

## Bioluminescence: Mechanism and Mode of Control of Scintillon Activity

(dinoflagellates/*Gonyaulax*/luciferin/luciferase/control mechanisms)

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**ABSTRACT** Subcellular particles, termed scintillons, isolated from the luminescent dinoflagellate *Gonyaulax*, emit light when the pH is lowered from 8 to 5.7. The capacity to emit a second flash is regained by incubation of scintillons with the low molecular weight luciferin from *Gonyaulax*. This compound was previously demonstrated to be the substrate in the bioluminescent reaction catalyzed by a soluble luciferase isolated from the same cell. A model of the scintillon is presented that involves soluble elements structured within a membrane-bound particle.

Scintillons are bioluminescent particles isolated from cell-free extracts of *Gonyaulax polyedra* and other luminescent dinoflagellates (1, 2). These particles provide a useful model system to study the "on-off" type of control of the activity of a biochemical system that functions in living cells to give brief flashes of light in response to mechanical stimuli. Such a study is facilitated in this system by the fact that the extracts also contain soluble elements with the capacity for bioluminescence (3-5).

Both the soluble and particulate elements may be obtained in a form with the potential for bioluminescence activity by extraction of cells in cold 0.05 M Tris buffer (pH 8)-0.01 M EDTA-1 mM dithiothreitol, with a hand homogenizer (5). After removal of cell debris by centrifugation at  $2000 \times g$  for 5 min, the soluble and particulate fractions were separated by centrifugation for 10 min at  $27,000 \times g$ . In previous studies, there was no direct evidence that the two systems were related. Neither the soluble enzyme (luciferase) nor the substrate (luciferin) was found to stimulate the scintillon activity, and there was in turn no apparent effect of the particulate fraction on the luminescence of the soluble system (6). Moreover, the pH optimum of the scintillon reaction (pH 5.7) (1) appeared to be lower than that reported for the soluble system (pH 6.6) (4), but the two had not been compared in the same buffer.

In the present study, we show that in fact the low molecular weight substrate (*Gonyaulax* luciferin) is active for light emission by scintillons. Based on this finding, and taking into account the recent demonstration that there exists a specific substrate-binding protein (5), we suggest a model for the scintillon.

In the soluble fraction of extracts made as described above, the luciferase occurs in its higher molecular weight form (about 150,000), which at pH 8 is inactive for light emission. The substrate is found in the same extract, not as the free

low molecular weight species, but bound noncovalently to a high molecular weight (about 100,000) protein (4, 5). Light emission fails to occur in these extracts, even at 20°, for two reasons. As stated above, the luciferase itself is inactive at pH 8 (see Fig. 4); also, the luciferin (substrate) is unavailable in the reaction when tightly bound to its protein. When the pH is lowered, both of these restrictions are relieved; the luciferin is released and light emission occurs, continuing for many minutes.

The scintillon activity is distinct from soluble activity in that it occurs as a brief flash with a duration of only about 0.1 sec. (Fig. 1a), but similar in that it also is initiated *in vitro* by a shift of the pH of the medium. In fact, the flash of the living cell, which occurs *in vivo* upon mechanical stimulation, is kinetically similar to the *in vitro* scintillon flash (7).

"Discharged" scintillons, which have been caused to emit by the pH jump, do not emit more light when returned to pH 8 and exposed again to a pH jump to 5.7. However, when discharged scintillons were brought to pH 8 in the presence of free luciferin of low molecular weight (removed from its binding protein) and incubated at room temperature for a few minutes, a second flash (Fig. 1b) was obtained when the assay was repeated.

The "recharging" must take place at pH 8 rather than pH 5.7, and luciferin must be present during the incubation. Free luciferin is susceptible to a spontaneous activity loss, presumably due to autoxidation (3). Luciferin whose activity in the soluble assay had been lost in this way was inactive for recharging scintillons.

The response obtained in this recharging assay was greater both when larger amounts of luciferin were used and when incubation time was increased (Fig. 2). The curves suggest that 100% recovery might be obtained by the use of more luciferin and a longer incubation time.

Active scintillons could sometimes be caused to emit additional light by incubation with luciferin in the same fashion. Analysis by centrifugation in a sucrose gradient indicates that the less-dense scintillons are preferentially subject to this stimulation by luciferin. Upon centrifugation, scintillons band as illustrated in Fig. 3a, exhibiting a considerable density heterogeneity centered at about 1.23 g/cm<sup>3</sup>. The response of each fraction to incubation with luciferin revealed the differential stimulation shown in Fig. 3.

