THE EXISTENCE OF SCINTILLONS IN DINOFLAGELLATES: AN IN VITRO SIMULATION OF THE IN VIVO FLASHES

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1. Introduction

The flash of light emitted by numerous dinoflagellates like *Gonyaulax polyedra, Pyrocystis lunula, Noctiluca miliaris* originates in cytoplasmic particles termed 'scintillons' by Hastings et al. [1,2]. However, this particulate material has not been yet identified by electron microscopy, neither in the cells nor in subcellular fractions isolated by zonal centrifugation; the existence of scintillons has been the subject of several conflicting reports [3–8].

The in vivo flashes and the circadian rhythm of these light emissions are generally explained by two opposing hypotheses. According to the first hypothesis the flashes arise from the scintillons; these organites are converted into a soluble from during the photophase while the bioluminescence is no longer stimulable. However, the second hypothesis denies the existence of scintillons as luminous organites, but proposes a light mediated control of the bioluminescence to account for the circadian rhythm [5].

We suggest that a closer understanding of the flash mechanism requires an investigation of the flash generates when using purified luciferases from dinoflagellates. In order to demonstrate the eventual requirement of a particulate material for the production of flashes we have indicated, in this report, a procedure for the obtention of flashes by coupling various redox systems with the luciferase-luciferin reactions from *Gonyaulax polyedra, Pyrocystis lunula* and *Pyrocystic fusiformis*.

2. Materials and methods

The luciferases from *Gonyaulax polyedra, Pyrocystic lunula, Pyrocystis fusiformis* were purified according to the procedure described by the authors [9,10]. Each luciferin extract was prepared by boiling the cells 2 min in a 0.1 M, pH 6.7 phosphate buffer; the suspension was cooled in ice and then centrifuged for 20 min at 25 000 rev/min at 5°C. The supernatant was divided into 1 ml aliquots and kept in the freezer at −20°C.

The following procedure was used for the activity measurements: 10 to 100 μl of each luciferase (1 mg/ml) were added into 2 ml of 0.1 M, pH 6.7 phosphate buffer containing 0.8 M ammonium sulfate [11]. The light emission was triggered by a fast addition of 10 to 100 μl of luciferin extract with an Eppendorf pipette. The mixing was achieved in 0.1 sec by means of a magnetic stirrer. The light emitted by the sample was detected by an E.M.I. photomultiplier placed near the sample holder. The photocurrent was displayed on a Perkin–Elmer potentiometric recorder. All the kinetics were carried out at 18°C.

3. Results

When luciferin is added into the luciferase solution, the time course of the reaction does not appear as a flash but as a long glow, the duration of which varies according to the concentration of luciferase or luciferin. By varying the ratio of luciferin–luciferase, we do not observe a saw-tooth signal like in vivo. This result is obtained with luciferases and luciferins isolated from each species but also with luciferase from one species mixed with luciferin from another species [9–11]. Fig.1 summarizes this observation. Since

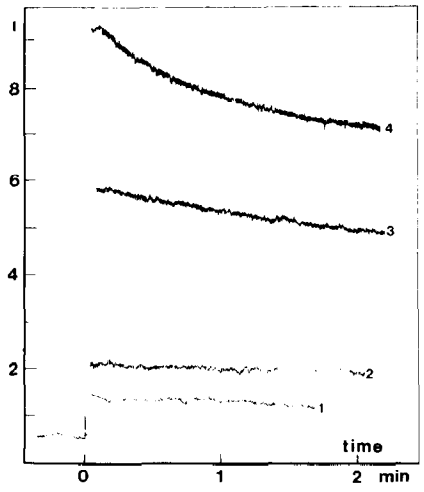

Fig.1. Kinetics of light emission. The luceferin concentration is constant and the luciferase concentration increases 12 μg/ml, (1), 25 μg/ml, (2), 70 μg/ml, (3), to 100 μg/ml, (4). Ordinate: light intensity in arbitrary units. Kinetic analysis of these curves: the luciferin concentration is proportional to the area under the curve. Each ordinate at time t is proportional to the rate of emission. A plot of $I_{(t)}$ versus $S_\infty - S_{(t)}$ is equivalent to the graph of the reaction rate versus the substrate concentration (see fig.6). At time t = o, the ordinate is proportional to the rate of luciferase–luciferin complex formation. A plot of the light intensity at time t = o versus the luciferase concentration or the quantity of luciferin extract is equivalent to the plot of v = f (s) or v = f (e) (see fig.6 and text).

