SOME OBSERVATIONS ON EFFECT OF FLUORESCENT SUBSTANCES ON LUCIGENIN CHEMILUMINESCENT REACTION

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The effect of several substances (e.g. fluorescein, eosin B, pyronin G, coumarin, glucose etc.) on the chemiluminescent reaction of lucigenin was examined. The best enhancer of the initial light intensity is eosin B (2.3 times). Fluorescein and pyronin G enhance the intensity of chemiluminescent reaction in time ($t_{1/2}$ = 8.6 versus $t_{1/2}$ = 7.49 min and $t_{1/2}$ = 12.6 versus $t_{1/2}$ = 7.09 min, respectively).

Lucigenin (10,10′-dimethyl-9,9′-biacridinium nitrate) is well known chemiluminescent (CL) agent. It provides the chemiluminescent reaction in strong alkali hydrogen peroxide observed in 1935 by Gleu and Petsch[1]. Since then, many researchers have studied the mechanism of this reaction especially[2 – 10].

It is known that the primary emitter of chemiluminescent reaction of lucigenin is *N*-methylacridone which can be produced[2,3] by: (i) reaction of lucigenin with H_2O_2 in strong alkali aqueous solutions (via 1,2-dioxetane intermediate); (ii) one electron transfer from the reaction products of lucigenin with various nucleophiles such as HO^-, $CF_3CH_2O^-$, CN^-, $CH_3CH_2NH_2$ – $(LG\text{-}Nu)^-$ to the another molecule of lucigenin in the presence of molecular oxygen; (iii) reaction of lucigenin with superoxide ($O_2^{-\bullet}$) which can be generated by reaction of molecular oxygen with the various radical species formed in the system.

The influence on the chemiluminescent reaction of lucigenin has been studied for many substances such as: dissolved oxygen[1,11], reducing agents (pyrocatechol, resorcinol, phenol[12]), oxidizing agents (osmium tetroxide, sodium hypochlorite, potassium ferricyanide[1], benzoquinone, hydroquinone[12]), metal ions, anions and complexing agents[13], cosolvents (methanol, ethanol, 1-propanol, butanol, 2-methylbutanol, DMSO, DMF (refs[12,14,15]) etc. It was found[14,15] that increased concentration of alcohol shifts the chemiluminescence to lower wavelengths and increases the initial intensity and the speed of its decay.

In recent years several systems have been proposed for chemiluminescent detection of DNA probes[16]. They have detection limit comparable with that of the radioisotopic method but they are not commonly used yet. Most of them are catalyzed by enzyme

(luminol, isoluminol, luciferin, 1,2-dioxetanes) and the luminescent reaction can be enhanced by fluorescent enhancers (e.g. fluorescein in oxalate esters system).

Acridinium esters are also used as the chemiluminescent detector[16,17], and it has been proved that their sensitivity is enhanced by the product of the chemiluminescent reaction, *N*-methylacridone, which is fluorescent. No effect of external fluorescent enhancer on the intensity of chemiluminescent reaction of lucigenin has been studied up to now. The main aim of this work was to study the effect of external fluorescent agents on chemiluminescent reaction of lucigenin in aqueous media.

EXPERIMENTAL

Intensity (number of emitted photons) of chemiluminescent reaction was measured on Packard–Tri Carb Liquid Scintillation Spectrometer, Model 3320.

The reagents – lucigenin (Aldrich), pyronin G (Loba Chemie), ethidium bromide (Serva), glucose and eosin B (Lachema Brno), coumarin and 6-hydroxychromone-2-carbaldehyde (Comenius University) were used as delivered. All solutions were prepared by dilution of stock solutions immediately before the experiments. Lucigenin was used as a $1 . 10^{-6}$ M solution prepared from $1 . 10^{-3}$ M stock solution in redistilled water.

Solutions ($1 . 10^{-3}$ M and $1 . 10^{-6}$ M) of pyronin G, ethidium bromide and glucose were prepared from $5 . 10^{-3}$ M stock solutions in redistilled water, ethanolic solutions of eosin B, coumarin and 6-hydroxychromone-2-carbaldehyde from $5 . 10^{-3}$ M stock solutions in ethanol. Fluorescein solutions were prepared from $5 . 10^{-3}$ M solution in 0.1 M KOH.

Experiments were done in two ways: to the basic solution of 0.1 M KOH and 3% hydrogen peroxide (v/v, 3 ml), $1 . 10^{-6}$ M aqueous solution of lucigenin was added and the intensity of produced light was measured. Afterwards the appropriate volume of the examined substance solution ($1 . 10^{-3}$ M and/or $1 . 10^{-6}$ M) was added. The other experiments were done by mixing the all reaction components in the very beginning of the reaction.

