*ON THE MECHANISM OF TEMPERATURE INDEPENDENCE IN A BIOLOGICAL CLOCK**

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Introduction.—Diurnal rhythms which persist under constant conditions have been found in a variety of plants and animals (see Bünning;[1] Pittendrigh[2]). Most of the evidence points to the conclusion that diurnal rhythmicity is endogenous and results from oscillations of physiological processes of one sort or another. An unusual feature of many rhythms, however, is their temperature independence. In instances where study has been directed toward the temperature characteristics (Wahl;[3] Brown and Webb;[4] Pittendrigh;[5] Bruce and Pittendrigh;[6] Bühnemann[7]), it has been found that the period, or duration of a cycle, is unaffected or only slightly affected by temperature changes. The way in which this is achieved in organisms which are otherwise markedly temperature-sensitive is not known. It is possible that some uncontrolled environmental variable might be involved, i.e., that there is an exogenous factor which plays a role in determining the period in rhythmic organisms. Another possibility is that temperature compensation is incorporated as a part of the rhythmic mechanism. In either case, it may be noted that temperature independence constitutes support for the idea that a diurnal rhythm could be related to an accurate biological timing device, or clock. If, in fact, the rhythms are related to a chronometer mechanism, the significance of temperature independence is clear.

We have recently demonstrated (Hastings *et al.*[8]) that the dinoflagellate *Gonyaulax polyedra* possesses a diurnal rhythm of luminescence which persists under conditions of constant low light and constant temperature. Since *Gonyaulax* is a unicellular form which may be grown readily in large quantities and studied biochemically, it is an advantageous material for the study of rhythmicity. In addition, the quantitative measurement of light intensity provides an easy and accurate method for following rhythmic changes. The experiments reported here describe the effect of temperature upon the luminescent rhythm. The results suggest that temperature independence is achieved by means of a compensation mechanism.

Materials and Methods.—*Gonyaulax polyedra* is an armored marine dinoflagellate which is both photosynthetic and luminescent. This organism was isolated from a net sample collected off the pier at Scripps Institution of Oceanography and has been maintained in a unialgal culture in a medium composed of 75 per cent filtered sea water, 2 per cent soil extract, 2×10^{-3} *M* KNO_3, 2×10^{-4} *M* K_2HPO_4, 6×10^{-6} *M* $FeCl_3$, and 2.7×10^{-5} *M* ethylenediamine-tetraacetic acid (EDTA) at pH 8.0–8.2. Under optimal conditions of light and temperature, a growth rate of about one division per day has been measured. Bacteria-free cultures have been found to have a reduced viability and growth rate, apparently due to the lack

of some unknown growth factor which may be supplied by the bacteria. In these experiments, therefore, bacteria-free cultures were not used.

Luminescence in *Gonyaulax* normally occurs only upon stimulation. The cell emits a flash of light having a duration of less than 0.1 second, a record of which has been published previously (Hastings and Sweeney[9]). In our experiments, luminescence was evoked by bubbling a fine stream of purified air through a 2-ml. aliquot of the cell suspension for 1 minute. With continued stimulation, the luminescent response declines sharply, so that after 1 minute, only a very small amount of light is emitted. Due to this fatigue characteristic, the luminescence of a suspension may be quantified by measuring the relatively brief bright burst of light emitted upon stimulation. The light emitted was measured with the photomultiplier apparatus previously described (Sweeney and Hastings[10]), which permits integration of light by accumulating the current output from the phototube on a capacitor. Our index of luminescence therefore is a fixed proportion of the total amount of light emitted by a cell suspension during a 1-minute period of stimulation. After each determination the cell suspension used for luminescence measurement was discarded.

The experiments illustrated in Figure 1 were carried out in the Earhart Laboratory of the California Institute of Technology, where eight different temperature-controlled rooms were available.[11] The temperatures of seven of these rooms and the extreme temperature variations recorded are noted in Figure 1. The eighth room was at 6° C. Unless otherwise stated, the only illumination in these rooms was provided by an overhead fluorescent fixture with two 40-watt "daylight" bulbs placed at a distance of 1 meter from the cultures, where the light intensity was about 100 foot-candles. Uniformity of illumination was achieved by means of a diffusing screen of white tracing paper placed under the fixtures.

