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Solvent Effects in the Reaction of Lucigenin with Basic Hydrogen Peroxide: Chemiluminescence Spectra in Mixed Polar Solvents

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Summary. The chemiluminescent reaction of lucigenin with basic hydrogen peroxide has been studied in several mixtures of water with the cosolvents methanol, ethanol, 1-propanol, dimethylformamide, and dimethylsulfoxide. The chemiluminescence spectra depend on the cosolvent and its concentration in the reaction medium. With increasing cosolvent concentration, the chemiluminescence shifts to lower wavelengths. For similar cosolvents, the size of this shift increases with decreasing dielectric constant. In high-cosolvent-concentration mixtures, the chemiluminescence matches the fluorescent emission of N-methylacridone. Chemiluminescence from low-cosolvent-concentration mixtures is explained as the sum of the lucigenin and N-methylacridone fluorescent emissions, the lucigenin emission probably being a consequence of energy transfer from N-methyl-acridone. The cosolvent inhibits this energy transfer. These observations, taken together with our previous kinetic results, indicate that the reaction mechanism is the same in all the studied reaction media.

Keywords. Chemiluminescence; Lucigenin; Solvent effects.

Lösungsmitteleffekte bei der Reaktion von Lucigenin mit basischem Hydrogenperoxid: Chemilumineszenzspektren in gemischten polaren Lösungsmitteln

Zusammenfassung. Die Chemilumineszenzreaktion von Lucigenin mit basischem Hydrogenperoxid wurde in verschiedenen Mischungen von Wasser mit Methanol, Ethanol, 1-Propanol, Dimethylformamid oder Dimethylsulfoxyd untersucht. Die Chemilumineszenzspektren hängen vom organischen Kosolvens und dessen Konzentration im Reaktionsmedium ab. Mit ansteigender Konzentration ergeben sich in der Chemilumineszenz Verschiebungen zu größeren Wellenlängen. Für ähnliche Kosolventien steigt diese Verschiebung mit kleineren Dielektrizitätskonstanten an. Bei hohen Kosolvenskonzentrationen gleicht die Chemilumineszenz der Fluoreszenzemission von N-Methylacridon. Die Chemilumineszenz bei kleinen Kosolvenskonzentrationen kann als die Summe der Fluoreszenzemission von Lucigenin und N-Methylacridon erklärt werden, wobei die Lucigeninemission vermutlich eine Folge eines Energietransfers von N-Methylacridon ist. Das Kosolvens verhindert diesen Energietransfer. Diese Beobachtungen, zusammen mit früheren kinetischen Resultaten, erlauben den Schluß, daß der Reaktionsmechanismus in allen Reaktionsmedien der gleiche ist.

Introduction

Lucigenin (10,10′-dimethyl-9,9′-biacridinium nitrate) is a classic chemiluminescent agent [1]. Intense chemiluminescence (CL) is observed when lucigenin (L) is treated

with hydrogen peroxide in alkaline solutions. This reaction is important because of its ability to quantitate hydrogen peroxide and certain enzymes or metal ions based on their catalytic or inhibitory effect on the reaction [2].

The reaction has been extensively studied in aqueous solutions [3–6]. N-methylacridone (*NMA*) is the major fluorescent product but the CL spectrum does not match the fluorscence spectrum of *NMA*. Maskewicz et al. [4] explained this difference as a consequence of the reabsorption by lucigenin of *NMA* fluorescent emission, but the difference also appears at very low lucigenin concentrations.

In recent years, this reaction has also been characterized in aqueous solutions of membrane mimetic agents such as anionic surfactants and cyclodextrins [2, 7–10]. Their presence enhance the solubility of lucigenin and *NMA* and can alter the CL spectra and intensities. Thus, cyclodextrins increase CL intensity [7, 8] and some surfactants also change the CL spectrum [9]. These results reveal the importance of the physical and chemical nature of the reaction media in this reaction.

Previously we have studied the effect of reaction media composition on lucigenin-hydrogen peroxide reaction kinetics [5]. Light emission decay as well as lucigenin disappearance were recorded in mixtures of water with the cosolvents: methanol, ethanol, 1-propanol, dimethylsulfoxide (*DMSO*), and dimethylformamide (*DMF*). The kinetic results appear to indicate that the fundamental step in the disappearance of lucigenin and in light emission decay is the addition of HO_2^- to lucigenin. The cosolvent acts as a catalyst for the reaction with HO_2^- and increases both the initial CL intensity and the decay rate constant.

The purpose of this work is to analyse the hydrogen peroxide-lucigenin reaction to determine the effects of the above cosolvents on the CL spectra.