Scintillons that have been discharged and recharged by incubation with luciferin band in essentially the same pattern as "native" particles (Fig. 3b). The experiment also demonstrates that the luciferin is actually bound to the

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scintillons during the incubation, for all soluble unbound components remain at the top of the gradient.

Finally (Fig. 3c), discharged and inactive scintillons band at approximately the same density as do native or recharged scintillons. After centrifugation, their distribution can be

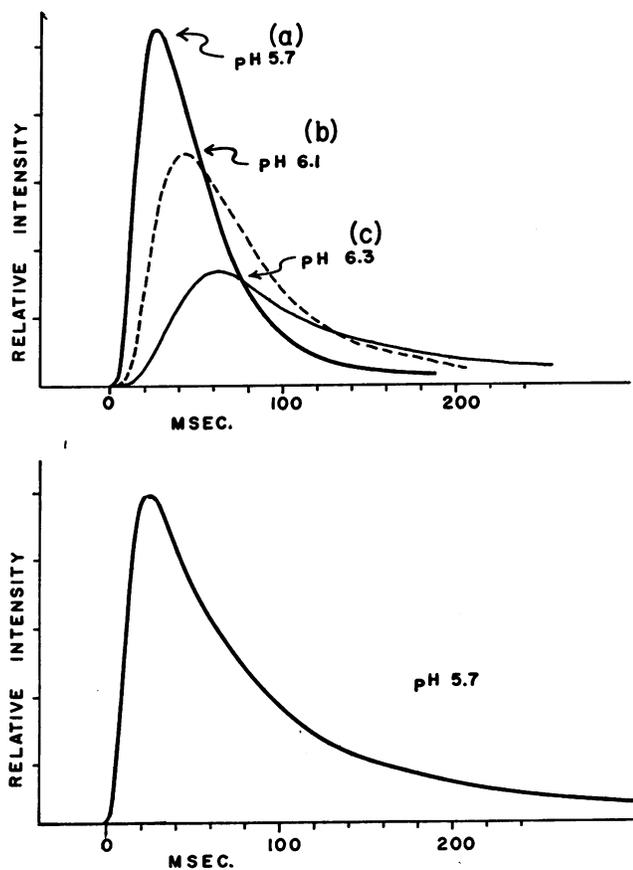


FIG. 1. *In vitro* flashes, obtained by rapid mixing of scintillons with acid in the Gibson stopped-flow apparatus as described (10). The two ordinate scales are not the same; less than 100% "recharging" was usually obtained.

*Top:* (a) Native crude scintillons [1:10 in buffer: 0.05 M Tris-0.01 M EDTA (pH 8), 20°] were mixed at zero time with 0.2 M sodium citrate buffer at pH 5.4, bringing the pH of the mixture to 5.7. In (b) and (c), the pH of the citrate was 5.75 and 5.9, respectively, giving final values of 6.1 and 6.3. Similar results were obtained with purified scintillons. 1 ml of crude scintillons was obtained per liter of cell culture (2).

*Bottom:* "Discharged" crude scintillons were prepared as described above, except 0.03 M citrate was used to lower the pH to 5.7. The pH was then adjusted to 8 with 2 volumes of 0.05 M Tris-0.01 M EDTA (pH 8.5) and the scintillons were "recharged" by incubation with 0.1 ml of luciferin (per ml) for 20 min at 20°. The pH was then lowered to 5.7 by mixing in the stopped-flow apparatus as described above, and the flash was recorded. To prepare luciferin, the supernatant from the crude extract was treated with ammonium sulfate to precipitate the protein-bound luciferin (as well as luciferase) between 35 and 60% saturation. After resuspension in 0.05 M Tris-0.5 mM dithiothreitol (pH 8) and overnight dialysis against 2 mM phosphate buffer (pH 8), the material was heated at 70° for 30 sec, releasing the luciferin from the binding protein. 1 ml of this luciferin was obtained from cells from 2 liters of culture.