Experimental Procedure.—Gonyaulax is an obligate photoautotroph. When cultures are grown with alternating light and dark periods of 12 hours each (= LD), their luminescence is much greater during the dark period than during the light period. If such cultures are then transferred either to total darkness or to continuous dim light (100–200 foot-candles), this rhythm of luminescence persists, retaining in both cases the period of approximately 24 hours. It has been shown (Sweeney and Hastings[10]) that in continuous darkness the amplitude of the rhythm is damped as a result of the depletion of its energy supply. In dim light, however, the luminescent rhythm persists with undiminished amplitude and has been observed for as long as fourteen consecutive days (Hastings *et al.*[8]).

The plan of the experiment was as follows: Two-milliliter aliquots from a culture of *Gonyaulax* which had been growing at 22° C. in LD conditions (light from 6:00 A.M. to 6:00 P.M. at an intensity of 800 foot-candles) were pipetted into each of 1,300 test tubes. The cell density at this time was 3,600 cells per milliliter. The tubes were placed in beakers and returned to the previous LD conditions at 22°. Two days later, just prior to 6:00 A.M., cultures were removed from the dark at 22° and placed in the dark in each of the eight rooms, at different temperatures. A few minutes later, at 6:00 A.M., the lights were turned on in all rooms. The cells therefore were exposed to the transition from dark to light almost simultaneously with the transition from 22° to a second temperature. Thereafter, at