Experimental

Lucigenin (Ega-Chemie) was recrystallized once from absolute ethanol and dried at 110°C. *NMA* was synthetized from lucigenin [4] and recrystallized once from ethanol. UV-vis absorption and fluorescence spectra of these substances were in good agreement with those reported in the literature [4, 7]. Doubly distilled (in glass vessels) water was employed. The other reagents and cosolvents were of analytical or spectroscopic grade and were used without further purification.

Excitation and emission fluorescence spectra were obtained using a Perkin-Elmer LS-5 luminescence spectrometer. CL spectra were recorded on the same instrument, with the excitation source off, employing fast scanning rates and wide slits. The emission spectra were corrected for the emission monochromator and photomultiplier tube spectral response. UV-vis absorption spectra were obtained using either a Cary-17 spectrophotometer or a Pye-Unicam SP-1800 spectrophotometer

To record fluorescence and CL spectra of the reaction mixture, 3 ml of the selected solvent mixture containing the desired concentrations of hydrogen peroxide and lucigenin were placed in a special glass cuvette. The injection of 1 ml of aqueous sodium hydroxide at the appropriate concentration simultaneously initiated the CL reaction and the record. This procedure minimizes the undesirable reaction of lucigenin with ambient oxygen in alkaline solution.

Moreover, CL spectra in lucigenin reaction depend on the initial lucigenin concentration, probably due to reabsorption phenomena caused by lucigenin. To avoid these effects, an initial lucigenin concentration lower than $10^{-5}\,M$ was always used.

Results

When lucigenin reacts with hydrogen peroxide in mixtures of water with polar cosolvents (such as aliphatic alcohols, *DMF* or *DMSO*), the CL spectra depend on the composition of the mixture (Fig. 1).

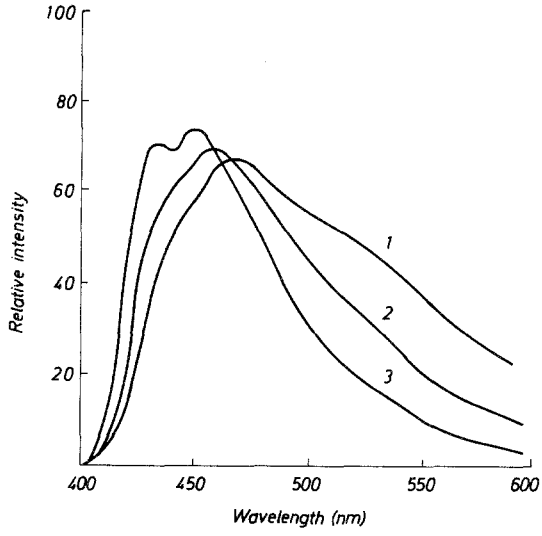

Fig. 1. CL spectra of the lucigenin reaction in ethanol-water mixtures. Solvent composition, % (*v*/*v*) ethanol: *1*: 4; *2*: 20; *3*: 35. Initial concentrations (*M*): Lucigenin, $6.25 \cdot 10^{-6}$; NaOH, $6.5 \cdot 10^{-2}$; H_2O_2, $5 \cdot 10^{-2}$

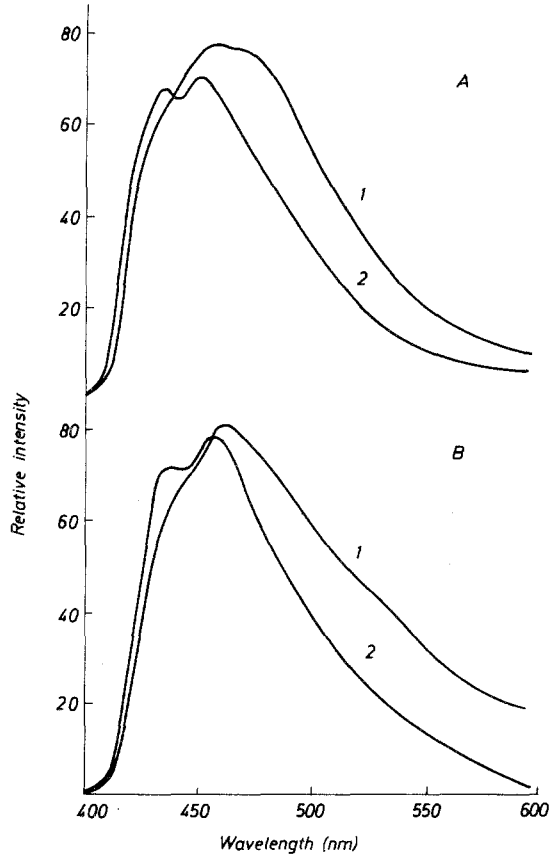

Fig. 2. CL spectra in: **A** 35% (*v*/*v*) alcohol – 65% water mixtures. Alcohol: *1*: Methanol; *2*: 1-Propanol. **B** 20% (*v*/*v*) cosolvent – 80% water mixtures. Cosolvent: *1*: *DMSO*; *2*: *DMF*. Concentrations: see Fig. 1

