mately 1 percent of instances two nuclear bodies were observed to be in close proximity or in contiguity. A nucleus cut approximately through the middle usually contained portions of one or two bodies in the plane of the section. In no instance was more than one observed in an individual section of a lymphocyte.

The nuclear bodies were judged to be most numerous and complex in structure in the parenchymatous cells of the adrenal cortex, particularly in the zona fasciculata, and also, though to a lesser degree, in the epithelial cells of renal-collecting tubules and in lymphocytes. They appeared considerably less frequently and were more simple in structure in endothelial cells and fibroblasts.

The majority of nuclear bodies were approximately spherical and measured 0.8 to 1.2 μ in diameter. Typically, they consisted of a peripheral fibrillar portion, and a central or core area, approximately 0.4 to 0.6 μ in size, which contained electron-opaque particulate matter (Fig. 1). This central area appeared to be best developed in the parenchymatous cells of the adrenal cortex. The particulates measured approximately 100 to 300 angstroms in diameter and were arranged into aggregates of irregular sizes and shapes, or into short strands. Occasionally the central area was substantially larger and contained particulate matter in random arrangement. In some types of cells, particularly in lymphocytes and endothelial cells, the central or core area of the nuclear body was considerably smaller, or in many instances unobservable. In the latter instances the peripheral fibrillar portion, which consisted of concentric, thin interanastomosing strands, appeared to be continuous to the center of the body. Accurate measurements of the thickness of the fibrillar material were not obtainable since these structures varied considerably in size and appeared to be uniformly thinner in Maraglas (*6*) embedded tissue.

Previous reports (*1–4*) indicate in general that this body may be substantially smaller (0.33 to 0.62 μ) and at times not spherical in other species. From the descriptions and photographs presented before (*1–4*) it is not clear as to whether or not the nuclear bodies contain component parts similar to those described here. The reports of Brody, however, do mention the presence of "concentrically arranged membranes" (*1*).

Difficulties were at first encountered with staining of the particulate matter of the central or core area. A series of experiments with single and combination stains indicated that satisfactory results could be obtained using a uranyl acetate-lead monoxide sequence. Thus, tissue sections embedded in Vestopal W (*5*), or Maraglas (*6*), were first stained with saturated uranyl acetate for 1 to 3 hours, blotted dry, and subsequently subjected to Karnovsky's lead monoxide mixture "A" (*7*) for 5 minutes, with subsequent washing in distilled water free of carbon dioxide. All tissues were subjected to fixation with buffered osmium-sucrose at *p*H 7.2 to 7.4 according to the method of Caulfield (*8*).

The significance of the nuclear body described here cannot be ascertained at present. It would appear, in cells of the calf, that this body, although of a similar nature to that described in other species, is a more complex structure. It would also appear that this structure may be concerned with specific metabolic processes which are characteristic of certain cell types in the calf, since it has a more prominent central electron-opaque portion in parenchymatous cells than in interstitial cells (*9*).

ALVIN F. WEBER
STEPHEN P. FROMMES
Department of Veterinary Anatomy, College of Veterinary Medicine, University of Minnesota, St. Paul 1

References and Notes

1. I. Brody, *J. Ultrastruct. Res.* **6**, 304 (1962); *ibid.*, p. 324.
2. H. Latta and A. B. Maunsbach, *ibid.*, p. 562.
3. M. G. Farquhar and G. E. Palade, *J. Cell Biol.* **13**, 55 (1962).
4. J. G. Lafontaine, *J. Biophys. Biochem. Cytol.* **4**, 777 (1958).
5. E. Kellenberger, E. Schwab, A. Ryter, *Experientia* **12**, 421 (1956).
6. J. Freeman and B. Spurlock, *J. Cell Biol.* **13**, 437 (1962).
7. M. Karnovsky, *J. Biophys. Biochem. Cytol.* **11**, 729 (1961).
8. J. B. Caulfield, *ibid.* **3**, 827 (1957).
9. This research was supported in part by U.S. Public Health Service grants A-2462(C2) and GM-7009(C4), and Atomic Energy Commission grant AT (11-1)-910.

10 June 1963

Daily Rhythm of Luciferin Activity in Gonyaulax polyedra

Abstract. *Extracts prepared from* Gonyaulax *cells harvested late in the 12-hour dark period of an artificial 24-hour day contain as much as four times more luciferin activity than similar extracts prepared during the light period. If cultures are not treated with heat or light to reduce the flashing associated with handling, the time of maximum activity is obscured.*

The control mechanism governing persistent daily rhythms observed in many organisms has been referred to as a biological clock (*1*). A number of previous studies were concerned with the biochemical pathways through which this clock functions and their relation to the more ultimate control mechanism (*2*). Several biochemical changes immediately responsible for the diurnal rhythm of bioluminescence observed in *Gonyaulax polyedra* have been studied (*3*). We now report on the activity of luciferin, a substrate for the enzyme *Gonyaulax* luciferase, which catalyzes the luminescent reaction (see *4*).