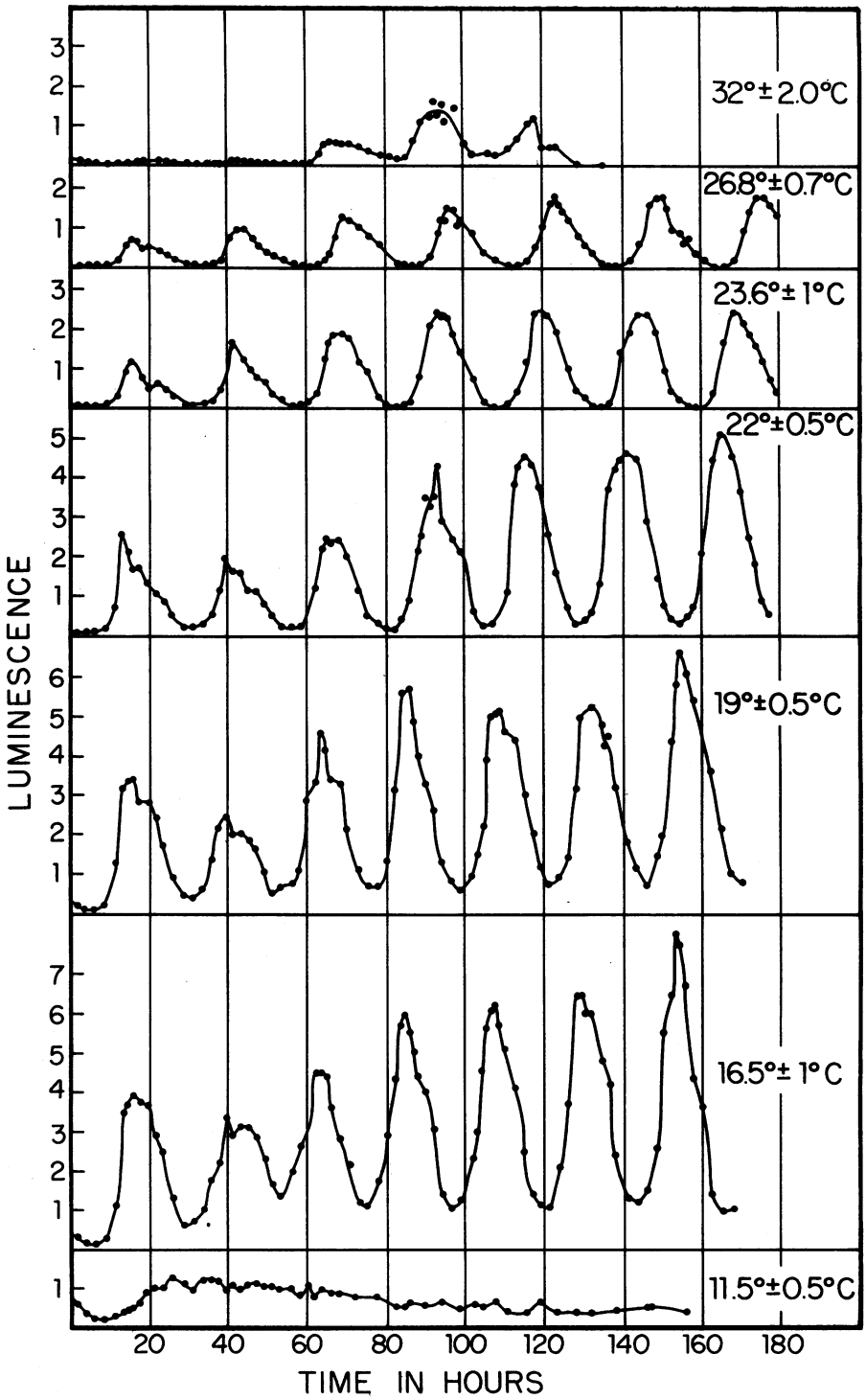

FIG. 1.—Characteristics of the persistent rhythm of luminescence at several different temperatures. The cells were kept at 22° C. in LD conditions prior to the beginning of the experiment shown on the graph. At that time the cells were transferred from the dark at 22° to the various temperatures, all in dim light, and left in these conditions throughout the experiment. The luminescence of the cells was determined approximately every 2 hours. At the dim light intensity used, there is little cell division, even at the optimal temperature for growth. The number of cells therefore remained essentially the same in all tubes at all temperatures.

approximately 2-hour intervals, two tubes were removed from each of the rooms, assayed for luminescence, and then discarded.

Results.—The results of this experiment are shown in Figure 1. At the five temperatures between 16.5° and 26.8° C. the experiments were continued for seven cycles. It is quite evident that the higher the temperature, the longer is the duration of a period. We have estimated the average time of a period at each temperature by taking the time at which the first and last peaks of luminescence occurred and dividing by the number of periods. The data from this computation are plotted in Figure 2.

