The Effects of Inhibitors of Macromolecular Biosynthesis upon the Persistent Rhythm of Luminescence in *Gonyaulax*

MARLENE W. KARAKASHIAN and J. WOODLAND HASTINGS

From the Biochemistry Division, University of Illinois, Urbana. Dr. Karakashian's present address is Department of Biology, Reed College, Portland, Oregon

ABSTRACT Certain inhibitors of nucleic acid and protein synthesis, namely actinomycin D, mitomycin C, and puromycin, have been found to block the expression of a persistent daily rhythm of bioluminescence. The action does not inhibit luminescence *per se* but rather the rhythmicity. Exposure of the cells to these inhibitors for only a few hours, which might be expected to thereby delay the rhythm by a few hours, does not in fact have this effect. Chloramphenicol and amethopterin do not inhibit the rhythm. It is proposed that the functioning of the clock-like rhythmic mechanism depends upon the cell's normal ability to synthesize RNA.

INTRODUCTION

We have recently reported that the daily rhythm of bioluminescence in the marine dinoflagellate, *Gonyaulax polyedra*, is inhibited by the antibiotic actinomycin D (1). In view of the known specificity of action of this compound, an important role for DNA-dependent RNA synthesis was suggested. Considering the biochemical interrelationships of DNA, RNA, and protein, it is important to investigate the effects of various other inhibitors which act at different steps in the biosynthesis and metabolism of these macromolecules. The present paper describes experiments with several such inhibitors and includes additional studies with actinomycin D, puromycin, and chloramphenicol. The results confirm our previous experiments and lend further support to the conclusion that the functioning of the clock-like rhythmic mechanism is dependent upon the cell's unimpaired ability to synthesize RNA.

MATERIALS AND METHODS

The organism used was the photosynthetic and luminescent marine dinoflagellate, *Gonyaulax polyedra*. Cultures were grown in a modified sea water medium (2) at 21°C
under conditions of alternating light and dark periods of 12 hours each. Illumination
was from fluorescent bulbs at an intensity of about 1000 foot-candles. When cultures
had attained a density of about 3000 cells per ml, they were dispensed in 12 ml
aliquots into vials suitable for the Packard liquid scintillation counter turntable. A
modification of this instrument permitted the repeated measurement and recording
of the spontaneous glow of luminescence (3) of the cells in each of the vials.

Vials were transferred to the turntable from conditions of alternating light and
dark at the end of a normal light period; this is referred to as zero time in all experi-
ments. The cells were then kept on the turntable in constant dim light (125 foot-
candles) and constant temperature (21°C). Inhibitors were added at various times
as specified. When desired, the inhibitor was removed by centrifuging and resus-
spending cells in fresh medium. In some of the experiments the action of an inhibitor was
terminated by dilution. In these cases 2 ml of a more concentrated culture (usually
about 11,000 cells per ml) were dispensed into vials, inhibitor was added at the
specified times, and the aliquots were diluted sixfold with fresh medium at the end
of the exposure period. These alternative means of ending inhibitor action yielded
virtually identical results with respect to whether the agent modified the subsequent
appearance of the glow rhythm.

Gonyaulax sensitivity to the inhibitors was tested by measuring effects upon cell
division. Cell counts were made using an electronic particle counter, the Coulter
counter (4). The cells were also observed visually at intervals after the addition of
inhibitor and effects upon motility and cell morphology were noted.

Puromycin dihydrochloride was obtained from Nutritional Biochemicals Corp.,
Cleveland, and amethopterin from American Cyanamid Co., Pearl River, New
York, Mitomycin C and actinomycin D were kindly donated by the Cancer Chem-
otherapy Division of the National Institutes of Health. Novobiocin (albamycin sodium
salt) was a gift from the Upjohn Company, Kalamazoo, Michigan.

RESULTS

Inhibition of Growth A number of presumptive inhibitors were tested for
their effect upon cell division in Gonyaulax. The results of experiments with
some of the compounds which were effective in this regard are presented in
Fig. 1. Cell division stopped promptly in the presence of mitomycin C and
amethopterin, two inhibitors of nucleic acid synthesis.

