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A Differential Effect of Heavy Water on Temperature-Dependent and Temperature-Compensated Aspects of the Circadian System of *Drosophila pseudoobscura*

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ABSTRACT **D_2O is the only "chemical" agent that consistently affects the frequency of circadian oscillations: its effect is now known to be so widespread and predictable that its action merits closer study as a potential clue to the currently obscure concrete nature of circadian oscillators. The great diversity of D_2O effects on biological systems in general is briefly reviewed and the need for rejectable hypotheses concerning the action of D_2O on circadian clocks is stressed because current speculation on its action yields "predictions" expected from almost any hypothesis. We consider the hypothesis that it "diminishes the apparent temperature" of the cell and proceed to test this by examining the effect of D_2O on temperature-dependent and temperature-compensated aspects of the circadian system in *Drosophila*. We find these components respond as differentially to D_2O as they do to temperature; we conclude, however, with a warning that this result may be equivocal if, as we now suspect, the frequency of circadian oscillations is generally homeostatically conserved—not only in the face of temperature change, but change in any variable to which it is sensitive. More crucial tests of the temperature-equivalence hypothesis for D_2O action are defined.**

Deuterium oxide (heavy water) stands out as the only "chemical" agent that consistently and reproducibly affects the period (τ) of circadian oscillations. In 1960 Bruce and Pittendrigh (1) first reported that heavy water has a pronounced effect on the period of the freerunning circadian rhythm of phototaxis in *Euglena gracilis*. In populations of that flagellate adapted for long periods (months) to D_2O, the period of the freerunning rhythm was lengthened from its normal value (close to 23 hr) to 28 or 29 hr. Many subsequent studies, the most important of which are those of Suter and Rawson (2) and Enright (3), indicate this effect of D_2O is widespread: it lengthens τ in unicellulars (1), green plants (4), isopods (3), insects (Caldarola, in preparation), birds (5, 6), mice, and hamsters (2, 5, 7, 8). The effect is clearly widespread and since no exceptions have been found in 12 cases, it is likely to be truly general. As several authors have noted, it therefore merits closer study as a potential clue to the physical nature of the cellular oscillation responsible for circadian rhythmicity.

The diversity of D_2O effects on biological systems

It is customary (9–11) to discuss the effects of D_2O on biological systems under three categories. (*1*) *Primary isotopic substitution:* here D substitutes for H at the reaction site in a molecule with a consequent change in the zero-point energy of the bond to be broken in subsequent reactions. In the terms more generally used by biologists, the activation energy of the bond is raised and reactions involving it proceed more slowly at a given kT. (*2*) *Secondary isotopic substitution:* here D substitutes for H at sites not directly involved in the reaction. The effect on reaction rates is less predictable, but not necessarily negligible. Probably the principal biological interest in "secondary" substitutions of D for H relates to their effect on the stability of protein structure—on that of the α-helix itself as well as tertiary and quaternary structure. There is widespread opinion (e.g., ref. 12, p. 130) that hydrogen bonds play a significant role in stabilizing that structure and that the bond is usually strengthened when D substitutes for H with a consequent enhancement of structural stability. (*3*) *Solvent effects* are those attributable to the properties of D_2O acting (instead of H_2O) as the general solvent in which cellular processes take place. There are many separable effects: the viscosity of D_2O exceeds that of water; the increased mass of the deuteron slows its diffusion; the dissociation constant for D_2O differs from that of water (pD is not equivalent to pH), changing the electrochemistry of the whole system with the result, among others, that ion mobilities will change. One of the most important solvent effects of D_2O appears to be on the so-called hydrophobic "bonds" (or interactions) that develop when proteins assume their tertiary configuration. Nonpolar side groups, in rotating ("hydrophobically") away from the water in which they lie, are brought into close proximity to each other, and their mutual attraction by van der Waals forces creates a "bond" that contributes significantly to the stability of the folded configuration. Such hydrophobic interactions are thought to be strengthened when D_2O substitutes for H_2O as the protein solvent (13, 14).