Although bioluminescence in the living cell is maximum during the night, we found that the luciferin activity was considerably greater in extracts prepared during the day. Subsequent experiments indicate that this result reflects the differential utilization of the endogenous supply of luciferin during the process of harvesting the cells. Manipulations associated with collecting the cells stimulate them to emit flashes of light, and the emission was much greater when cells were handled during the dark period.

By appropriate changes in the way the *Gonyaulax* cultures were treated before extraction, much more luciferin activity could be obtained from cells harvested during the night than from similar harvests during the day. This result is consistent with the hypothesis that the luciferin concentration in vivo is higher during that portion of the day when luminescence is maximal.

Gonyaulax polyedra is a marine dinoflagellate which is both photosynthetic and luminescent. Cells were cultured at 22°C in 750 ml of a modified seawater medium in Fernbach flasks. Illumination was provided either by natural daylight or by fluorescent lights at an intensity of 9900 lu/m^2 (900 ft ca), day-night conditions being simulated with artificial light by alternating 12-hour periods of light and dark (LD: 12–12).

Crude cell-free extracts containing

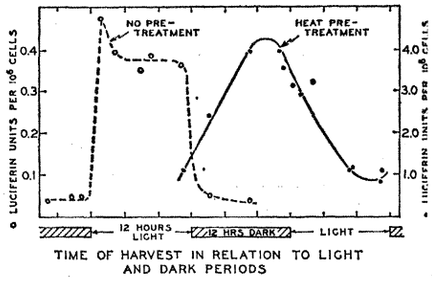

Fig. 1. The ordinate scales are different for the two curves, which depict the luciferin activity measured in extracts of cells harvested by different means. Time of day is the time of harvesting in relation to the environmental light and dark periods; it does not relate the order in which the experiments were carried out. A unit of luciferin activity corresponds to an initial rate of 10^7 quanta sec^{-1}.

luciferin activity were obtained by extracting cells with water at 95°C for 2 min. The solution was rapidly cooled and used as substrate in the bioluminescent reaction after centrifugation at 2000*g* for several minutes to remove cellular debris. Luciferin activity was estimated by measurement of the initial rate of the reaction with the partially purified enzyme, *Gonyaulax* luciferase. Reaction rate is given directly by the intensity of the light-emitting reaction and was measured with a photomultiplier photometer whose output was recorded on a graphic instrument. The values for luciferin are based on measurements made in the concentration range where light intensity was a linear function of concentration. In some instances, total light (yield) measurements were also performed. The enzyme preparation used in the assays was purified by fractionation with ammonium sulfate and stored at −15°C (*4*).

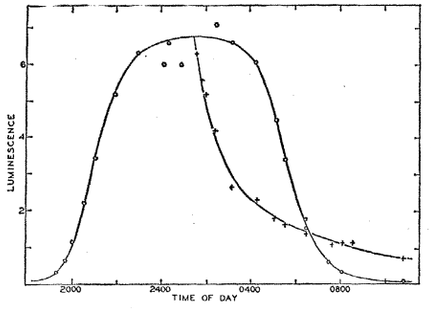

Fig. 2. This illustrates the inhibition of the luminescence which occurs in vivo when *Gonyaulax* cells are exposed to bright light 7700 lu/m² (700 ft ca), during the middle of the dark period. The nature of the diurnal rhythm of luminescence in vivo is evident from the curve (circles). When samples of the culture were placed in the light, they rapidly lost the capacity to emit light when assayed at the later times as indicated (crosses). This is due to the inhibition of flashing. In the assay procedure the cells were vigorously agitated by bubbling a stream of air through the culture and measuring the total amount of light emitted (*3*). Units are relative. The cultures were maintained in natural illumination. Abscissa, time of day. Sunrise, 0630; sunset, 1935. Note that the figure is for only a portion of the day.

The results initially obtained, when cells were simply harvested by filtration on a Büchner funnel, scraped from the filter paper, and extracted with hot water, are shown in Fig. 1 (dotted line and left ordinate scale). Note that the luciferin activity of the extracts increased rather abruptly after the dark-to-light transition, maintained a high level during the day, and promptly decreased again very early in the dark period. The "spike" in the curve was repeatedly encountered. In contrast, a diurnal rhythm is usually best represented by a sinusoidal-like function. Furthermore, it could not be assumed that the exposure to light per se caused concentrations of luciferin to be higher in the daytime, since cultures grown in constant light did not yield extracts with high luciferin activity (Table 1).