Certain inhibitors which are known to interfere with normal protein syn-
thesis in other systems were found to be inhibitors of growth in Gonyaulax,
namely chloramphenicol and puromycin. Finally, the antibiotic novobiocin,
whose site of inhibition is not known, was also effective in stopping growth in
Gonyaulax. Actinomycin D has previously been shown to inhibit division at
concentrations as low as 0.08 µg/ml (1).

Effects upon Persistent Rhythm As seen in Fig. 2, some of the inhibitors
were quite effective in blocking the occurrence of rhythmicity while others

1 Packard Instrument Company, La Grange, Illinois.
were not. Confirming previous studies (1), both actinomycin D and puromycin inhibited, the former only after a delay of 1 day, whereas with chloramphenicol the rhythm persisted with a considerably increased amplitude. The effects of mitomycin C are particularly interesting because the rhythmicity was inhibited only after a delay of 3 days, and in addition, because the actual level of luminescence which remained after that time was quite high. Finally, it can be noted that amethopterin and novobiocin allowed the rhythm to persist, although at a rather decreased amplitude.

\[\text{Figure 1. Effects of inhibitors on cell division in Gonyaulax. Inhibitors were added at the time indicated by arrows. Concentrations: mitomycin C (0.52 \mu g/ml), amethopterin (10^{-5} M), novobiocin (5.1 \times 10^{-4} M), chloramphenicol (1.5 \times 10^{-4} M), puromycin (2.0 \times 10^{-4} M). Ordinate, cell number on a logarithmic scale; abscissa, time. Cultures were maintained on a cycle of alternating light and dark periods (light and dark bars at bottom of figure) of 12 hours each at 21^\circ C.} \]

In all of these experiments the inhibitor was left in the culture for the duration of the experiment and, moreover, the cells were not exposed to daily light-dark cycles. The cultures were placed in constant dim light at constant temperature at the beginning of the experiment, zero hours on the graph.

**Effects of Brief Exposure to Inhibitors** The inhibition for only a short time of a biochemical pathway directly involved in the rhythmic function might be expected to result in a permanent phase shift. Thus, a brief blocking of a relevant biochemical pathway might delay the occurrence of the subsequent maximum and the rhythm might persist thereafter with this altered phase.

On the other hand, if a given inhibitor was acting only upon processes which were remote from the rhythmic mechanism (even though these proc-
esses might be controlled by the mechanism), then a transient interruption by the inhibitor might not produce any permanent effect upon the timing.

These considerations prompted an investigation into the effects of short (8 hour) exposures to the various inhibitors. In order to check the possibility that the organism has a differential sensitivity in time, inhibitors were routinely added at 4, 12, and 20 hours after the onset of dim illumination. After the 8 hours of treatment, the inhibitor was either removed by dilution or by centrifugation.
Figure 3. Effects of inhibitors on phase of glow rhythm. The times at which maxima occurred in individual cultures on successive days are indicated by black triangles along a horizontal line. The time of treatment for any given culture is shown by a bar along the horizontal line. Control cultures received distilled water rather than an inhibitory agent. Exposures to all agents except chloramphenicol were terminated by a sixfold dilution of cultures (see Materials and Methods). Chloramphenicol treatments were concluded by centrifuging and resuspending cells in fresh medium. Also shown for purposes of comparison are cultures which were subjected to 6 hours of light at various times in the cycle (indicated by bars) and cultures which were not disturbed during the experiment. Cells were transferred from conditions of alternating light and dark periods to constant dim light at the end of a light period, zero time in the figure.
The results are presented in Fig. 3. Since phase is the point of interest in these experiments, we have simply indicated the time at which the peak occurred with a triangular symbol in lieu of drawing a complete curve for each experiment. In no case was there a permanent phase shift comparable in duration to that of the inhibitor treatment. However, the phase shifts following exposure to puromycin (Fig. 3), though relatively small, are characteristic and significant, suggesting that puromycin may act at a step closely related to the rhythmic system.

Moreover, the cells were fully sensitive in the presence of these inhibitors to phase shifting by a light pulse (5, 6). Fig. 4 illustrates similar experiments which demonstrate that none of the inhibitors used in the present study prevent the induction of a phase shift by a pulse of light.

Light intensity changes constitute the way whereby phase is established under natural conditions and an “inhibitor pulse” can also be viewed as an attempt to chemically mimic a “light pulse.” It may be noted that in pre-
Previous studies of this kind a number of other inhibitors also induced little or no phase shift (7).