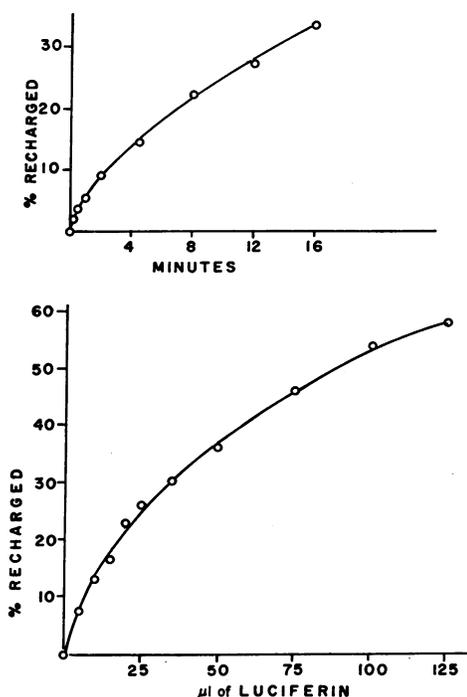


FIG. 2. Recovery of activity of "discharged" scintillons upon incubation with luciferin in a volume of 1 ml at 22°. The experiments illustrate the effect of time of incubation with 50  $\mu$ l of luciferin (*top*), and of luciferin concentration with an incubation time of 17 min (*bottom*). "Discharged" scintillons and luciferin were prepared as described in the legend to Fig. 1. Assays were performed by injection of the buffer used to lower the pH into a vial placed in front of a photomultiplier tube which recorded the total light (2). 100% recharging would have yielded  $10^{10}$  quanta per sample.

determined by incubation with luciferin in the recharging assay.

pH-Activity profiles for the soluble system are compared in Fig. 4 with those for scintillons. Although these are similar to the curves previously reported (1, 4), they have been determined here under the same conditions for both soluble and particulate systems, and with three buffers instead of one.

We now emphasize that the soluble and scintillon pH profiles have significant similarities. Both have a narrow pH range for activity and are subject to a characteristic inhibitory effect of maleate. The pH optima differ by about only 0.8 pH units in citrate and phosphate buffers, and by even less if yield (*bottom graph*) is used as the basis for comparison.

It was suggested (7) that control of the scintillon flash might involve protonation of the luciferin molecule. This postulate was based on the observation that luciferin behaves as an anion above pH 7 on ion-exchange resins, together with the fact that the reaction does not occur above pH 7.1. However, it is now known that luciferin is active up to a pH of at least 9. Luciferase occurs not only in the high molecular weight form (A), but also in a lower molecular weight form (B). The B form is fully active for light emission over a broader pH range, utilizing the same free luciferin molecule (4, 5). The pH "control" thus appears to be a feature of the "A" luciferase molecule, apparently involving a part of the enzyme molecule that can be removed without destroying its catalytic function.

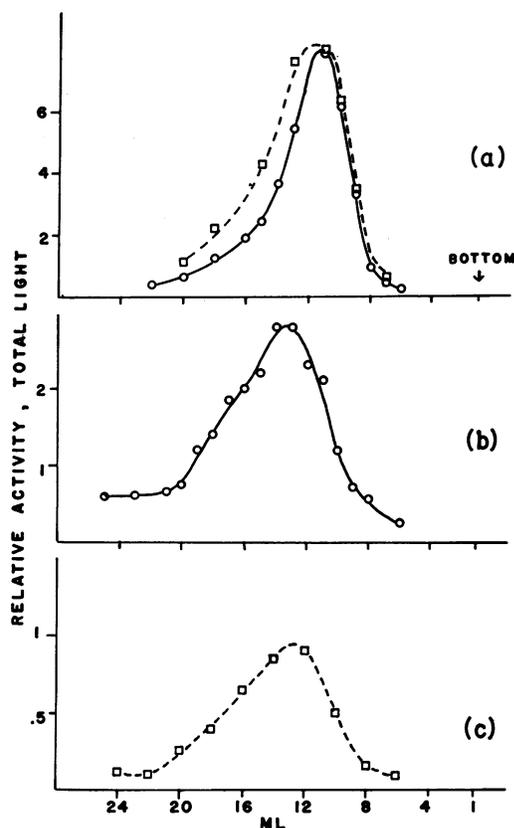


FIG. 3. Sucrose gradient centrifugation of (a) native scintillons (b) recharged scintillons, and (c) discharged scintillons. Gradients were formed from six layers, 4.2-ml each, of sucrose solutions having densities of 1.14–1.29 g/cm<sup>3</sup>, and allowed to “smooth” for 3 days. The sucrose solutions contained 0.05 M Tris–0.01 M EDTA (pH 8). Centrifugation was at 19,000 rpm for 5 hr at 5° in a Spinco 25.1 rotor. Fractions were assayed either by rapidly lowering the pH of a sample to 5.7 by injection of buffer (O—O) or (in a and c) by first incubating at pH 8 with 0.1 ml of crude luciferin (3) for 20 min and then lowering the pH to 5.7 (□—□).

