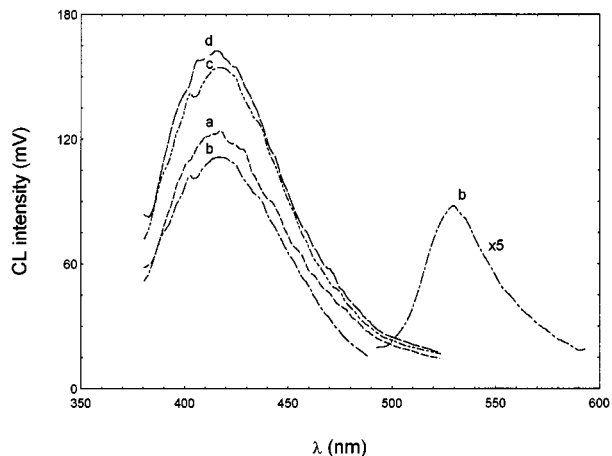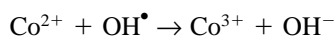
Fig. 5. CL spectra for aminophthalate dianion in the presence of 1.2 μM Mg^{2+} (a) and with various concentrations of fluorescein: 8 μM (b), 2.4 μM (c), and 1.6 μM (d).

$$Co^{2+} + H_2O_2 \rightarrow Co^{3+} + OH^- + OH^{\bullet}$$

$$Co^{2+} + OH^{\bullet} \rightarrow Co^{3+} + OH^-$$

The important increase in CL in the case of Mg^{2+}, without modification of the reaction kinetics, may be a result of one complex reaction mechanism. Its presence in this CL system ensures a chemical amplification, possibly owing to the reactive species of the oxygen production, especially $O_2^{\bullet-}$.

Thus, in the case of Co^{2+} the prooxidant character is stronger than in the case of Mg^{2+}. Compared with

the reference system, their presence ensures a stronger, prolonged, and stable light emission.

The Effect of Fluorescein Addition as the Overall Intensity (Fig. 2). One can note that the addition of fluorescein produces a complex effect on the overall CL intensities. One can observe a decrease in the intensity initially (up to 100 s) and then a slight increase in the I_{CL}, more obvious in the case of 2 μM fluorescein. For this concentration, the enhancer effect of fluorescein is assigned to the energy transfer process, more efficient in this case than with lower concentrations (0.2 and 0.4 μM). The effect of fluorescein at a higher concentration, 20 μM, is basically a decrease in I_{CL} because of the important absorption of the APhD emission and the autoabsorption of fluorescein emission (filter effect, concentration quenching).

The Effect of Fluorescein Addition on the Spectral Distribution of APhD CL. The kinetic data on CL intensity were completed with spectrophotometric measurements, following the CL intensity variation as a function of λ_{em}, in other words, determining the CL emission bands. Figure 3 shows the CL spectrum of APhD in the absence and presence of fluorescein (8 μM). One can note that in the presence of fluorescein the emission spectrum consists of two bands having a maximum at 415 and at 520 nm, respectively. One can affirm that the energy transfer process occurs, the band with the maximum at 529 nm being specific to fluorescein emission. At the same time, the presence of fluorescein shifts the APhD fluorescence band (from 425 to 415 nm).
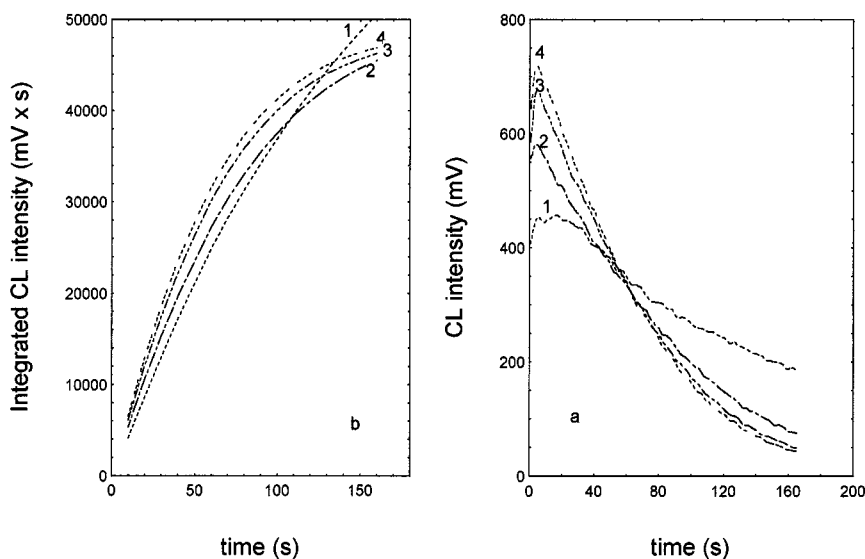