CL spectra recorded in low-cosolvent-proportion mixtures (i.e., when the volume percentage of cosolvent is lower than 5%) are very similar to those recorded in aqueous solutions. In such mixtures a small shift to lower wavelengths can be observed in the CL spectra as the reaction progresses. On the other hand, as the cosolvent percentage increases, the CL is also found to shift gradually to lower

wavelengths. As can be seen in Fig. 1, this shift appears to be due to a decrease in light emission intensity at high wavelengths. Moreover, in high-cosolvent-proportion mixtures, no shift is observed during the reaction.

All the cosolvents used act similarly, but the size of the shift depends on the cosolvent, being greater for cosolvents of smaller dielectric constant within a homologous series (Figs. 2 *A*, *B*),

$$\text{1-propanol} > \text{ethanol} > \text{methanol} > H_2O\,, \qquad DMF > DMSO\,.$$

The same order was observed for the enhancement of the rate of lucigenin disappearance (followed spectrophotometrically at 368 nm) and initial CL intensities and decay rates.

To analyze these solvent effects on CL spectra, the fluorescence emission and excitation spectra of *NMA* and lucigenin were determined in mixed solvents. The fluorescence emission spectrum of *NMA* in these mixed solvents is less intense than in water, and a small shift is observed, from highest intensities at 434 and 453 nm in water to 424 and 443 in pure ethanol. This shift appears to indicate that the dipole moment of *NMA* in the excited state is higher than in the ground state. The fluorescence emission spectrum of lucigenin in mixed solvents is also less intense than in water (Legg and Hercules [11] observed that the fluorescence yields of lucigenin are 0.34 in water and 0.09 in ethanol) but no shift in wavelength is observed.

However, in basic solution of lucigenin in high cosolvent concentration mixtures a new fluorescence emission (Fig. 3) can be detected (emission maximum at 502 nm, excitation maximum at 406 nm in 35% ethanol − 65% water). This emission may be due to dimethylbiacrilidene (*DBA*), since Legg and Hercules [11] observed that *DBA* is produced in a two-electron reduction of lucigenin by hydroxide ions in ethanol or *DMF*, and Maeda et al. [12] found the emission and excitation maxima of *DBA* in ethanol to be at 504 and 423 nm respectively.

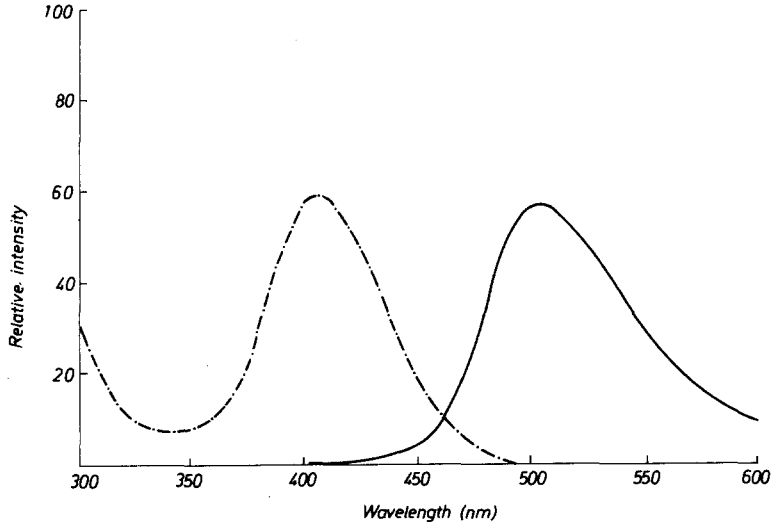

Fig. 3. Excitation (−.−, $\lambda_{em} = 520$ nm) and emission (——, $\lambda_{ex} = 350$ nm) spectra of lucigenin ($6.25 \cdot 10^{-6}$ *M*) in 35% ethanol − 65% water (base concentration: *M*)

Discussion

As can be seen in Fig. 4, the CL spectra recorded in high-cosolvent-proportion mixtures closely resemble the *NMA* fluorescence emission spectra in the same reaction media. This good agreement reveals that *NMA* is the only emitter for the lucigenin-hydrogen peroxide CL reaction in such mixtures.