Phenomenologically D_2O has two widespread effects on biological material. First, partial deuteration slows virtually every biological process to which it has been applied, ranging from the rate of single reactions to the respiration of complex systems and the growth rate of whole organisms (11). This widespread rate-depression is clearly explainable by many different *potential* causes; and especially in the more organized systems studied it is not known precisely which of these is responsible for the retardation. Increased viscosity and the greater mass of the deuteron will reduce the diffusion rate of many cellular constituents; the mobility of some ions will be decreased because of the changed electrochemical environment; reactions will proceed more slowly because of increased

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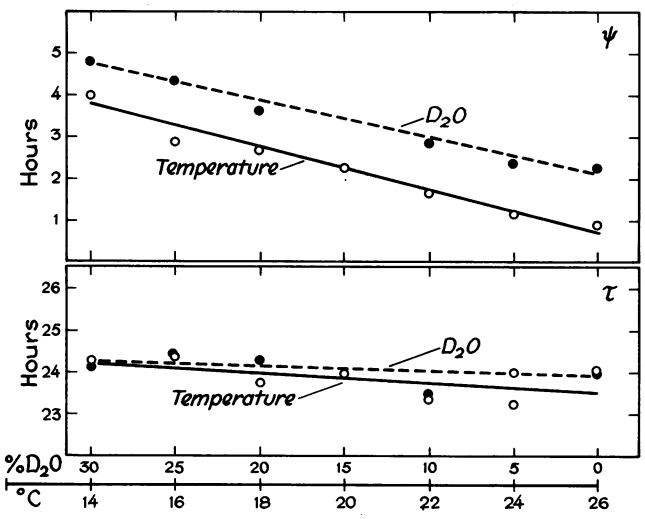

FIG. 1. The differential dependence of τ and ψ on temperature and D_2O. See *text;* and Table 3 for statistical summary.

activation energies; in the majority of enzymatic systems reviewed by Katz and Crespi (11) and Thomson (10), deuterium depresses the maximum velocity (V_m) under a specified set of reaction conditions and the Michaelis constant (K_m), which in some sense reflects enzyme–substrate affinity; the "mismatch" of rate constants in different subsystems of a *partially* deuterated cell could well impair the regulatory mechanisms that assure optimal rates in the normal system; and it is possible that the reduced flexibility of protein configuration due to the enhanced strength of hydrogen bonds and hydrophobic interactions will reduce their enzymatic efficacy and change membrane properties.

Second, in a number of cases deuteration has been shown to raise the temperature necessary for the thermal denaturation of proteins. This is almost surely traceable to the effect of D_2O on the hydrogen bonds and hydrophobic interactions contributing to the stability of the molecule's tertiary configuration.

How does D_2O affect circadian oscillations?

Given the purely phenomenological result that D_2O retards the rate of virtually all other biological processes, it is not surprising that it lengthens the period of (slows) all the circadian oscillations that have been deuterated. And in trying to elucidate this phenomenon we are again embarrassed by a richness of potential explanations that are not readily unconfounded.

TABLE 1. *The dependence of τ and ψ on temperature*

(at 0% D_2O)	Temp.(°C) 14°	16°	18°	20°	22°	24°	26°
$\bar{\tau}$	24.3	24.4	23.8	24.0	23.4	23.3	24.1
SD	1.19	0.78	1.23	0.52	1.26	0.56	0.81
SE	0.42	0.26	0.39	0.20	0.56	0.28	0.40
n	8	10	10	7	5	4	4
$\bar{\psi}$	4.0	2.9	2.7	2.3	1.7	1.2	0.9
SD	1.01	1.38	1.31	0.56	0.33	0.27	0.21
SE	0.36	0.40	0.27	0.21	0.12	0.12	0.09
n	8	12	12	7	7	5	5

Enright (3) has recently reported that D_2O lengthens the period of many noncircadian oscillations in marine organisms, ranging from the high-frequency discharge of electric fish (millisecond periods) to the lower frequency (seconds) cardiac and respiratory rhythms of marine invertebrates. On the two premises (*1*) that the concrete mechanism of the pacemaker for all these noncircadian rhythms depends crucially on ion mobilities and membrane properties, and (*2*) that circadian oscillations respond comparably to deuteration, he adopts the view that membranes and ion mobilities are also strong features of the concrete mechanism of circadian oscillations. Although we share with Enright and many other workers the intuition that membrane phenomena are very probably involved in circadian oscillations, we find his own evidence constitutes little cogent support for it. Thus, we see no reason to believe that *any* oscillatory system in cells should escape the rate retardation universal in nonoscillatory systems. Nor do we perceive why the diversity of possible explanations is in any way less for oscillatory than for nonoscillatory systems; the "predictions" Enright developed as tests of his hypothesis are as readily derived from *any* hypothesis about D_2O action that we can conceive of.