Fig. 6. The time variation of CL for the LH_2–H_2O_2–Co^{2+} system (1) with various concentrations of fluorescein: 8 μM (2); 16 μM (3); 24 μM (4). λ_{em} = 430 nm; $[Co^{2+}]$ = 0.12 μM.

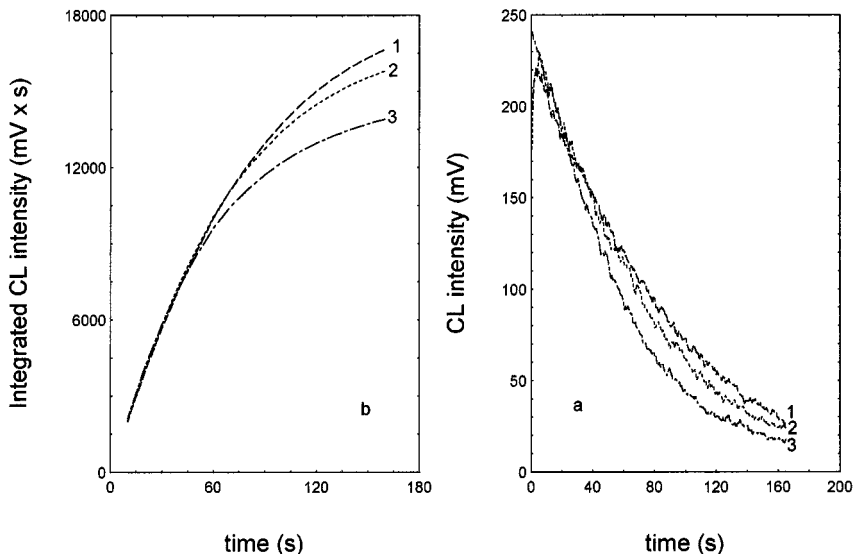**Fig. 7.** The time variation of CL for the $LH_2-H_2O_2-Co^{2+}$ system with various concentrations of fluorescein: 8 μM (1); 16 μM (2); 24 μM (3). λ_{em} = 520 nm; $[Co^{2+}]$ = 0.12 μM.

The Effect of Mg^{2+} on the Energy Transfer Process. Figures 4A and 4B show the time variation of the integrated I_{CL} at two emission wavelengths, in the presence of Mg^{2+} (1.2 μM) and the effect of the fluorescein concentration variation. In this case the energy transfer is not as efficient as in the previous case (with Co^{2+} present), in the sense that the integrated I_{CL} values, measured at both 430 and 520 nm, are very low. Fluorescein addition does not modify the CL kinetics. The integrated intensity at 430 nm (Fig. 4A) increases proportionally with the integration time. The energy transfer process is evidenced by the decreasing integrated intensity, the effect being higher with increasing fluorescein concentration. One would expect that the integrated intensity measured at 520 nm (Fig. 4B) would be maximum at the maximum fluorescein concentration (24 μM), but for 16 μM, as well as for 24 μM, the integrated intensity is lower than for 8 μM, owing to the reabsorption of emission (or concentration quenching).

