A Test of the Hypothesis That D₂O Affects Circadian Oscillations by Diminishing the Apparent Temperature

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ABSTRACT The period (τ) of a circadian pacemaker in the cockroach Leucophaea maderae is a nonmonotonic function of temperature. The slope of the curve (τ as a function of temperature) is negative at 20° and positive at 30°. When these insects are deuterated at 20° and 30° the period (τ) of the pacemaker lengthens in both cases, although there is a marked temperature dependence of D₂O action. The increase in τ is nearly three times greater at 20° than 30°. This observation is a flat contradiction of a prediction made earlier that when D₂O affects circadian pacemakers it does so by diminishing the apparent temperature of the cell. That prediction, however, involves an assumption that may well be unfounded. Unless D₂O acts nonselectively on all the components in the system regulating τ, the prediction we sought to test is unfounded; and if D₂O does not act nonselectively, the observed temperature dependence of D₂O action is understandable in terms of simulating a lower temperature for those components it does affect.

Several recent papers (1–3) from this laboratory have developed the hypothesis that when D₂O affects the frequency of circadian pacemakers, its action simulates a lowering of cellular temperatures. The papers reviewed and extended other instances of D₂O action on biological systems where that interpretation of heavy water action is clearly indicated. They stressed especially (1) the fact that D₂O has a differential impact on temperature-dependent and temperature-compensated aspects of the circadian system in Drosophila: its effect on the temperature-compensated period (or frequency) of the pacemaking oscillation is less than its effect on the phase (relative to a light/dark cycle) of temperature-dependent processes whose initiation is timed by the pacemaker. It was, however, stressed that the meaning of this particular parallelism between the action of D₂O and temperature was open to another interpretation because the period of circadian oscillations is evidently subject to a general homeostasis (3). The well-known temperature-compensation of the period (τ) of freerunning circadian pacemakers is only a special case reflecting the operation of a homeostatic mechanism that tempers the change in frequency that would otherwise be caused by any agent in the cell. Thus the fact that the Drosophila circadian pacemaker was found to be "D₂O-compensated" does not, of itself, necessarily mean D₂O was simulating low temperature.

The "low-temperature equivalence" hypothesis of D₂O action on circadian clocks nevertheless escapes the weakness of total untestability. Pittendrigh et al. (1) noted [following an older suggestion by Bruce and Pittendrigh (4)] that known differences between species in the details of τ's temperature-compensation provide the opportunity for a more crucial test of the hypothesis: some circadian pacemakers have negative, and others positive, temperature-coefficients. Thus where the action of lowering the temperature is to increase frequency, D₂O should shorten τ. The experiments reported here pursue that suggestion. The work of which they are part (5;*) was based on an observation that at 20° two cockroach species (Leucophaea maderae and Byrsotria fumigata) responded differently to a 10° step-up. In Leucophaea the Q₀ (ratio of responses at two temperatures 10° apart) was less than 1.0; in Byrsotria it was greater than 1.0. In the course of the ensuing experiments it was found that in Leucophaea the dependence of τ on temperature was clearly nonmonotonic (Fig. 2). This fact provides an especially attractive opportunity to test the hypothesis within a given species: the action of D₂O should be different at two specifiable temperatures in Leucophaea.

METHODS

The experiments we report here test the effect of D₂O on the period (τ) of the freerunning circadian rhythm of running-wheel activity of Leucophaea in constant darkness. The use of small Lucite running-wheels whose rotations are registered on an operations recorder has been described elsewhere (5;*). The apparatus yields data of the type exemplified in Fig. 1. The reliability of τ estimates from such data is good. An empirical test was made in which four different people estimated τ in 128 different free-runs. The eye-fitting of lines to activity onsets (whose periodicity is taken as reflecting that of the rhythm's pacemaker) yielded estimates that did not vary by more than 0.12% (5). The insects are left undisturbed in their running-wheels for weeks and are free to eat (Purina chow attached to the wheel's stationary faceplate) and drink from a cotton wick that draws water from a Lucite reservoir external to the faceplate. All the experiments reported were performed in constant darkness and constant temperature.

RESULTS

A long series of experiments reported fully elsewhere (5;*) yielded the data summarized by the top panel of Fig. 2. τ is, as noted, a nonmonotonic function of temperature. At 20°, e.g., the slope of the curve is negative, but at 30° it is positive.

Doubt about the Biological Significance of Low-Temperature Insect Dehydration

The lower panel indicates the logical basis of the experiments reported here. If $D_2O$ action is to "diminish the apparent temperature" [using Lwoff's (6) expression], then the entire curve should be displaced to the right on the temperature axis. The value of $\tau$ at a given temperature when the insect drinks $D_2O$ should be that expressed at a lower temperature when the insect drinks $H_2O$. Several predictions suggested by the lower panel have been pursued (5;*), but the two experiments on Leucophaea we report here yield a satisfactorily unequivocal test of the hypothesis as initially formulated.

Samples of individual male Leucophaea (Table 1) were subjected to "deuteration-steps," by which we mean that following weeks of drinking pure $H_2O$ they were abruptly switched to $25\% D_2O$ as a drinking supply. Before and after that step $\tau$ was monitored in each animal. Such steps were imposed on 14 insects at 20° and on 12 at 30°. As the lower panel of Fig. 2 indicates, the temperature equivalence hypothesis predicts that at 20° the effect of $D_2O$ should be to lengthen $\tau$; at 30° it should shorten $\tau$.

Table 1 indicates no such difference was found in the sign of the $\Delta \tau$ ($\tau$ in $D_2O - \tau$ in $H_2O$) caused by the deuteration step. Moreover, the data are sufficiently extensive to leave no doubt about the statistical validity of the result.

**DISCUSSION**

There can be no equivocation in concluding that an apparently crucial prediction of the low-temperature hypothesis was not fulfilled. Nor can there be equivocation, therefore, in concluding that the hypothesis yielding the prediction is invalid.

As so often happens, however, the outcome of experimental work is to raise new questions that leave doubt—not about the validity of the hypothesis *as stated*—but whether the general intuition that led to it was adequately represented in the detailed formulation of the hypothesis. As Table 1 shows, there is a striking temperature-dependence of $D_2O$ action. The $\Delta \tau$ effected by the same concentration of $D_2O$ is significantly greater at 20° than at 30°. A similar dependence of $D_2O$ action on temperature has been found in the cockroach *Byrsotria fumigata* (5,*). We shall not pursue here the details of these other relevant facts; we wish only to emphasize that the specific hypothesis we began with (and now reject) included (implicitly) the proposition that $D_2O$ would have access to, and affect, all the components of the homeostatic mechanism whereby $\tau$ is temperature-compensated. Clearly all such components are affected when temperature changes. That implicit assumption is a major element in the hypothesis and may well be unfounded. Certainly, unless $D_2O$ is as nonevaporative as temperature in its action, the predictions incorporated in the lower panel of Fig. 2 do not, in fact, test the general proposition that heavy water simulates...
a lowering of temperature when it affects, selectively, those components of the mechanism to which it does have access.

The temperature-dependence of the D$_2$O effect we report here is clear and poses a new problem in itself. It could well be (5;*) that it derives from a selective action of D$_2$O on only one (or less than all) of the components involved in the homeostasis of r. In any case, all that these experiments rigorously exclude is that D$_2$O has (i) a nonselective impact on all the constituent processes in a circadian pacemaker, and (ii) in affecting them, does so by effectively lowering the temperature. Other aspects of this problem are addressed more fully elsewhere (5;*).

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