This agreement does not appear in water or low-cosolvent-proportion mixtures (see Figs. 1 and 2). Moreover, the fluorescence spectra obtained during the CL reaction in these mixtures present only two emissions (Fig. 5), both different from CL. The fluorescence emission at low wavelengths, which may be clearly assigned to *NMA*, increases during the CL reaction (*NMA* being the only fluorescent species at the end of the reaction), whereas the emission at high wavelengths decreases. This second emission may be assigned to lucigenin or a similar species such as the

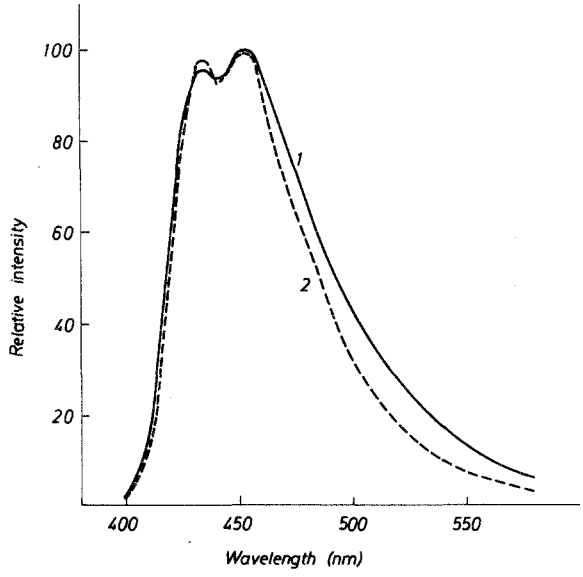

Fig. 4. CL (——) and *NMA* fluroescence emission (---, $\lambda_{ex} = 350$ nm) spectra in 35% ethanol – 65% water. Concentrations: see Fig. 1

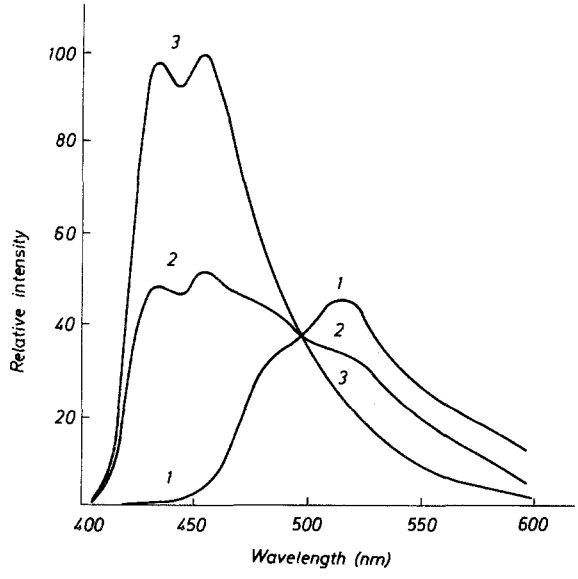

Fig. 5. Fluorescence emission spectra of the reaction mixture ($\lambda_{ex} = 350$ nm) in 4% ethanol – 96% water. Reaction time (min): *1*: 0; *2*: 6; *3*: 12 (final spectrum). Concentrations: see Fig. 1

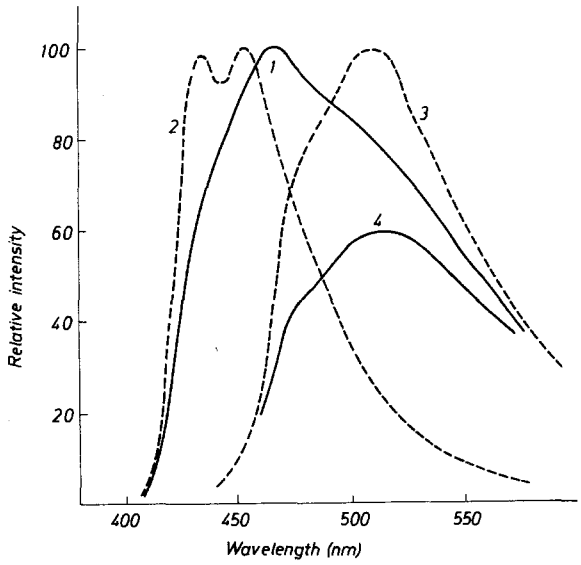

Fig. 6. CL and fluorescence spectra in 4% ethanol – 96% water. *1*: Initial CL spectrum; *2*: *NMA* fluorescence emission ($\lambda_{ex} = 350$ nm); *3*: Lucigenin fluorescence emission ($\lambda_{ex} = 350$ nm); *4*: Difference spectrum $I_4(\lambda)$ (see text). Concentrations: see Fig. 1

pseudo-base. The rate of disappearance of this emission increases with increasing cosolvent concentration, due to cosolvent catalysis of lucigenin disappearance [5].