RESULTS AND DISCUSSION

We started our study with the mild oxidating agent – glucose. No clear correlation between glucose/lucigenin concentration and intensity of luminescence was found. More intensive luminescence (the best result is increasing of initial CL intensity of lucigenin by 3.14 times in 6.5 min after mixing) was usually observed at a relative higher glucose concentration (at the ratio of lucigenin–glucose 100 and/or 50, 20 to $200 . 10^6$). It was found that the optimal concentration of reaction components was needed to achieve the highest intensity of luminescence. Such behavior is very familiar in the case of synergetic systems.

In the next step we studied the effect of further, mainly fluorescent substances to the lucigenin reaction. First we have examined fluorescein as the very common fluorescent agent. Experiments were carried out in two ways:

1) To the basic aqueous solution of potassium hydroxide and hydrogen peroxide, lucigenin was added and the intensity of the light emission was measured. Afterwards, an appropriate volume of fluorescein was added (Fig. 1).

2) All reaction components were mixed in the very beginning of the reaction.

The results showed (Fig. 1) that fluorescein (in our reaction conditions) did not increase intensity of chemiluminescent reaction in any of two above mentioned reaction modifications immediately after mixing. It has no effect or even inhibits the chemiluminescent reaction (Fig. 1, line *2*). This situation is changing in the course of the reaction when the beneficial effect of fluorescein begins and lasts at least 80 min (lines *2* and *3*, $t_{1/2}$ = 11.0, and 12.6 min against $t_{1/2}$ = 7.49 min of pure lucigenin reaction).

The change of fluorescein concentration has a small effect on the light intensity (Fig. 2). Again, its influence is increased in time and is obvious in the highest concentrations of fluorescein (ratio of lucigenin–fluorescein 20 : 50 . 10^3 and 20 . 10^3 v/v) especially.

These results were confirmed by the comparative study of fluorescein and another fluorescent substances. As far as the immediate effect on the light intensity is concerned, fluorescein is not the most feasible substance but it prolongs the interval in which the intensity of the reaction is increased. The following substances, beside fluorescein, were examined: eosin B, pyronin G, ethidium bromide, coumarin, 6-hydroxychromone-2-carbaldehyde. Initial experiments showed that eosin B was the best enhancer of the chemiluminescent intensity immediately after mixing (Fig. 3 line *2* and Fig. 4). In this reaction composition (Fig. 3, ratio lucigenin–additive 20 : 100 . 10^3) no other substance had such a beneficial effect. Except fluorescein above mentioned, eosin B and pyronin G have also a good prolongation effect on the chemiluminescent reac-

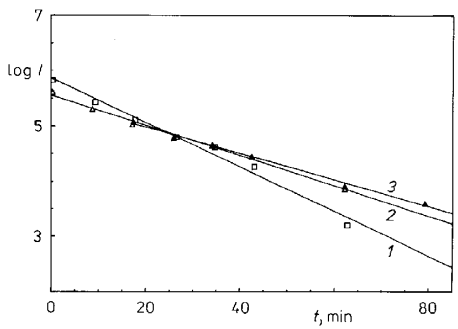

FIG. 1

The effect of fluorescein on the CL reaction of lucigenin. To 3 ml of 0.1 M KOH and 3 ml of 3% H_2O_2 was added: *1* 20 μl of 1 . 10^{-6} M lucigenin, *2* 20 μl of 1 . 10^{-6} M lucigenin and 100 μl of 1 . 10^{-3} M fluorescein, *3* 20 μl of 1 . 10^{-6} M lucigenin and in 18th min of reaction 100 μl of 1 . 10^{-3} M fluorescein

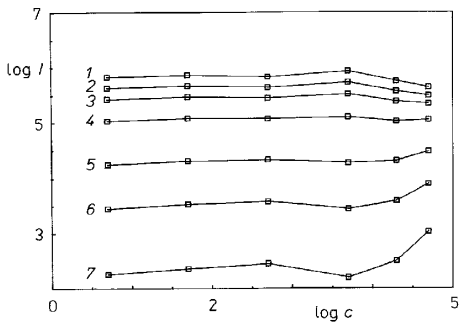

FIG. 2

The change of CL intensity with the change of fluorescein concentration. To 3 ml of 0.1 M KOH, 3 ml of 3% H_2O_2 and 20 μl of 1 . 10^{-6} M lucigenin, 5 (50, 500) μl of 1 . 10^{-6} M fluorescein and/or 5 (20, 50) μl of 1 . 10^{-3} M fluorescein was added and CL intensity was measured. Data are calculated for time *t* (min): *1* 0, *2* 5, *3* 10, *4* 20, *5* 40, *6* 60, and *7* 90

tion ($t_{1/2}$ = 8.57 and 8.6 min against $t_{1/2}$ = 7.09 min of pure lucigenin reaction). For eosin B, the light intensity is gradually increased with increased concentration (Fig. 5) and this effect lasts in time. Ethidium bromide seems to be without any effect, coumarin and 6-hydroxychromone-2-carbaldehyde have a very mild enhancing effect. They increase initial CL intensity of pure lucigenin reaction 1.35 and 1.27 times at the ratio lucigenin–additive 20 : 50 . 10^3 (v/v). This confirmed that the optimal composition of reaction mixture was very important.