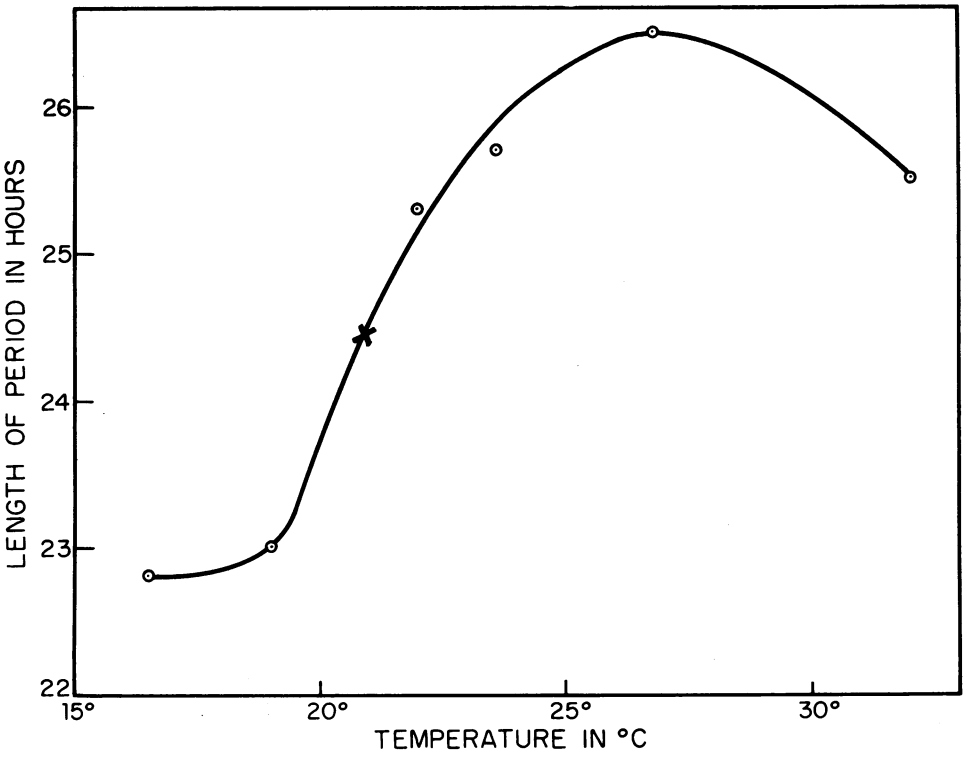

FIG. 2.—The effect of temperature upon the length of a period in the rhythm of luminescence. Data taken from the experiment shown in Fig. 1, except for the point indicated by the symbol X. This was obtained in an experiment (similar to those shown in Fig. 1) carried out in Evanston several months later at a temperature of 21.0° ± 0.1° C.

The temperature control in the room at 32° C. was poor, and the cells were killed on the sixth day of the experiment, when the temperature rose to 36°. The irregularity of the luminescence is probably due in part to the temperature fluctuations and in part to an adverse effect of the high temperature upon the cells. In spite of this, there is a clearly apparent rhythmicity with a period not far from 24 hours, the average duration being about 25.5 hours.

At a temperature of 11.5° C. the rhythmicity was lost, although the cells retained luminescence and viability. From previous experiments it is known that essentially no growth occurs at 11°. When the cultures at 11.5° were returned to a

temperature of 22°, the rhythmicity was regained, but it was not determined whether or not the rhythmicity would persist for more than one cycle. It has been reported by Brown and Webb[4] that in the fiddler crab, *Uca*, a low-temperature treatment is equivalent to "stopping the clock" and that when the organism is brought back to a higher temperature, the rhythmicity resumes, with the phase displaced by a time period corresponding to the time of low-temperature treatment. This does not occur in *Gonyaulax*. The phase is determined by the time at which the cultures are returned to the higher temperature. Some cultures were removed from the 11.5° room and taken to the 22° room after 58 hours, and others after 67 hours at the lower temperature. In both cases, pronounced rhythms were initiated, having their first maximal luminescence 30 hours following the transfer. This suggests that a temperature shock may serve as a timing or setting device for rhythms.

Cell suspensions kept in constant dim light at 6° C. showed no measurable luminescence, even when warmed to 22° before assaying. Nevertheless, the cells remained viable, as evidenced by the fact that cultures transferred from 6° to continuous light at 22° regained the ability to luminesce after about 12 hours. The luminescence of such cultures showed no evidence of regaining a rhythmic character. It appears that cells which are kept at such a low temperature will not respond to the temperature shock as a stimulus to initiate rhythmicity.

The important finding in these experiments, namely, that the length of the period of the endogenous rhythm is greater at 26° than at 16° C., was confirmed by a later experiment carried out at La Jolla. This experiment was performed in essentially the same manner as described above, utilizing, however, only two temperature rooms (at 15.9° ± 0.2° and 26.6° ± 0.5°). In each case, only two periods were measured; at the higher temperature the durations of the periods were 27 and $26^1/_2$ hours, and at the lower temperature, 23 and 22 hours. This result confirms the previous finding. The cultures in these two rooms were then exchanged without any change in illumination. The persisting rhythm was somewhat irregular, but it was easily possible to measure the periods. In the cultures moved from 26° to 16°, three periods having durations of 20, 21, and 20 hours were observed. In those moved from 16° to 26°, two periods having durations of $24^1/_2$ and 24 hours were observed. The period may therefore be altered slightly by a temperature change alone.

From these experiments it is evident that, although the period does vary with temperature, the effect is not very great. The rates of various processes in chemical and biological systems usually differ by a factor of two or three at temperatures 10° C. apart. In our experiments the length of the period varies by only about 15 per cent at temperatures 10° apart. Since the temperature effect is relatively small, we may speak of this "clock" as being temperature-independent and comparable to the other cases of temperature-independent rhythms which have been reported.