The ultimate loss of apparent rhythmicity following a brief exposure to either actinomycin or mitomycin (Figs. 3 and 4) suggests that these agents were not removed by the methods used. This interpretation is supported by experiments in which cells exposed to actinomycin D (0.33 μg/ml) for 8 hours were washed and isolated individually into a large volume of fresh medium. In this case 96 per cent of the cells failed to grow, whereas an equal number of control cells isolated in the same manner exhibited a 60 per cent viability. A more direct evaluation of the effects of these inhibitors upon the rhythmic mechanism is therefore not feasible until a way is found to reverse their inhibitory action.

**Figure 5.** Effect of actinomycin D (0.16 μg/ml) added at various times in the cycle. This experiment shows the limits of the actinomycin-sensitive stage on the 2nd day in constant conditions. The format for the presentation of data is similar to that used in Figs. 3 and 4. It will be noted that the earliest exposures to actinomycin prevent the occurrence of the third maximum and that exposures initiated too late to actually inhibit the third maximum nevertheless delay its appearance. These results suggest that an actinomycin-sensitive stage begins 21 to 27 hours prior to any given glow maximum and ends 6 to 12 hours later, or, about 15 hours before peak light emission.

The delayed effect of actinomycin noted in the preceding experiments could signify that the drug was added after a critical "actinomycin-sensitive" stage in the cycle. This conclusion was supported by the demonstration that even very high concen-
trations of actinomycin D (1.0 μg/ml, final concentration) added at a time such as is indicated in Fig. 2 (8 hours prior to the glow maximum) failed to abolish the luminescence peak or alter the time of its appearance (although no subsequent peaks occurred) (1). By experiments similar to those shown in Fig. 5, it was concluded that actinomycin inhibits processes which normally occur between 21 and 27 hours prior to a given glow peak. That these processes recur daily was demonstrated by the fact that such a sensitive period has been detected by a similar means both on earlier and later days in constant conditions. It is not likely that the effect may be explained by assuming that the compound penetrates only very slowly in view of the results of pulse experiments described above.

Although a high concentration of actinomycin added 21 hours prior to the time of maximum luminescence fails to abolish the appearance of that peak, it does alter the phase slightly. This effect upon phase is quite clear in a group of cultures to which inhibitor was added at various times as indicated (Fig. 5). Additions made 15 or fewer hours prior to the third peak illustrated had little or no effects on its phase, whereas exposures at about 21 hours or earlier produced an effect, giving progressively greater phase delays.

**DISCUSSION**

Studies of daily rhythms in a large number of different organisms have led to the generalization that the cellular mechanisms controlling rhythmicity has a clock-like function, and that it serves to regulate with respect to time of day the physiological and biochemical capacities of various cellular functions (8, 9). The insensitivity of this clock-like mechanism to many inhibitors (7, 10) might be interpreted as an adaptively significant feature of a functional biological clock, analogous to the characteristic feature of temperature independence (11, 12).

It is evident that interpretations of the experiments presented in this paper must rest primarily upon our knowledge concerning the chemical action of these drugs, which is indeed still quite inadequate. It will also be necessary to prove that the action of these compounds on *Gonyaulax* is similar to their reported effects on other cells in order to verify the interpretations given.

By the use of certain inhibitors, it has been possible to stop growth and division in *Gonyaulax* without causing a change in the phase or period of the rhythm of luminescence. Novobiocin is possibly an example of one such compound (13). On the other hand, there are compounds such as actinomycin D which have a clear effect upon both growth and rhythmicity. All known effects of this drug upon intact cells at the low concentrations which we have used indicate that it acts by inhibiting DNA-dependent RNA synthesis (14–16). Hurwitz et al. (17) have reported that low concentrations of actinomycin inhibit purified RNA polymerase in a cell-free system, and that the
The site of the inhibition is the DNA primer. The drug binds strongly and specifically to the guanine residue of DNA (18, 19), and its action may be reversed, at least in cell-free systems, by the addition of excess DNA (20).

