LIGHT YIELDS FROM SOLUBLE VERSUS INSOLUBLE EXTRACTS OF THE BIOLUMINESCENT MARINE DINOFLAGELLATE, GONYAULAX POLYEDRA

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The importance of particle-bound bioluminescence ("scintillons") in extracts from marine dinoflagellates such as Gonyaulax polyedra has been the subject of recent conflicting reports (Sweeney and Bouck, 1966; Hastings et al., 1966). Initial observations suggested that of the total light yield obtainable in vitro by far the major portion was associated with the scintillon fraction rather than the soluble portion (Hastings et al., 1966). From this and other evidence it was inferred that the scintillon probably was identical with the in vivo state of bioluminescence. Sweeney and Bouck (1966) pointed out however that if the soluble fraction is assayed under optimal conditions many times more photons could be accumulated than from the scintillons. Additionally the crystalline-like particles observed in electron micrographs of Gonyaulax could be observed in non-luminous as well as luminous species but appear to be absent in the bioluminescent species Noctiluca miliaris (Eckert and Reynolds, 1967).

An investigation into the bioluminescence mechanism in dinoflagellates and particularly the influence of diurnal rhythms requires the role of the scintillons to be established. Three independent questions must be considered: 1. Are the crystals observed in electron micrographs of Gonyaulax to be identified with the "flashing units" visualized in Noctiluca (Eckert and Reynolds, 1967; Eckert, 1966)? 2. Do the crystals when extracted become

the particulate enzyme system, i.e., "scintillons"? 3. Is the particulate enzyme system the origin of the *in vivo* bioluminescence? This present report bears only on the third question.

By carrying out extraction and assay according to published procedures (Sweeney and Bouck, 1966; Hastings *et al.*, 1966), we can confirm Sweeney's result that the particulate contribution is negligible in comparison to that from the soluble system. However, these procedures yield extracts containing only a few percent of the maximum stimulable *in vivo* light and conclusions based on such low yields could be misleading.

We wish to report here an improved procedure which can result in over 30% of the *maximum* light obtainable *in vivo* in the sum of scintillon and soluble extracts. The total light yield from the soluble portion assayed under optimal conditions still exceeds that from the scintillon system. The significant prescence of scintillons however, opens again the question of their function in dinoflagellate bioluminescence.

Experimental

A stock culture was obtained from B. M. Sweeney and grown in 1 liter quantities in a supplemented sea-water medium (Sweeney and Hastings, 1957) adjusted to pH 8.0 and contained in 2.8 liter Fernbach flasks. Growth was in a 12-12 hour day-night cycle, 20-23^{o}C with an illumination of about 400 foot candles from "cool-white" fluorescence lamps. The results reported here were unchanged by using a chemically defined medium (Hastings *et al.*, 1966).

The cells were harvested at about 20,000 cells/cc by taking the culture in the sixth hour of darkness and illuminating for a given time (400 foot candles), a procedure known to improve extraction yields of luciferin (Bode *et al.*, 1963). The culture was then filtered off in a Buchner funnel, scraping the cells off the paper and grinding at room temperature in a Ten-Broeck homogenizer with a deoxygenated solution containing sucrose (0.1M) tris-maleate (0.05M, pH 8.2), EDTA (1 g/liter), bovine serum albumin (0.3 g/liter) and 2-mer-

captoethanol (.02M). The debris was centrifuged off (200-500xg, 3 min) and reground several times following the procedure approximately as previously described (Hastings et al., 1966). It is largely the presence of mercaptoethanol in the grinding buffer which results in the high yield. This was used after finding that in this concentration it increases the half-life of a scintillon preparation (2^{o}C) from 10 hours to several days.

The light measuring apparatus was calibrated absolutely using the luminol chemiluminescence standard previously described (Lee et al., 1966). The maximum in vivo light was estimated by bubbling a 2 ml sample with a stream of air and integrating the light obtained over several minutes. The sample was withdrawn from the culture about 18 hours before the assay which was made at the sixth hour of darkness, just prior to the illumination.

The scintillon light yield was obtained by injecting the crude scintillon suspension (0.2 ml) into dilute acetic acid (1.8 ml, final pH 5.7) and integrating the light obtained (30 sec.). The soluble assay was made by injecting the crude soluble preparation (0.5 ml) into an ammonium sulfate (1M), tris-maleate (0.2M) mixture (1.5 ml, pH 6.5). The resulting light emission was integrated over 30 minutes.