The energy transfer process is also evident in Fig. 5, which shows the CL emission spectra of APhD in the presence of Mg^{2+} (1.2 μM) and fluorescein. At low fluorescein concentrations (1.6 and 2.4 μM) the CL emission band of APhD is rather higher, due to the acceleration of the luminol conversion to aminophthalate dianion, but at 8 μM the intensity of this band is lower following an energy transfer. Therefore, to make energy transfer possible it is necessary to have an optimal fluorescein concentration. Within the sensitivity limits of the apparatus employed, no emission bands in the spectral range

490–580 nm were observed for the other fluorescein concentrations tested.

The Effect of Co^{2+} on the Energy Transfer Process. Figures 6 and 7 show the time variation of CL intensity emitted by the luminol–hydrogen peroxide–Co^{2+}–fluorescein system at two emission wavelengths, 430 nm (Fig. 6) and 520 nm (Fig. 7). The effect of the fluorescein concentration in the presence of Co^{2+} (0.12 μM) on the energy transfer and CL kinetics was investigated.

Figures 6a and b present the complex effect of fluorescein addition on the I_{CL} (measured at time intervals of 550 ms; Fig. 6a) and integrated I_{CL} by means of computer at different time intervals from the beginning of the reaction (Fig. 6b). One can note that during the first moments of the reaction the I_{CL} increases (up to about 60 s), and then the effect is the reverse, that is, the I_{CL} decreases proportionally with the fluorescein concentration. The CL kinetics is also modified, the time decay being more rapid. Figure 6b shows the increasing integrated I_{CL} proportional to the fluorescein concentration. The increase in CL at 430 nm may be assigned to the electron transfer process from fluorescein to luminol, determining the formation of luminol radical. A similar phenomenon was observed in the case of a comparable CL system, but with horseradish peroxidase instead of Co^{2+} [2]. It is known that fluorescein becomes chemiluminescent in the presence of this enzyme [23,24], and this is probably why the enhancer effect of fluorescein, at higher concentrations, is greater in the case of the
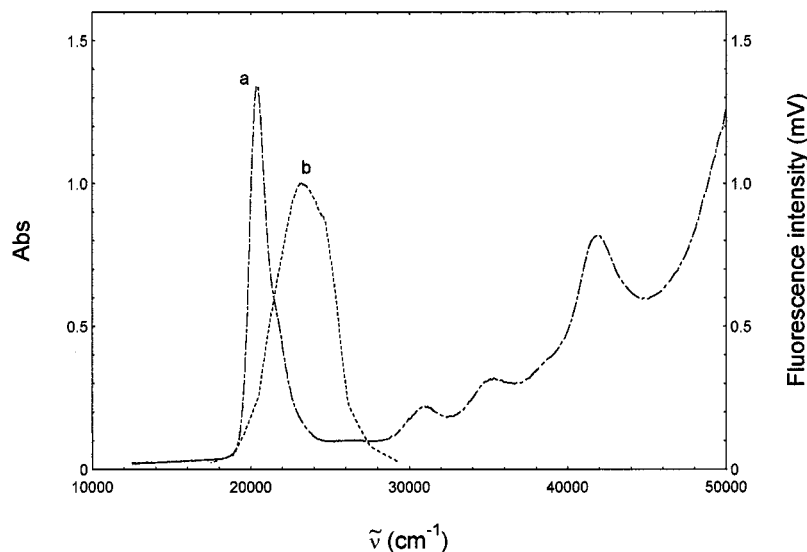
Fig. 8. The spectral distribution of the aminophthalate dianion fluorescence (a) and of fluorescein absorption (b).

system investigated by Diaz *et al.* [2]. If the integration is carried out over a time interval greater that about 100 s (Fig. 6b), the curves become flat and located below the curve of the reference system (curve 1), pointing to a decrease in the integrated intensities because of the energy transfer process and the more rapid CL decay.