If the action of D_2O on circadian oscillations is to provide some insight into the nature of circadian oscillations, we must find hypotheses that will yield some predictions other than simple rate-retardation, which is attributable to too many different causes to be, of itself, a useful hint: we need rejectable hypotheses. They will be the more attractive if they yield the otherwise unlikely prediction that deuteration of the oscillation will *decrease* τ, i.e., apparently accelerate rates.

Deuteration often simulates a depression of cellular temperature

Bruce and Pittendrigh (1) had such an hypothesis in mind in the closing comments of their paper. They noted that there are suggestive similarities between the action of D_2O and *very* low temperatures on the circadian oscillator in *Euglena:* both agents cause an extreme lengthening of τ (whose small Q_{10} is above 1.0 in the physiological range) and a general damping of the system, as though the compensating mechanism that regulates τ in the physiological temperature range cannot cope with either very low temperature or very large D_2O effects. Are they in fact the same? They explicitly noted that it would be useful to examine the effect of D_2O on circadian oscillations whose Q_{10} was less than 1.0. The implicit point was that if D_2O was indeed effectively lowering cell temperatures, it should *shorten*—not lengthen—the value of τ in such cases.

TABLE 2. *The dependence of τ and ψ on D_2O concentration*

D_2O concentration (at 20°C)	0%	5%	10%	23%	25%	30%
$\bar{\tau}$	24.0	24.0	23.5	24.3	24.5	24.1
SD	0.64	0.57	0.78	0.92	0.79	1.05
SE	0.11	0.10	0.32	0.38	0.15	0.61
n	33	31	6	6	26	3
$\bar{\psi}$	2.3	2.4	2.8	3.6	4.3	4.8
SD	0.43	0.39	0.40	0.45	0.43	0.41
SE	0.09	0.09	0.13	0.14	0.13	0.20
n	21	17	10	10	11	4

This "low-temperature equivalence" hypothesis for D_2O action has since become more attractive for several reasons. Lwoff and Lwoff (15, 16) explicitly equated the effect of deuteration with a lowering of cellular temperatures in their study of polio-virus replication rate, which shows a pronounced temperature optimum; below that optimum addition of D_2O further depressed the rate, but above the optimum temperature, D_2O increased it. They closed their first paper with the sentence: "All (other) conditions being equal, heavy water increases the effect of low temperatures and tempers those of high temperatures"; that is to say, "*It diminishes the apparent temperature.*" [Translated from the French; parenthetic insertion and italics added.]

Jung (17) has published very similar results on the interaction of D_2O and temperature in *Escherichia coli* B. He finds the inhibitory action of D_2O on growth rate of this prokaryote is ameliorated by raising the temperature; and conversely the deleterious effects of high temperature are offset, to a truly remarkable extent, by addition of D_2O to the medium in which the bacteria grow. At 55° survival of the deuterated cells is increased by a factor of 10^5.

The proposition that D_2O also acts on circadian oscillations by diminishing the apparent temperature of the cell is fully consonant with a strong feature of Enright's data (3) that did not attract his own attention. Thus, the effect of 20% D_2O on the noncircadian oscillators he treated was generally very much greater than its effect on the circadian oscillation he studied and the others he reviewed: the period of noncircadian oscillators is temperature-dependent, but the period of circadian oscillators is conspicuously temperature-compensated.

The "low-temperature equivalence" hypothesis itself embraces more than one primary physical action of D_2O but it at least excludes others: diffusion effects and ion mobilities are not expected to change significantly as a function of temperature. Thus, were the hypothesis more fully validated, the current wide array of potential explanations of D_2O action on circadian oscillations would at least be narrowed. The experiments reported in this and subsequent papers (Caldarola, in preparation) were undertaken to test and explore its utility. We describe here a differential effect of D_2O on temperature-dependent and temperature-compensated aspects of the circadian system gating the emergence of adult *Drosophila pseudoobscura* from their puparia.

Temperature-dependent and temperature-compensated aspects of the circadian system in *Drosophila*

In populations of developmentally asynchronous pupae maintained in constant light (LL) the emergence activity of adults is distributed aperiodically as a function of time. If, however, such populations are transferred to constant darkness (DD) and constant temperature, a circadian rhythm of emergence activity immediately develops. The LL/DD transition initiates—in all individuals synchronously—circadian oscillations in the brain that gate the emergence act of the developed adult to a narrow (90°; 6^h) fraction of the oscillator's cycle. It is a remarkable property of the oscillating system that its circadian period (τ) is strongly temperature-compensated: Pittendrigh found in 1954 (18) that the Q_{10} for the range from 16° to 26° did not exceed 1.02.

