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Heavy Water Slows the *Gonyaulax* Clock: A Test of the Hypothesis That D_2O Affects Circadian Oscillations by Diminishing the Apparent Temperature

(rhythms/bioluminescence/dinoflagellates)

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ABSTRACT **In order to test the hypothesis that deuterium oxide acts in circadian systems by simulating a lower environmental temperature, we have examined the effects of D_2O on *Gonyaulax polyedra*, where the period of the circadian rhythm of bioluminescent glow in constant environmental conditions is shorter at 16° than at 22°, and the phase of the glow peak relative to a light-dark cycle is advanced at 16° relative to 22°. Addition of low concentrations of D_2O to *Gonyaulax* does not produce the same effects on period and phase as lowering the temperature; the free running period is lengthened and the phase is delayed. These results show that in the *Gonyaulax* rhythm, the effect of added D_2O is not equivalent to lowering the temperature.**

Although there are many potential causes for the effects of heavy water in biological systems, its action, phenomenologically speaking, is frequently comparable to the effect of lowering the temperature (1–3). In fact, in experiments with the rate of replication of polio virus, Lwoff and Lwoff (4, 5) concluded that D_2O in that system "diminishes the apparent temperature."

This "low temperature equivalence" hypothesis for D_2O action is of particular interest in circadian systems, for it is here that one finds a rather unusual effect of temperature (6): the frequency (or period) of the daily oscillations is about the same at different temperatures, in otherwise constant conditions. This has been postulated (7, 8) to be the result of a biochemical compensation mechanism, the functional importance of which is hypothesized to be related to the timekeeping or "biological clock" role of the system (9).

An important test of the "low temperature equivalence" hypothesis for D_2O is to see whether or not circadian oscillating systems that exhibit temperature compensation in period also exhibit a similar compensation with added D_2O. Pittendrigh *et al.* (3) recently posed and examined this question, prompted in part by data from Enright (10) which showed that the periods of circadian oscillators were less affected by 20% D_2O than were those of noncircadian biological oscillating systems. Their results, in experiments with the *Drosophila* eclosion rhythm, supported the low temperature equivalence hypothesis: two different features of the rhythm were affected similarly by D_2O and by temperature. The period of the rhythm in constant conditions is almost temperature independent, and is similarly unaffected by D_2O. The phase angle of the rhythm, however, is markedly delayed at lower temperatures, which effect can be duplicated by D_2O.

For several reasons, including their own concern that circadian systems might exhibit general homeostasis (11) not specifically related to the mechanism of D_2O action, Pittendrigh *et al.* (3) were still wary of the conclusion, and anxious to further test this hypothesis. In particular, they suggested that a circadian system that exhibits "over-compensation" be examined, i.e., one in which the frequency is higher at lower temperature. We have carried out such experiments and find that their caution was justified: D_2O and temperature do not appear to be equivalent in the *Gonyaulax* system.

MATERIALS AND METHODS

Cultures of the bioluminescent marine dinoflagellate *Gonyaulax polyedra* were grown at either 22° or 16° on cycles of 12 hr of light and 12 hr of darkness (*LD*:*12*,12) on a supplemented sea water medium ("31" medium) as previously described (12). For deuteration, a salt medium containing different amounts of D_2O, as desired, was added to make up 24% of the final volume. For this salt medium, to 100 ml of H_2O or D_2O were added 2.54 g of NaCl, 100 μl of nitrate stock (75 mg of $NaNO_3$ per ml of deionized distilled water), 10 μl of "31" vitamin mix (12) and 100 μl of "TW" trace elements. One liter of "TW" trace elements contained 800 mg of $Na_2MoO_4 \cdot 2H_2O$, 23 mg of $CoCl_2 \cdot 2H_2O$, 11 mg of $CuCl_2 \cdot 2H_2O$, and 6 mg of KI. From two stocks of this salt medium, one made with deionized distilled water and the other with 99.8% D_2O (Bio-Rad), appropriate quantities of each were added to give the desired percentage of D_2O. Other details are given in the thesis of McDaniel (13).

The bioluminescent "glow" (a continuous, low-intensity, spontaneous light emission) was recorded from 10-ml cultures of cells in vials, by means of a modified automatic scintillation counter (14, 15). To determine the growth rate, we removed aliquots of cells from a culture and determined the cell concentration with the aid of an electronic cell counter.

RESULTS AND DISCUSSION

The "anomalous" effect of temperature on the period length (i.e., shorter periods at lower temperatures) of the circadian rhythm of bioluminescence (7) was confirmed and can be seen from the two experiments at 16° and 22° in Fig. 1 in which there was no D_2O added. The Q_{10} (ratio of period lengths at a 10° temperature interval) calculated from these two points

Abbreviation: *LD*:*12*,12, 12 hr of light and 12 hr of darkness.

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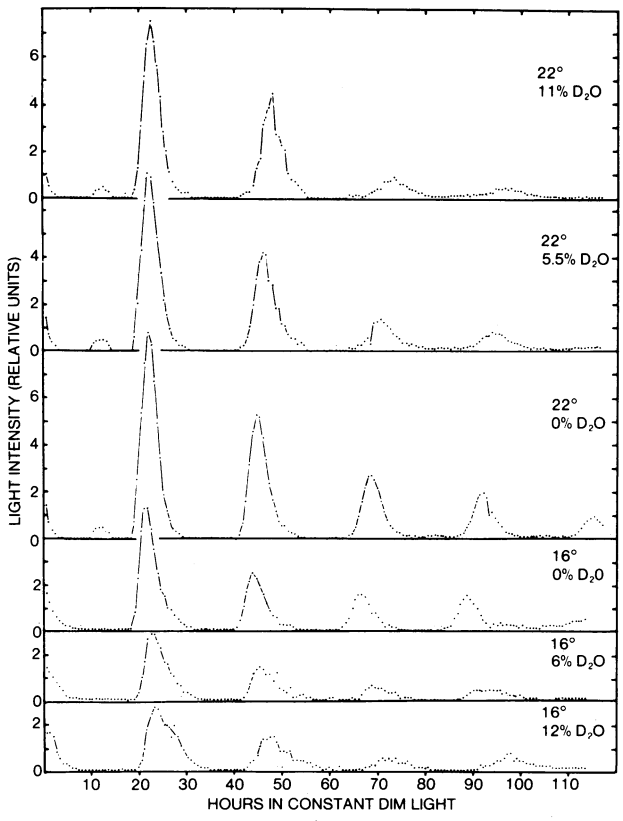