In considering these results, we were aware that an enormous amount of light was emitted during the process of harvesting. Individual cells emit light as flashes (like the firefly), and flashing is induced by any mechanical agitation. It was evident from visual observation during filtration that the light emitted by this flashing was far greater during the night than during the day. This cellular rhythm is known to be associated with changes in enzyme activity (*6*).

In addition, however, there might occur either a daily fluctuation in the cellular level of luciferin, or a daily change in the susceptibility of the cell to flash, thereby emitting light, or both. If the second situation obtained, then it would be reasonable to expect lower apparent luciferin activities in extracts from cells harvested during the night, solely because a greater percentage of the substrate present would be utilized in the light emission stimulated by the harvesting process. Moreover, such differential luciferin utilization might actually obscure a possible change in the concentration of luciferin in vivo.

Table 1. The effect of heat treatment prior to harvest upon the yield of luciferin activity in extracts. Luciferin activities are given in arbitrary units based on equal numbers of cells.

Illumination during growth*	Time of harvest	Relative luciferin activity	
		Normal	Heat-treated
LD: 12–12	Dark	4.0	100
LD: 12–12	Light	63.	69
Constant light	Arbitrary	5.0	64

* Cells grown on LD: 12–12 were harvested in the middle of either the dark or the light period, the yield of luciferin being greatly increased by heat.

Attempts were then made either to prevent the flashing, and hence the differential loss which was postulated to occur during harvesting. A number of presumptive inhibitors and other chemical agents were added to cultures in an effort to inhibit flashing, but none was effective. It was discovered, however, that a marked increase in the yield of luciferin occurred if the temperature of the culture was rapidly increased to 34°C before harvesting (*5*). As shown in Table 1, the amount of luciferin extracted from cells harvested during the dark period can thereby be increased as much as 25-fold, but this temperature treatment does not appreciably change the yield from cells harvested during the light. Moreover, with cells grown on constant light, the heat treatment increased the yield of luciferin to a level comparable to that from cells harvested during the light portion of a light-dark regime. Visual observation confirmed that the temperature shock had indeed decreased flashing during the harvesting of cells.

The nature of the diurnal rhythm of luciferin activity was then reinvestigated by utilizing this temperature treatment before harvesting. As shown in Fig. 1

Table 2. Luciferin activity in extracts of cells exposed to heat and bright light. Cultures were grown on LD: 12–12 and experiments begun in the middle of the dark period. Luciferin activities are given in arbitrary units for equal numbers of cells.

Duration of light exposure (mins)	Relative luciferin activity	
	Light	Light and heat
0	10.1	123
10	12.2	104
30	26.5	102
90	103.	117

(solid line and right-hand ordinate), the maximum amount of luciferin activity was then obtained from cells harvested during the dark period. In this case, a sinusoidal-type function was observed, with the increase in extractable luciferin beginning before the end of the light period. Likewise, the decrease anticipated the onset of the light period and was, therefore, not triggered by it. With this technique of harvesting, the changes in luciferin activity in extracts continue to occur in the absence of an environmental light-dark cycle with cells kept in constant dim light (*6*).

An apparently analogous inhibition of flashing and concomitant conservation of luciferin can also be caused by exposing the cells to bright light (Table 2). If cells are exposed to bright light during the middle of the dark period (that is, at the time when the capacity is maximum for emitting light by flashing in response to agitation), the light-emitting capacity of the living cell drops sharply (Fig. 2). The present results demonstrate that this effect is not due to a destruction of luciferin by light—which had been considered to be one of the likely possibilities—but to an inhibition of the ability of the cell to respond to stimulation. The amount of luciferin extractable from cells harvested in the middle of the dark period thus increases dramatically upon exposure to a very bright light (Table 2), in parallel with the inhibition of the ability of the living cell to emit light following stimulation.

It is well known that extraction procedures often initiate chemical changes which can obscure certain aspects of cell biochemistry. The example encountered here seems analogous to one in muscle where glycolysis may be stimulated during extraction and cause production of lactic acid equivalent to that following exhaustive stimulation in vivo (*7*). The breakdown of certain compounds during extraction also is encountered and can be frequently attributed to the release or activation of specific enzymes, or to both. Similarly, a differential loss of enzyme activity during extraction may occur. For example, changes in the extractable activity of specific enzymes correlated with stages in the developmental pattern of the slime mold were attributable to differential inactivation of particular enzymes during the extraction process (*8*).