Our scintillon model, which is consistent with the facts now available, includes the “A” luciferase molecule and the luciferin-binding protein, either structurally associated<sup>‡</sup> or adjacent to one another, located within a membrane-bound structure (8). An internal pH change results in a conformational change of both proteins. Oxidation of the luciferin released from the binding protein is then catalyzed by the active form of the luciferase. Proton penetration occurs in the *in vitro* system by virtue of an imposed pH gradient. In the *in vivo* situation, we postulate that the membrane controls proton movement in response to a cellular excitatory event, presumably a membrane potential change, such as described for *Noctiluca* (9).

<sup>‡</sup> We have evidence that the luciferase and the substrate-binding protein may form a complex, as indicated by the fact that there occurs in the soluble fraction a material that mimics the scintillon flash. When this putative complex is subjected to a pH jump, there occurs a scintillon-like flash, superimposed upon the slow-decaying bioluminescence characteristic of the soluble system. The exact conditions required for the formation and stabilization of this postulated complex have not been explored.

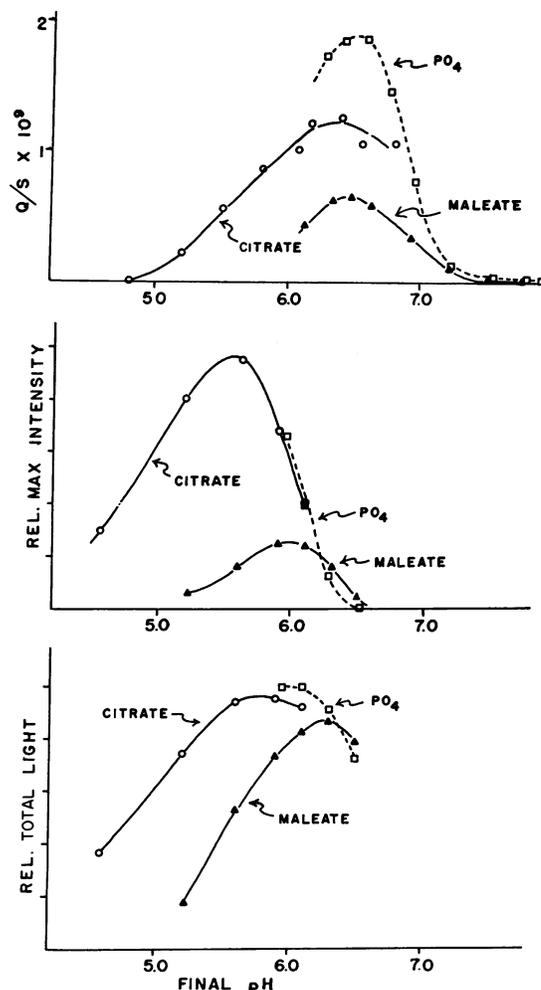


FIG. 4. Effect of pH upon the bioluminescent reaction. *Top:* Free enzyme (*A-luciferase*) and substrate (purified luciferin): Intensity. The partial purification of *A-luciferase* on Sephadex G-100 and of luciferin by DEAE-cellulose chromatography were as described (5). Vials contained 1 ml of 0.01 M Tris–0.01 M EDTA–0.2 mg of bovine serum albumin, and 1 ml of either 0.2 M sodium citrate, phosphate, or maleate. Assays were performed by addition of 25  $\mu$ l of *A-luciferase* to the buffer at the pH specified, and, after one min, 100  $\mu$ l of purified luciferin. The initial (maximum) intensity was recorded as the rate.

*Middle and Bottom: Scintillons: Flash intensity and total light.* Crude scintillons were diluted 10-fold into 0.02 M Tris–0.01 M EDTA (pH 8) at 20° and mixed in the stopped-flow apparatus with either 0.1 M sodium citrate, 0.2 M sodium phosphate, or 0.2 M sodium maleate to achieve the final pH indicated. Intensity and total-light measurements were made simultaneously.

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