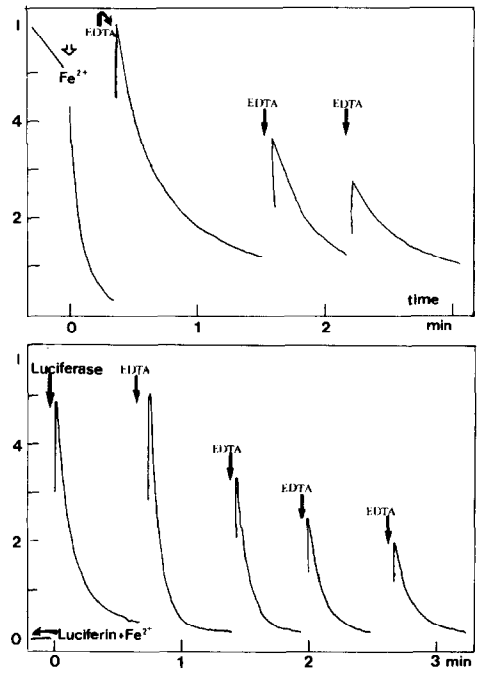

Fig.2. Upper curve: Effect of ferrous ions and EDTA on the light emission. Fe^{2+} (chloride or sulfate), final concentration: 1.4 10^{-3} M is added during the time course of the emission; after extinction, EDTA is added; each arrow is an addition of 8 mM EDTA. Lower curve: Luciferin is preincubated with ferrous ions and luciferase initiates the first flash. All the concentrations are the same as in the upper figure. The luciferase and luciferin concentrations are the same as in the third kinetic indicated in fig.1.

the in vitro luminous reactions do not give rise to flashes, the influence of coupled redox systems has been tested on the kinetics of the light emission.

When Fe^{2+} ions are added into the reaction mixture, during the course of the emission, a quenching of the light emission is observed. After the extinction of the emission, the addition of small amounts of EDTA give rise to light pulses which mimic the in vivo emissions and those observed on particulate material. A sequence of several flashes

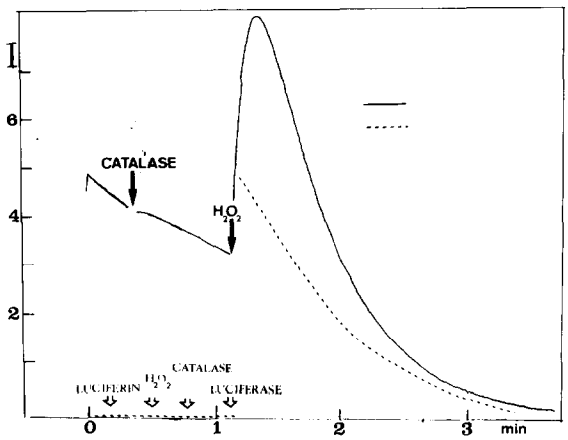

Fig.3. Effect of catalase and H_2O_2 on the light emission. Full line: Successive additions of 3 10^{-8} M catalase and 20 μl of 6% H_2O_2. Dotted line: Luciferin, H_2O_2 and catalase are preincubated and the emission is triggered by luciferase. The concentrations are the same as those used in the upper experiment.

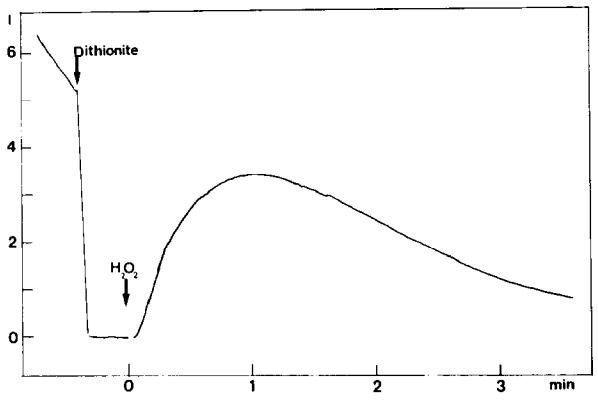

Fig.4. Effect of dithionite and H_2O_2 on the light emission. Dithionite is added up to the extinction; light is triggered by H_2O_2. The luciferase and luciferin concentrations are those used in fig.1 (third kinetic). Dotted line: preincubation of luciferin with dithionite and H_2O_2.

is also triggered by successive additions of EDTA, when luciferase is added to luciferin pre-incubated with ferrous ions (fig.2). Moreover, instead of EDTA, small variations of the pH induced by HCl also trigger short light pulses. This behaviour is similar to observations made in the isolated scintillons [11,12].

Hence the light pulses may be generated using a soluble system. In order to go further into the comparison, we shall consider the duration of the emission in relation with other redox systems since

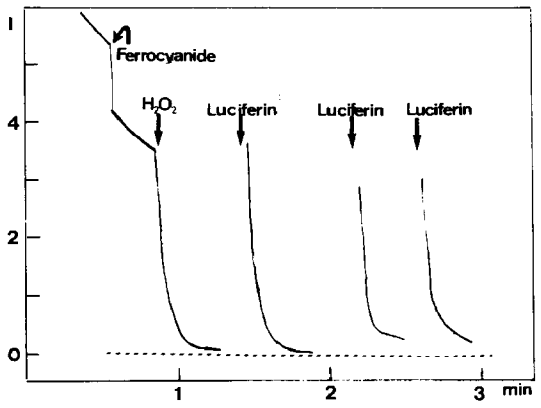

Fig.5. Effect of ferricyanide and H_2O_2 on the light emission. Ferricyanide 3.5 10^{-3} M and 50 µl of 6% H_2O_2. After extinction the bioluminescence is triggered by small amounts of luciferin extracts.