However, the dependence on temperature which we do observe is worthy of consideration. First of all, it essentially eliminates the possibility that the period of the rhythm is regulated directly by some uncontrolled periodic variable in the environment. Second, in view of the fact that the observed Q_{10} is less than 1.0, it definitely suggests that a temperature-compensation mechanism is involved.[12]

If we were to assume that the duration of the period is governed by the rate of a single chemical reaction, then an increase in temperature should result in a period of shorter duration. The effect we observe in this case is precisely the opposite, i.e., the Q_{10} between 16.5° and 26.8° C. is 0.86. This result can be readily explained if we assume that temperature independence is achieved by means of compensation, and that this situation is an example of overcompensation.

There are several other compelling reasons to conclude that temperature independence, in biological clocks generally, results from a temperature-compensating mechanism. The pertinent considerations have been discussed in detail by Bruce and Pittendrigh[6] and by Pittendrigh.[2] For example, the effects upon endogenous rhythms in several organisms of repeated and nonrepeated temperature shocks, and the initiation of rhythms by single temperature shocks, favor the interpretation of a temperature-compensating mechanism.

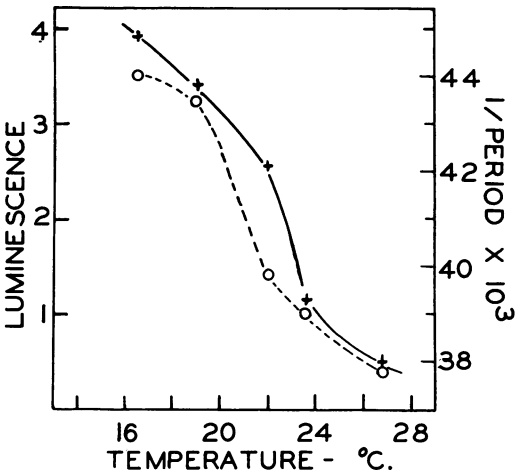

FIG. 3.—Plot showing the effect of temperature upon the amount of luminescence (measured 16 hours after the beginning of the experiment; data plotted with crosses, *solid line*), as compared with the temperature effect upon period (plotted as the inverse of period duration; data plotted with circles, *dotted line*). Data taken from the experiments shown in Fig. 1.

A temperature-compensation model can be expressed very simply in terms of two chemical reactions, A → B and C → D, both of which are temperature-dependent in the usual way. Let it be assumed that the rate of the first reaction controls the duration of the period, while D is an inhibitor of this reaction. If the two reactions have appropriate temperature coefficients, the rate of the first reaction, A → B, will remain essentially constant with temperature changes. If the temperature coefficient of the second reaction was too high in relation to the coefficient of the first reaction, an apparent Q_{10} for A → B of less than 1.0 would be expected. Also, the fact that the average period length in the experiment at 32° C. is less than at 26.8° and 23.6° may be understood in terms of the model reactions by assuming them to be enzymatic and to have different optimal temperatures. The observed result would be expected if the temperature optimum of C → D was somewhat lower than that of A → B.[13]

There is additional evidence for the suggestion that an inhibitor is acting in the luminescent system. In the experiments shown in Figure 1, it can be seen that the intensity of luminescence is less at higher temperatures. In Figure 3, the intensity of luminescence, measured 16 hours after the beginning of the experiment, is plotted against the temperature. On the same graph are plotted the data from Figure 2, using the inverse of time on the ordinate. The parallel between the two implies that both result from the action of the same postulated inhibitor system, even though the intensity of luminescence is changed by a greater degree than is

the periodicity with the temperature change. The unusual relationship between luminescence and temperature has been confirmed in many experiments, the details of which will be published elsewhere. We have found that light emission in the living cell increases with decreasing temperature to as low as 5° C.

In contrast to these findings, we have previously reported[9] that the luminescent reaction in cell-free extracts is temperature-dependent in the usual way. Luminescence increases with increasing temperature to an optimum at 25° C. and decreases thereafter as a result of enzyme denaturation. The growth rate of the cells is also temperature-dependent in a similar way, the optimum being at about 25°. It appears that the temperature relationships of both the periodicity and the intensity of luminescence in the living cell result from some particular and perhaps unique mechanism. It is at least certain that the entire metabolic pattern of the cell is not temperature-dependent in this unusual way.