To explain the CL spectra recorded in water, two hypotheses have been proposed. Maskiewicz et al. [4] suggested that *NMA* is the primary emitting species and that the observed CL spectra result from the absorption of *NMA** emission by lucigenin. However, this hypothesis cannot be applied in this case since lucigenin absorption at *NMA** emission wavelengths would be very small at lucigenin concentrations below $10^{-5}\,M$ ($\log\varepsilon = 4.01$ at 430 nm in 4% ethanol – 96% water). Moreover, this hypothesis does not explain the gradual variation of the initial CL spectra with varying cosolvent concentration in the mixture.

On the other hand, most of the authors [2, 9, 11, 13] have proposed that *NMA* is the primary emitter in this reaction, but that its energy is transferred to a compound undergoing fluorescence at longer wavelengths. This compound must be lucigenin (or the pseudo-base) since lucigenin is the only other fluorescent species in these reaction mixtures (Fig. 5).

We have verified that the CL spectra recorded in these low-cosolvent-proportion mixtures can be explained as the sum of *NMA* and lucigenin fluorescence emissions in variable proportions. Curve 4 in Fig. 6 (for a 4% ethanol – 96% water mixture) was obtained by subtracting the *NMA* fluorescence emission spectrum from the initial CL spectrum

$$I_4(\lambda) = I_{CL}(\lambda) - [I_{FL}(\lambda)]_{NMA}\,,$$
$$[I_{FL}(440)]_{NMA} = I_{CL}(440)\,, \qquad (1)$$

where $I_{CL}(\lambda)$ is the initial CL intensity in this mixture and $I_{FL}(\lambda)$ is the *NMA* fluorescence intensity, calculated by assuming that *NMA* fluorescence is the only emission that contributes to CL at 440 nm. The similarity between Curve 4 and that for lucigenin fluorescence (Curve 2) supports the above hypothesis. Similar results (with only the proportions of the emissions varying) can be obtained for other mixtures and other reaction times.

If we consider that *NMA* is the only emitter in high-cosolvent-proportion mixtures, the above results appear to indicate that the CL spectra recorded in low-cosolvent-proportion mixtures are the result of an energy transfer from *NMA** to lucigenin (L^{++}) (Scheme 1).

$$L^{++} + H_2O_2 + NaOH \rightarrow NMA + NMA^*$$

$$NMA^* + L^{++} \rightarrow NMA + (L^{++})^*$$

$$\left.\begin{array}{l} NMA^* \rightarrow NMA + h\nu \\ (L^{++})^* \rightarrow L^{++} + h\nu' \end{array}\right\} \text{CL}$$

Scheme 1

This energy transfer, whose mechanism has not been elucidated yet, explains the observed variations of the CL spectra. The small shifts observed in CL spectra as the reaction progresses could be due simply to lucigenin disappearing during the reaction, since the extent of energy transfer is dependent upon the lucigenin concentration.

The observed shifts in CL spectra as the cosolvent proportion in the mixture increases can be attributed principally (Fig. 1) to a decrease in the contribution of lucigenin fluorescence to CL emission. Several factors may contribute to this decrease:

(i) The fluorescence yield of lucigenin in these mixtures decreases as the cosolvent proportion increases.

(ii) In high-cosolvent-proportion mixtures, a new fluorescent emission can be detected. This emission has been assigned to *DBA* (formed from lucigenin) which apparently does not act as an acceptor in energy transfer from *NMA**.

(iii) According to the generally accepted mechanism [4], singlet state *NMA* is obtained by decomposition of a 1,2-dioxetane intermediate (an uncharged species). In a mixture of water with less polar cosolvents, *NMA** may be formed and solubilized in a less polar microenvironment. This different solvation may reduce the efficiency of energy transfer from *NMA** to lucigenin. Paleos et al. [9] have proposed a different solubilization mechanism to explain the fact that CL spectra recorded in the lucigenin reaction in micellar media present light intensities at *NMA* fluorescence wavelengths higher than those recorded in water.

Furthermore, this energy transfer inhibition by the cosolvent would be higher for cosolvents of lower dielectric constant in a homologous series (such as aliphatic alcohols), which would account for the observed relative solvent effects (Fig. 2 *A, B*).

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