We can summarize that eosin B is a good enhancer of the lucigenin chemiluminescent reaction. It increases its intensity immediately after mixing (2.3 times is the best case) and this effect lasts in time at least 60 min.

The overall concentration of lucigenin in these experiments was 3.35 and 3.33 . 10^{-9} M (Fig. 4) that is approximately 10^{-9} less than is the detection limit for a label based on acridinium esters detected by luminometer[16]. In any case, such a direct comparison is not very reliable because of different sensitivity of both equipments, that is scintillation spectrometer and luminometer.

Pyronin G does not change intensity of chemiluminescent reaction immediately after mixing and fluorescein even inhibits it. Their beneficial effect begins approximately in 30 min time of the lucigenin chemiluminescence and depends on the order of mixing. Addition of fluorescein to the reaction mixture after some time (18 min in our case) seems to be more suitable from this point of view.

Coumarin and 6-hydroxychromone-2-carbaldehyde are very mild enhancers of the initial intensity of chemiluminescent reaction at very special ratio of lucigenin–additive

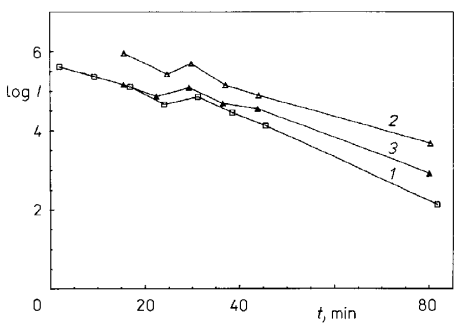

FIG. 3

The effect of eosin B and pyronin G to the CL reaction of lucigenin. To 3 ml of 0.1 M KOH and 3 ml of 3% H_2O_2 was added: *1* 20 μl of 1 . 10^{-6} M lucigenin, *2* 20 μl of 1 . 10^{-6} M lucigenin and in 16th min 100 μl of 1 . 10^{-3} M eosin B, *3* 20 μl of 1 . 10^{-6} M lucigenin and in 15th min 100 μl of 1 . 10^{-3} M pyronin G

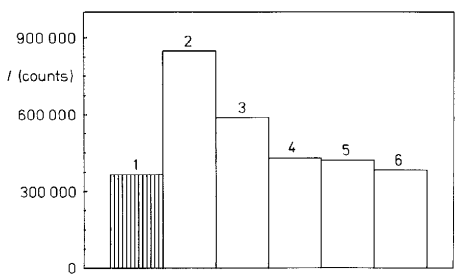

FIG. 4

The immediate change of the initial CL intensity (eosin B). To 3 ml of 0.1 M KOH, 3 ml of 3% H_2O_2 and 20 μl of 1 . 10^{-6} M lucigenin was added: 1 0, 2 50, 3 20, 4 5 μl of 1 . 10^{-3} M eosin B and/or 5 50, 6 5 μl of 1 . 10^{-6} M eosin B

20 : 50 . 10^3 (v/v) and they are worthless for our aim. Almost all experiments confirmed that the optimal composition of reaction mixture is necessary to obtain the optimal effect of fluorescent substance.

Our results proved that some substances have a beneficial effect on the intensity and the life-time of lucigenin CL reaction especially. This can be very helpful at using a lucigenin derivative as the chemiluminescent probe. For this reason, several new aspects of lucigenin CL reaction as well as an effect of another substances on this reaction will be studied.

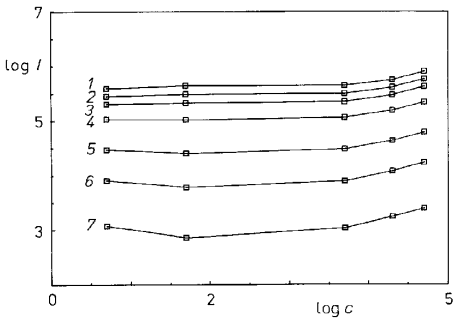

FIG. 5
The change of CL intensity with the change of eosin B concentration. To 3 ml of 0.1 M KOH, 3 ml of 3% H_2O_2 and 20 μl of 1 . 10^{-6} M lucigenin, 5 (50) μl of 1 . 10^{-6} M eosin B and/or 5 (20, 50) μl of 1 . 10^{-3} M eosin B was added and CL intensity was measured. Data are calculated for time *t* (min): *1* 0, *2* 5, *3* 10, *4* 20, *5* 40, *6* 60, *7* 90

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