Figures 7a and b show the decay curves, at 520 nm, of fluorescein emission. One can observe that simultaneously with the fluorescein concentration increase, the kinetics is more rapid, showing the same effect as the fluorescein concentration on the I_{CL} measured at 430 nm. For short time intervals, e.g., 10–15 s, the intensity of fluorescein decreases in the sequence 24, 16, 8 μM. The curves with integrated intensity variation offer some observations: up to about 50 s the values are similar. Differences show up at longer integration times, that is, a different decrease in the integrated CL intensity owing to the acceleration of the reaction, but also probably due

to an autoabsorption process of the fluorescein emission at higher concentrations.

An attempt was made to demonstrate an energy transfer in DMSO. Therefore hydrogen peroxide was replaced with superoxide anion, solubilized in this solvent by means of corona ether [25]. The CL emission is much smaller and the reaction kinetics is more rapid than in the case of buffered solutions. The effect of fluorescein addition was evidenced, at two fluorescein concentrations: 2.4 and 8 μM. The CL intensity of APhD was increased in these two cases. The second band, specific to fluoresceine emission, was not observed, the energy transfer being inefficient. In another publication more details on the CL of this system will be described.

The qualitative results were supplemented with quantitative data, determining the CL quantum yield, Q, the half-intensity time, $t_{1/2}$, and the critical transfer distance, R_0. One can note that Q in the presence of Co^{2+}

Table I. The Quantum Yield, Q, Half-Intensity Time $t_{1/2}$, and Critical Transfer Distance, R_0, in Different CL Systems

System	Quantum yield (Q)	$t_{1/2}$ (s)	R_0 (Å)	k_1 (s^{-1})^a
LH$_2$–H$_2$O$_2$ (in buffered Tris–HCl, pH 8.32)	0.01	13.8046	19	5.03×10^{-2}
LH$_2$–H$_2$O$_2$–Co^{2+}–Fl^b (8 μM) (in buffered Tris–HCl, pH 8.32)	0.07	41.4137	26.3	1.67×10^{-2}
LH$_2$–H$_2$O$_2$–Mg^{2+}–Fl (8 μM) (in buffered Tris–HCl, pH 8.32)	0.04	33.9293	23.9	2.04×10^{-2}
LH$_2$–O2$^{\circ-}$–DMSO		10.7991		6.42×10^{-2}
LH$_2$–O2$^{\circ-}$–DMSO–Fl (2.4 μM)		19.5842		3.54×10^{-2}
LH$_2$–O2$^{\circ-}$–DMSO–Fl (8 μM)		17.4012		4×10^{-2}

^{a} k_1 = rate constant ($k_1 = \ln 2/t_{1/2}$).
^{b} Fluorescein.

is seven times greater compared to the reference system, and four times greater in the presence of Mg^{2+}. At the same time, $t_{1/2}$ increases in the presence of catalysts, as well as the R_0 value (Table I).

The $t_{1/2}$ value in DMSO is lower than in buffered water; in the presence of fluorescein, the value increases (Table I).

Fluorescence Energy Transfer in APhD–Fluorescein. This was demonstrated by the appearance of a fluorescein fluorescence band (λ_{max} at 520 nm) with simultaneously decreasing APhD fluorescence, by excitation at 330 nm. The fluorescence intensity (4 h after the mixing of the solutions) measured at 520 nm increases with the tested fluorescein concentrations, 0.8, 1.6, 2.4, and 8 μM. The influence of Co^{2+} and Mg^{2+} on fluorescein fluorescence was studied. It was found that these ions, at low concentrations, do not modify the fluorescein fluorescence. Figure 8 presents the spectral distribution of APhD fluorescence and fluorescein (8 μM) absorption. The intersection of the two spectra afforded the determination of the spectral overlap [Eq. (3)], $J = 8.14 \times 10^{-15}$ M^{-1} cm^3. The value of R_0 for fluoresence energy transfer is about 30 Å [from Eq. (4)].

CONCLUSIONS

(1) The chemiluminescence energy transfer of aminophthalate dianion to fluorescein in buffered solutions (pH 8.32) was demonstrated by the appearance of a second luminescence band, specific to fluorescein and a decrease in the aminophthalate dianion CL intensity.