FIG. 1. Free-running period of the *Gonyaulax* glow rhythm. The top three curves illustrate the glow rhythm for cells grown in *LD:12,12* at 22° and assayed at 22°. For the three bottom curves the cells were grown and assayed at 16°. D_2O was added at the end of the dark period, at which time the cells were transferred to constant dim light [110 foot-candles (1180 lux)]. Each of these curves represents the average of the glow of four separate vials. The percent deuteration for each culture is given above each curve on the figure.

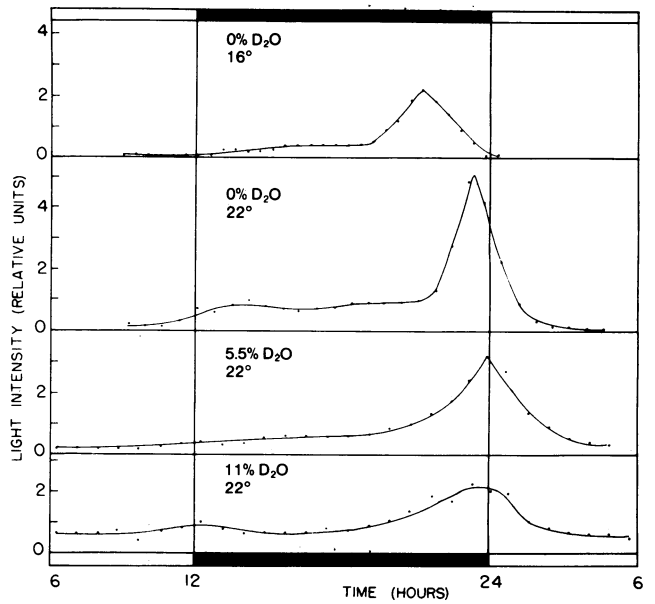

FIG. 2. The phase angle of the *Gonyaulax* glow rhythm with respect to an *LD:12,12* cycle. The top curve illustrates the average glow of three separate vials of cells taken from a culture grown at 16° in "31" medium and assayed at 16° in *LD:12,12*. The bottom three curves illustrate the glow (average of five separate vials) of cells taken from a culture grown at 22° in medium of 0, 5.5, and 11% D_2O, from top to bottom, respectively, and assayed at 22° in *LD:12,12*. The shaded areas at the top and bottom of the figure represent the dark part of the light–dark cycle.

was 0.93. At both temperatures the effect of added D_2O (at all concentrations tried) was to increase the length of the period. The cultures with D_2O added appeared somewhat less healthy, and there appeared to be more scatter in the data, making the measurements less precise than we would have wished. At 16° (bottom three curves) the measured periods were 22.3, 23.2, and 24.6 (±0.7, standard error of the mean) hr for 0, 6, and 12% D_2O, respectively. At 22° (top three curves) the corresponding values were 23.3, 24.2, and 25.3 (±0.7) hr for 0, 5.5, and 11% D_2O, respectively. With 16.5% D_2O at 22° (not illustrated) the period was 25.5 (±0.7) hr. These experiments have been repeated several times with similar results.

Pittendrigh *et al.* (3) also compared the effect of temperature with that of D_2O on the phase angle of the *Drosophila* rhythm with respect to the light–dark cycle. They found that there was a delay (i.e., the peak occurs later, relative to the onset of light) both at lower temperature and with D_2O. With the *Gonyaulax* glow rhythm under a light–dark cycle, there is instead an advance in the phase cycle at a lower temperature (Fig. 2). D_2O does not cause such an advance; at 22° both 5.5 and 11% D_2O result in a phase delay. D_2O may actually cause a larger phase delay on the *Gonyaulax* clock, but this is difficult to measure with the glow rhythm, since *Gonyaulax* bioluminescence is photoinhibitable and at 22°, with no D_2O added, the glow peak occurs just before the lights come on. Regardless of the absolute magnitude of the phase delay, the effect of D_2O is not similar to that of low temperature.

In summary, we have tested the hypothesis that D_2O affects circadian oscillations by diminishing the apparent temperature, and obtained evidence to reject it. At 22° added D_2O causes an increase in the period of the oscillation and a delay in the phase angle of the rhythm, whereas lowering the temperature to 16° results in changes in the opposite direction.

With regard to its mode of action, Pittendrigh *et al.* (3) have recently noted that D_2O stands out as the only chemical agent whose action is uniformly similar and widely (if not universally) effective in circadian systems. Its mechanism of action may be too complex to be of help in elucidating the biochemical basis of circadian rhythms. On the other hand, D_2O may have a common and possibly even a relatively simple mode of action, such as one involving solvent effects [see Pittendrigh and Cosbey (16) for a discussion and interpretation of rapid effects of D_2O]. Analysis of the effects of D_2O may be of help in evaluating models for the circadian clock, such as the one involving membranes proposed recently by Njus *et al.* (8).

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