The oscillation (O) can be entrained by light (L) cycles, and in steady state it assumes a unique phase relationship ($\psi_{O,L}$) to the light cycle. The phase-relation between the driving oscillator and the light cycle is itself invariant with temperature.

TABLE 3. *Regression and correlation coefficients for the dependence of τ and ψ on temperature and D_2O*

	Temperature		D_2O	
	Coefficient	P	Coefficient	P
$\bar{\tau}$				
Regression	−0.057	>0.1	0.015	>0.2
Correlation	−0.582	>0.1	0.524	>0.1
$\bar{\psi}$				
Regression	−0.245	<0.001	0.086	<0.001
Correlation	−0.984	<0.001	0.986	<0.0001

Computations were based on the mean values ($\bar{\tau}$ and $\bar{\psi}$) given in Tables 1 and 2. The statistical significance (P) of all coefficients is indicated.

The actual gating of the flies' emergence is, however, effected not by the light-sensitive oscillator but by a second component, believed to be a second oscillator (19), driven by the temperature-compensated (light-sensitive) pacemaker. Whatever the detailed nature of this driven, gating component in the system may be, it is an empirical fact that the position of the gate (*its* phase) relative to the temperature-compensated pacemaker is temperature-dependent. Thus, while the phase-relation ($\psi_{O,L}$) of the pacemaker relative to the light cycle is temperature-invariant, the phase-relation ($\psi_{O,R}$ and thus $\psi_{R,L}$) of the observed rhythm of gates is markedly temperature-dependent. The lower the temperature the more negative is $\psi_{O,R}$. In other words the gate occurs later, relative to the onset of light, when the temperature is lowered.

It is this pronounced difference between τ and $\psi_{R,L}$ in their dependence on temperature that yields a first chance to test and reject the hypothesis that deuteration amounts to cooling the circadian system.

METHODS

We have used the same strain of *Drosophila pseudoobscura* (PU 301) that the senior author has used since 1954; it was collected in 1947 from Mather, Calif. To assay rhythmicity of adult emergences in populations of developmentally asynchronous insects, it is necessary to use thousands of pupae in a single experiment. To obtain such numbers, cultures are raised in large circular transparent plastic tubs measuring 5 inches in diameter and 4 inches deep. Each has a screw-on plastic top equipped with a rubber gasket that ensures against escape of young larvae, and a nylon-net-covered air vent. Culture medium is poured into the tubs to about 1 inch in depth. About 750 parents are allowed to mature in a single tub and are transferred three times to fresh tubs at 2-day intervals. These 6-day-old parents, at the height of their egg-productivity, are then transferred to tubs containing a different culture medium susceptible of easy deuteration. For this we used "Carolina Instant Drosophila Medium" which consists of a dehydrated nutrient to which cold water is mixed in 1:1 proportions. The water we added was either 100% H_2O or H_2O/D_2O mixtures of various proportions. A few grains of active dry yeast were sprinkled on the surface of the medium while it was still wet. This procedure avoids the expense of evaporative loss of D_2O in the boiling of normal medium. The tubs were kept at 20° in *LD 12*:12. When pupation is about to begin, the food surface is covered by cheesecloth and this in turn is covered by sheets

of lightly crumpled thin plastic sheeting. After 4 days, during which pupation occurs preferentially on them, the plastic sheets are removed from the tubs and agitated in a 20° water-bath where the freed pupae float to the surface from which they are removed by a seive and dried on paper towelling. The harvested pupae are then attached (Elmer's casein glue) to the surface of aluminum plates that are inserted into a multichannel fraction collector in which flies emerging each hour are trapped in separate wells of detergent where they die and are subsequently counted. Data consist of flies emerging each hour over a period of up to a week. The fraction collector can be operated in total darkness or exposed to desired light/-dark cycles. All cultures were reared at 20° in *LD 12*:12; some were reared on undeuterated medium; the medium of others contained 5, 10, 20, 25, or 30% D_2O. All were assayed at 14, 16, 18, 20, 22, 24, and 26°. (The undeuterated sample to be tested at 14° was lost.)

RESULTS

Tables 1 and 2 summarize our observations on the dependence of τ and ψ on both temperature and D_2O concentration. Fig. 1 and Table 3 give the regressions of both parameters of the circadian system on temperature and D_2O concentration.