Results and Discussion

At the time of harvest the in vivo total stimulable light per cell was in the range 1-1.5 x 10^{8} quanta. This is several times greater than previously reported due to the difference in absolute standards used (Hastings and Reynolds, 1966). The spontaneous emission during a 12 hour dark cycle amounts to about 1 x 10^{8} quanta per cell and up to 1.5 x 10^{8} for a dark period extended a few hours. This gives some assurance that the total luminescence capacity of the cell can be stimulated out by the technique used. This total is also reasonably independent of the method of stimulation, e.g., vigorous bubbling versus slow stirring.

The time rate of stimulated emission, however, is dependent on a

number of factors. Using a constant method of bubbling, however, the time course is reproducible and appears to consist of two portions; a "fast" portion was complete in the first few seconds of stimulation, while a "slow" portion took up to twenty minutes for completion.

In the table the maximum light yield which can be stimulated from the unilluminated cells is set at 100, on a per cell basis. With no illumination all but a few per cent of the light is obtained in the initial flash and the extractable yield is negligible. With increasing illumination

Table

Light yields extractable from Gonyaulax polyedra in the sixth hour of darkness after illumination (400 foot-candles, cool-white fluorescence lamps, 22°C). Ratio of light yields in the soluble and scintillon fractions is shown. The in vivo light yield per cell is arbitrarily set at 100 for the unilluminated case. Each result is the mean of three determinations.

Illumination Time	Light Yield in vivo		Extractable Light Yield	Ratio
(min.)	Total	"Slow" portion	(Soluble + Scintillon)	Soluble/Scintil
0	100	5	0	-
15	95	40	2	10
30	70	45	6	5
70	50	40	18	5
90	30	25	30	2

time the "slow" portion of the in vivo light increases in proportion and absolute magnitude along with the extractable luminescence.

The results although qualitative show that with increasing illumination time the total stimulable luminescence decreases (Haxo and Sweeney, 1955), the sensitivity to stimulation decreases and the total extractability of both soluble and scintillon components increase (Bode et al., 1963). There are two alternate models which will explain this and previous data:

1. The particulate system is an artifact introduced by the extraction procedure, or has some other function un-

related to the actual *in vivo* emission mechanism. The soluble system is an intermediate in some sort of light-mediated relationship to the bioluminescence system.

2. The in vivo emission originates from the particulate enzyme reaction under light-mediated control. Light decouples this control and the uncoupled scintillons revert to the soluble form, where the bioluminescence is no longer stimulable.

The second model is similar to that proposed for the sea pansy, *Renilla reniformis* (Kreiss and Cormier, 1967). The soluble form is extracted as the soluble system and is proportional to the in vivo light decrease on illumination. Only the decoupled scintillons are extractable and are represented by the slow part of the *in vivo* luminescence, which arises from the slow rate of return of the scintillons to the coupled condition.

The first mechanism suggests that only the light lost on illumination from the *in vivo* emission is available for extraction. The extractable light yield does not exceed this loss. The illumination removes the components from the bioluminescence center, the slow portion of the stimulable emission representing their partial return. This model does not explain the similarity in rate of light emission from the scintillons and the stimulated cells and why if an artifact, the scintillons are present in such quantity.

The second mechanism needs to explain why as the cells are illuminated and become less stimulable, the scintillons become more extractable. Preliminary experiments done in the early hours of darkness when the cells are less stimulable to begin with show after illumination and extraction the same high yields and high proportion of scintillons.

If the particulate system cannot be extracted from other species of bioluminescent dinoflagellates or is present during the daylight hours for *Gonyaulax*, then the first model would seem to be the more appropriate.

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References

Bode, V.C., DeSa, R. and Hastings, J.W., Science, 141, 913 (1963).
Eckert, R., Science, 151, 349 (1966).
Eckert, R., in "Bioluminescence in Progress, " F. H. Johnson and Y. Haneda, Eds. (Princeton University Press, Princeton, N.J., 1966) p. 269.
Eckert R. and Reynolds, G.T., J. Gen. Physiol., 50, 1429 (1967).
Hastings, J.W. and Reynolds, G.T., in "Bioluminescence in Progress," op. cit., p. 45 (1966).
Hastings, J.W., Vergin, M. and DeSa, R., ibid., p. 301 (1966).
Haxo, F. T. and Sweeney, B. M., in "The Luminescence of Biological Systems," F. H. Johnson, Ed. (American Assoc. Adv. Science, Washington, D.C., 1955) p. 415.
Kreiss, P. and Cormier, M. J., Biochim. Biophys. Acta, 141, 181 (1967).
Lee, J., Wesley, A.S., Ferguson, J.F. and Seliger, H.H., "Bioluminescence in Progress," op. cit., p. 35 (1966).
Sweeney, B. M. and Bouck, Y. B., in "Bioluminescence in Progress," op. cit., p. 331 (1966).
Sweeney, B. M. and Hastings, J. W., J. Cell. Comp. Physiol., 49, 115 (1957).