Our experiments, as well as the previous studies of Bruce and Pittendrigh[6] with *Euglena gracilis*, demonstrate that a clock system may occur in unicellular forms. It is known, from studies which we have reported previously,[9] that luminescent rhythmicity in *Gonyaulax* is associated with diurnal fluctuations in the extractable components of the luminescent system—with respect to both enzyme and substrate. Pittendrigh[2] has pointed out that this is to be expected and that fluctuations of this sort, or indeed any overt and measurable manifestations of rhythmicity, may be merely "outputs," or reflections of the activity of some undefined but master cellular clock mechanism. In contrast to this view, we would suggest that rhythmicity may occur in particular and essentially discrete systems, such as the luminescent system, and that no special clock need be invoked to account for luminescent rhythmicity or, indeed, any other rhythmic process. In support of this idea, we point to the evidence that the temperature-compensation mechanism apparently acts in the luminescent system directly. If there were some other clock to which luminescence was tied, we would expect the clock itself rather than the luminescent system, to be temperature-compensated.

It seems that the general nature of persistent diurnal rhythms must eventually be understood in terms of oscillating chemical systems. On the assumption that discrete biochemical systems may undergo sustained autonomous oscillations, it appears worthwhile to investigate the biochemistry of such systems in detail.

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[10] B. M. Sweeney and J. W. Hastings, *J. Cellular Comp. Physiol.*, **49**, 115–128, 1957.

[11] We are grateful to Professor F. W. Went for his kindness and co-operation in making the space available to us.

[12] It is interesting to note that this is the second instance in which a Q_{10} of less than 1.0 has been reported for the length of the period in a rhythmic process. Bühnemann found that the rhythm of sporulation in *Oedogonium* had a period of longer length at higher temperatures.

[13] E. N. Harvey (personal communication) has recently suggested a model based on similar assumptions. We are not of the opinion that the intracellular reactions which are involved in rhythmicity and the temperature-compensation mechanism are as simple as those we have suggested. They are used merely for illustrative purposes. It does not seem worthwhile at the present time to suggest a more elaborate model, in the absence of more specific biochemical information. We would guess, however, that the chemistry of rhythmicity will be shown to involve a heterogeneous system rather than a homogeneous one.

CATALYTIC AND POLYMERIZATION-DIRECTING PROPERTIES OF MINERAL SURFACES

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Recent years have seen an extension of our knowledge of how organic chemical compounds may have been synthesized in preorganismal times. Urey's interpretation of chemical and physical data pertaining to planetary evolution[1] has provided the major stimulus for such work. Miller has demonstrated the ease with which synthesis of amino acids and other organic compounds can be effected in gas mixtures.[2] This paper deals with a possible mechanism of polymerization of such compounds. Polymerization in simple aqueous medium has but a low probability. Bernal[3] has suggested that clays might act as adsorbing catalytic surfaces. In the present work it will be shown that in certain instances rock and mineral surfaces can act as catalytic materials for polymerization reactions.

In earlier work on the synthesis of lignin it was shown that during the enzymatic peroxidation of eugenol, lignin is formed only when the reaction system includes an appropriate matrix such as cellulose, methylcellulose, or pectic acid.[4] It has now been found that several mineral silicates can serve as matrices for the formation of lignin-like products which are, however, different from those formed in the presence of polysaccharides.

METHODS

Exposed surfaces of all rocks and minerals used were removed and specimens treated successively with hot chloroform, ether, hot methanol and ethanol, cold dilute HCl, and boiling water. To provide a check on aromatic contaminants, washings were scanned for ultraviolet-absorbing substances between 220 and 380 mμ (Beckman Model DU spectrophotometer with silica absorption cells). Initial washings sometimes yielded absorbancies as high as 0.05, but later washings were free of such contaminants.

Rocks and minerals were used as individual pieces 1–3 cm.^{3} in volume and 8–10 cm.^{2} in area. Irregular surfaces of fibrous minerals were greater in area but of the