the decay time of the flashes varies from one species to another. When catalase and perhydrol are added during the light emission, a longer flash is observed than the one obtained by addition of the Fe^{2+}/EDTA system. Luciferins do not emit light in the presence of perhydrol or perhydrol plus catalase; in these conditions, addition of luciferases is required to trigger the emission (fig.3). The longest emission coupled with a redox system is observed when the emission is quenched by dithionite and triggered by further addition of perhydrol (fig.4).

On the opposite, the fastest light emission is observed when the ferrocyanide–perhydrol system is used as quencher and luciferins as triggers (fig.5).

4. Discussion

These observations show that specific organites like the scintillons are not a necessary condition for the existence of flashes in these three Dinoflagellates. Indeed, the in vivo emission can be kinetically explained by a two-step mechanism. The first step is the enzymatic reaction which transforms the luciferin in an oxidizable product. The second step may be a non enzymatic oxidization accompanied by a light emission. This is summarized by the following reactions:

$$E + LH_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ELH_2 \xrightarrow{k_2} E + L'H_2 \qquad (1)$$

$$L'H_2 + O_2 \xrightarrow{k_3} L' + h\nu \qquad (2)$$

This interpretation is supported by the following observations: the luciferins cannot be oxidized with ligth emission by perhydrol or catalase plus perhydrol. The presence of luciferases is necessary to initiate the light emission. As indicated in fig.6 the overall reaction rate is linear without saturation, whereas the plot of the initial intensity versus luciferase or luciferin concentration shows the classical shape of enzymatic kinetics (fig.6). This suggest that the reaction (1) is controlled by a luciferase but not the reaction (2). The shape of the flash depends on k_3 and on the mechanism of oxidization as shown

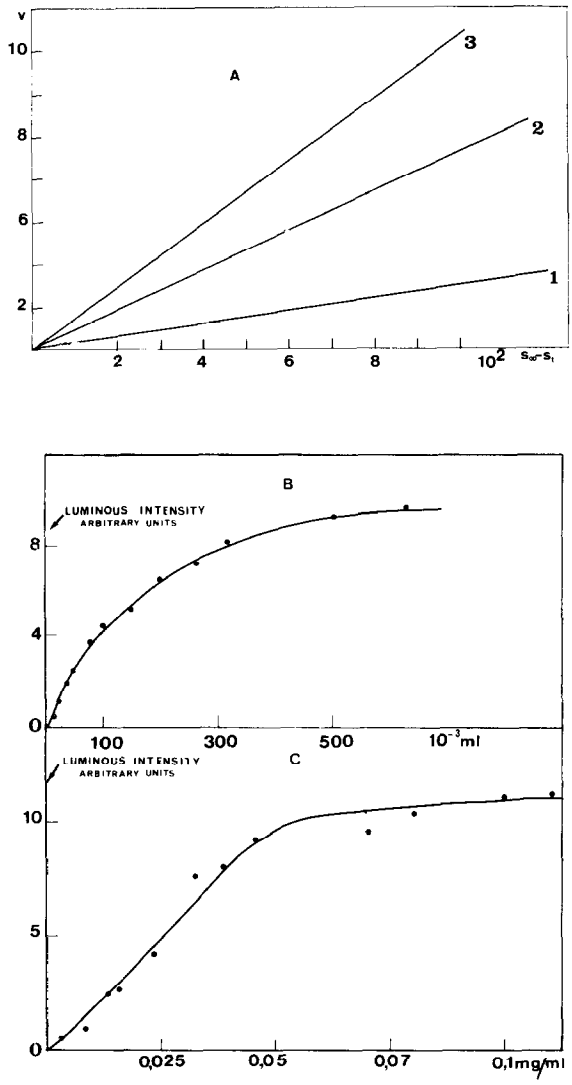

Fig.6. (A) Representation of the overall reaction rate versus luciferin concentration. (B) Initial rate of emission versus the quantity of luciferin extract. (C) Initial rate of emission versus luciferase concentration. These representations are obtained, according to the procedure described in fig.1. A, B, C, concern the luciferase and the luciferin isolated from *Pyrocystic lunula*. Similar curves are obtained with the other species.

in figs.1–5. These mechanisms are very important for the rate of light emission as it has been already shown by Michelson et al. [13–16].

These results do not deny the existence of the scintillons but demonstrate that a specific luminous membrane or particulate system is not required for the obtention of flashes. The redox systems used suggest that the reactions may be associated with the membrane (see figs.1 and 5 for instance). As a matter of fact, the knowledge of the flash mechanism at the cellular level requires further investigations on the redox system equivalent to reaction (2) which may exist within the cell.

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