The general result is unequivocally clear. The data reconfirm the known facts that while ψ is strongly temperature-dependent, τ is only trivially so. Indeed the regression of τ on temperature in this set of experiments is not significant: the Q_{10} over the measured range does not exceed 1.03. τ and ψ are similarly differentially responsive to deuteration: τ is only trivially affected by D_2O. Again, the slope of the regression of τ on D_2O concentration, while of similar sign to that of temperature, is not of itself significantly different from zero. However, D_2O *is* affecting τ if only trivially: the number of τ estimates at 0% and 25% D_2O are sufficiently large (pooling three separate experiments) to justify a one-way analysis of variance which shows the lengthening of τ at 25% D_2O is significant at the 0.025 level. On the other hand, the dependence of ψ on D_2O concentration is clearly greater and statistically significant.

Thus, τ and ψ *are* as differentially dependent on D_2O concentration as they are on temperature. And to this extent the temperature-equivalence of D_2O action is upheld.

DISCUSSION

It is noteworthy that the slope of τ on D_2O concentration is much smaller than that reported by Suter and Rawson (2) and Enright (3) for *Peromyscus* and *Excirolana*, respectively. Until measurements of the actual D_2O concentration in the deuterated pupae can be made, we cannot attach too much significance to this disparity and (if valid) its clear bearing on Enright's concern with the identity of slopes of τ on D_2O concentration in *Peromyscus* and *Excirolana*. Thus, for 8 days before emergence the pupae do not drink and we cannot estimate the extent to which the D_2O of mature larvae is exchanged for atmospheric H_2O in the course of pupal life. Nevertheless, this uncertainty about the *absolute* D_2O concentration in any of our insects is irrelevant as far as the *differential* effect of the D_2O (whatever its concentration) on τ and ψ of the same insects.

We were prompted to make these observations by noting in Enright's data (3) that 20% D_2O causes a much smaller $\Delta\tau$ in the circadian oscillator of *Excirolana* than in the great majority of the other (temperature-dependent) biological oscillators he studied. This differential response to D_2O becomes the more cogent when it is found in two processes (one temperature-compensated, the other not) *in the same animal*. Had we failed to find a difference, we could have discarded our temperature-equivalence approach to D_2O action.

The significance of our results for the temperature-equivalence hypothesis cannot, however, be fully evaluated until another issue, entirely overlooked in the current circadian literature, is clarified. This is the proposition (Pittendrigh and Caldarola, in preparation) that the frequency of circadian oscillations is homeostatically conserved within narrow limits in the face of *all* change that it may encounter in the cellular milieu. It was the hypothesis that circadian oscillations were indeed the *biological "clocks"* implicated in the discovery of time-compensated sun-orientation (20–22) that led Pittendrigh (18) to look for and find the invariance of their frequency in the face of temperature change. The temperature-compensation of τ has been stressed ever since 1954 as one of the most characteristic features of circadian oscillations generally. But in that emphasis, we seem to have lost sight of a more general proposition that the same teleonomic (functional) logic invites: that to function as a reliable clock, circadian oscillation should be homeostatically protected against frequency change by *any* variation in the cellular milieu. We must therefore await completion of further work before taking the results reported here as the clear evidence they seem to offer that D_2O action is equivalent to that of temperature: we may be seeing only another manifestation of a *general homeostasis* of τ. This caveat extends, of course, to the evaluation of all dose–response curves in the study of circadian oscillations: until we know more about even the formal properties of the homeostatic mechanism, we cannot fully evaluate dose–response relations as guides to the *primary* physical action of *any* agent on the oscillating system itself.

Progress in evaluating the temperature-equivalence hypothesis for D_2O action on circadian oscillations now depends crucially on assaying the effects of heavy water on such oscillators that are "overcompensated" for temperature in the sense of having Q_{10}s less than one. Were we here to find D_2O caused an *increase in frequency* (shortening of τ), we would escape the profound ambiguity of the common result that D_2O lengthens τ. It is noteworthy in this respect that in all the cases so far reported of D_2O lengthening τ, the Q_{10} (where it is known) is slightly greater than one. Experiments are now in progress in our laboratory (Caldarola, unpublished) to exploit this opportunity: Caldarola has found that in *Leucophaea maderae* τ is a non-monotonic function of temperature and she is deuterating insects at different temperatures where the first derivatives of the curve of τ on temperature are either positive or negative depending on the temperature.

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