In conclusion, a daily variation in the bioluminescent flashing which occurs during extraction obscures the phase and amplitude of a daily rhythm in cellular luciferin. When this variation is inhibited, the time of maximum intracellular luciferin activity corresponds closely to the time of maximum bioluminescence in vivo (*9*).

VERNON C. BODE*
RICHARD DESA
J. WOODLAND HASTINGS
Biochemistry Division,
University of Illinois, Urbana

References and Notes

1. *Biological Clocks, Cold Spring Harbor Symp. Quant. Biol.* **25**, **1960** (1960).
2. J. W. Hastings, *ibid.*, p. 131; M. W. Karakashian and J. W. Hastings, *Proc. Natl. Acad. Sci. U.S.* **48**, 2130 (1962).
3. B. M. Sweeney and J. W. Hastings, *J. Cell. Comp. Physiol.* **49**, 115 (1957); J. W. Hastings and B. M. Sweeney, *Biol. Bull.* **115**, 440 (1958).
4. ———, *J. Cell. Comp. Physiol.* **49**, 209 (1957); J. W. Hastings and V. C. Bode, in *Light and Life*, W. D. McElroy and B. Glass, Eds. (Johns Hopkins Univ. Press, Baltimore, 1961), p. 292.
5. The temperature must be increased with minimal agitation. The growth flask was placed in a 46°C water bath and the culture was warmed (without shaking) for about 4 minutes to 34°C. The temperature required to obtain the effect is confined to a relatively narrow range. Below 32°C no marked differences from controls were observed, and extracts of cells which had been brought to temperatures of 37°C or higher had less luciferin activity.
6. J. W. Hastings and V. C. Bode, *Ann. N.Y. Acad. Sci.* **98**, 876 (1962).
7. W. M. Fletcher and F. G. Hopkins, *J. Physiol.* **35**, 247 (1907).
8. B. E. Wright, *Proc. Natl. Acad. Sci. U.S.* **46**, 798 (1960).
9. Supported in part by a grant from the National Science Foundation and by a contract from the Office of Naval Research.
* National Institutes of Health predoctoral fellow; present address: Department of Biochemistry, Stanford University School of Medicine, Palo Alto, California.

22 May 1963

Isolation of a Viral Agent from Pseudocowpox Disease

Abstract. *A virus with characteristics similar to the pox group was isolated in tissue culture from bovine skin and oral lesions typical of pseudocowpox. The properties of this agent, certain of which differ from vaccinia virus, suggest that the isolate is the etiologic agent of the pseudocowpox syndrome.*

Pseudocowpox, or milker's nodules, or paravaccinia (*1*), is a disease distinguishable from cowpox in that it does not confer immunity to vaccinia (*2*). This report describes the first isolation of a cytopathogenic virus obtained from pseudocowpox in cattle.

Skin lesions were excised from a cow's teat and her calf's mouth. (Identical results were obtained with the excision of lesions from the teat alone.) A pool of three pseudocowpox lesions was ground and diluted with Earle's salt solution. The clarified suspension was inoculated into primary cultures of fetal bovine testes and a cytopathic effect was observed after 10 days. The destruction of the cell sheet was complete in 12 days. The agent has now been carried for eight consecutive passages in primary cultures of bovine testes. The initial appearance of the cytopathic effect is a rounding and increased granularity of the cells. This is followed by further degenerative changes with subsequent cellular detachment from the glass in a patchwork pattern.

Infected cells stained with hematoxylin and eosin show large cytoplasmic inclusion bodies similar to those seen with pox virus infection. Preparations stained with acridine orange and examined in a fluorescence microscope show that the cytoplasmic inclusion and cell nuclei fluoresce green (Fig. 1). Treatment of infected cells with 100 μg of desoxyribonuclease per milliliter for 60 minutes at 37°C eliminated the green cytoplasmic fluorescence. These results suggest that the virus contains DNA. The eighth passage in bovine testes cells had a titer of $10^{4.7}$ $TCID_{50}$/ml (tissue culture infectious dose). This titer was obtained 14 days after inoculation. The virus forms smooth, round plaques 4 to 5 mm in diameter 10 days after plating. No decrease in titer was noted upon exposure of the virus to 4°C for 1 or 24 hours; to 37°C for 20 or 60 minutes; and to 56°C for 30 minutes. Treatment at 60°C for 30 minutes resulted in a complete loss of activity. Treatment of a virus suspension with diethyl ether (final ether concentration, 20 percent) for 18 hours at 4°C caused no appreciable loss of virus activity.

Hemagglutination did not occur at 4°C or at room temperature with human O red cells, or bovine, rat, hamster, guinea pig, monkey, or vaccinia-susceptible chicken erthrocytes.

Infectious fluid from bovine testes