(2) The effects of Co^{2+} and Mg^{2+} ions on the intensity and kinetics of CL, as well as on the energy transfer, were compared, and we found that in the presence of Co^{2+}, the CL intensity is higher and the energy transfer is more efficient.

(3) In the presence of Co^{2+}, the effect of fluorescein is complex: Co^{2+} increases the CL intensity (at short time values) and accelerates the kinetics of the CL reaction.

(4) The CL energy transfer in DMSO is inefficient because of the very low quantum yield of fluorescein in this solvent and the CL reaction kinetics is much more rapid, compared with the buffered solution.

(5) The fluorescence energy transfer of aminophthalate dianion to fluorescein is efficient owing to the high quantum yield of fluorescein fluorescence.

(6) The critical energy transfer distance R_0 is about 19 Å (reference system), 26 Å (in the presence of catalyst Co^{2+}), 24 Å (in the presence of catalyst Mg^{2+}), and about 30 Å in the case of fluorescence energy transfer.

REFERENCES

1. A. K. Campbell (1988) *Chemiluminescence: Principles and Aplication in Biology and Medicine*, Ellis Horwood, Chichester, England.
2. A. N. Diaz, J. A. Gonzalez Garcia, and J. Lovillo (1997) *J. Bioluminesc. Chemiluminesc.* **12**, 199.
3. R. Olinescu (1987) *Chemiluminescenta si bioluminescenta*, Tehnică Bucuresti, Bucharest.
4. R. Olinescu (1982) *Peroxidarea in chimie, biologie si medicină*, Stiintifică si Enciclopedică Bucuresti, Bucharest
5. R. Olinescu (1990) *Revista Chim.* **41**, 865.
6. A. Patel and C. J. Davies (1983) *Anal. Biochem.* **129**, 1162.
7. K. Robards and P. J. Worsfold (1992) *Anal. Chim. Acta* **266**, 147.
8. C. Matachescu, R. Olinescu, and E. Baiulescu (1995) *Rev. Roumaine Chim.* **40**, 149.
9. K. Sasamoto *et al.* (1995) *Analyst* **120**, 1709.
10. O. Hayashi and K. Asada (1977) *Biochemical Aspects of Active Oxygen Species*, University of Tokyo, Tokyo.
11. S. Ribarov and P. G. Bochev (1983) *J. Biochem. Biophys. Methods* **8**, 205.
12. D. Slawinski and J. Slawinski (1980) *Photochem. Photobiol.* **28**, 456.
13. K. Yagi (1982) *Biochem. Methods* **15**, 212.
14. K. V. Dye (1988) *Bioluminescence and Chemiluminescence: Instruments and Aplications*, CRC Press, Boca Raton, FL.
15. O. Merenyl, T. E. Eriksen, and J. Lind (1986) *J. Am. Chem. Soc.* **108**, 7716.
16. G. Merenyl, T. E. Eriksen, and J. Lind (1990) *J. Bioluminesc. Chemiluminesc.* **5**, 53.
17. T. M. Myazawa *et al.* (1994) *Methods Enzymol.* **233**, 324.
18. V. I. Regin (1977) *Zh Anal. Chem.* **32**, 1925.
19. Y. Hass and Würzberg (1979) *J. Phys. Chem.* **83**, 2692.
20. Th. Forster (1951) *Fluoreszenz Organisher Verbindungen*, Vandenhoeck und Ruprecht, Gottingen.
21. J. R. Lakowicz (1982) *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.
22. M. Voicescu, M. Vasilescu, and A. Meghea (1999) *Proceedings of the XI International Conference on Chemistry and Chemical Engineering*, Bucharest, Sept. 30–Oct. 2.
23. T. Segawa, T. Kamidate, and H. Watanabe (1990) *Anal. Sci.* **6**, 763.
24. T. Segawa, T. Kamidate, and H. Watanabe (1993) *Bull. Chem. Soc. Jpn.* **66**, 2237.
25. J. S. Valentine and A. B. Curtis (1975) *J. Am. Chem. Soc.* **97**, 224.