A reversible inhibition of the clock system by actinomycin has not yet been possible in Gonyaulax cells. However, there is evidence for an actinomycin-sensitive stage in the cycle, namely, a period of 6 to 12 hours duration occurring about a day in advance of the peak of a given cycle. This period corresponds to the time in the cycle when the cells exhibit a maximum sensitivity to phase shifting by pulses of bright light (Fig. 3). These results suggest that clock-related RNA synthesis occurs intermittently. This specific RNA could presumably be detected and perhaps isolated by an appropriate technique. The occurrence of such a period also raises the interesting question as to whether other Gonyaulax rhythms which differ in phase, e.g. photosynthesis (21), have the same sensitive period.

If DNA-dependent RNA synthesis is involved in clock function, as has been suggested above, the role of DNA as a template in the synthesis of RNA would be a matter of concern. There is considerable evidence indicating that cell division and presumably DNA synthesis per se is a rhythm-controlled, rather than a rhythm-determining process (7, 22). This conclusion is supported by the fact that amethopterin fails to inhibit the rhythm. Its lack of effect upon the persistence of the rhythm may be explained by the fact that it acts by blocking precursor synthesis (23) and presumably does not have any deleterious effect upon the functional integrity of DNA and RNA already present.

The eventual loss of rhythmicity in cells subjected to mitomycin C is probably not the result of an inhibition of DNA synthesis per se. It could be due to the actual destruction of DNA, which would then block both DNA and RNA synthesis. Indeed, Reich and coworkers (24, 25) have shown with both mammalian and bacterial systems that mitomycin C suppresses DNA synthesis by fragmentation of the DNA. It is significant in this regard that the rhythm persists for several days in the presence of mitomycin C, suggesting that RNA synthesis can continue even though partial fragmentation of DNA primer may presumably already have occurred.

The experiments involving inhibitors of protein synthesis may also be explained in terms of RNA involvement in clock function. The insensitivity of the system to chloramphenicol was known (7). It was somewhat surprising to find such a marked inhibitory effect of puromycin. Studies with chloramphenicol were therefore repeated, and while the original findings were confirmed, a striking increase in the amplitude of the rhythm during exposure to the drug was noted. The explanation for the different effects produced by chloramphenicol and puromycin may reside in the fact that the compounds actually act in different ways. Although the precise actions of chloramphenicol...
and puromycin are not yet completely understood, recent reports indicate that they block different, but closely related steps in protein synthesis (26–29). Analyses of the polyuridylic acid–directed incorporation of phenylalanine into protein have demonstrated that puromycin inhibits amino acid incorporation whether it is added before or after the messenger (30). Chloramphenicol, on the other hand, is inhibitory only if it is present before polyuridylic acid is added to the system.

It might thus be inferred that chloramphenicol and puromycin modify the glow rhythm by virtue of their interference with one aspect or another of normal RNA activity. It is known, for example, that chloramphenicol stimulates the production of certain types of RNA, notably messenger RNA (31–33), a fact which might well account for the increased amplitude of the rhythm in the presence of this agent. With regard to the action of puromycin, it might itself act as a repressor of RNA synthesis. Kurland and Maaløe (34) have obtained evidence in a bacterial system which suggests that the rate of RNA synthesis is dependent on the internal amino acid concentration, and they propose a model where transfer RNA acts as a repressor and activated amino acids as inducers of transfer and ribosomal RNA synthesis.

Since all RNA synthesis in normal cells is presumed to be DNA-dependent (35–38), it is of particular interest to speculate upon the nature and possible means of identifying the RNA involved in rhythmicity. The evident necessity for a “daily synthesis” of RNA suggests that at least a part of the RNA involved is relatively short-lived, analogous in this regard to the messenger RNA described for other systems (39, 40) which functions in protein synthesis.

As has been pointed out (7, 41), a simple model of a rhythmic system can be constructed by analogy with the control mechanism described for certain microorganisms. Such mechanisms involve DNA, RNA, and other unspecified metabolites. While the evidence presented here does not establish the validity of a model system such as one of these, it does strongly support the view that systems akin to such known control mechanisms will prove to be responsible for the phenomenon of temporal regulation in biological systems.

Dr. Karakashian was a National Science Foundation Postdoctoral Fellow.

This work was supported by grants from the National Science Foundation and the Graduate College of the University of Illinois.

Received for publication, February 18, 1963.

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