Heavy water as a tool for study of the forces that control length of period of the 24-hour clock of the hamster  
(D\textsubscript{2}O/factors influencing)

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ABSTRACT In alternating 12-hr periods of light and darkness, start of the dark period entrains the hamster’s 24-hr clock. Blinding or constant darkness frees the clock of entrainment by allowing it to run faster or slower than 24 hr. Constant light frees the clock from entrainment and permits it to run slower than 24 hr—thatis, lengthening its period. Heavy water given in drinking water linearly lengthens the period of the 24-hr clock of blinded hamsters or of hamsters kept in constant darkness in direct proportion to concentration of heavy water (1–50%). Heavy water (1–35%) has very different effects on length of the periods of the 24-hr clock when given under conditions of alternating 12-hr periods of light and darkness. Under these conditions, length of the period is controlled by three factors: (i) heavy water which slows the 24-hr clock; (ii) constant light which also slows the 24-hr clock; (iii) the counteracting effects of entrainment. It is thus possible to observe the effects of all three forces simultaneously in the same animal. The clock slowed by heavy water (1–20%) showed a strong tendency to return to a 24-hr entrainment whenever possible. On a 50% concentration of heavy water, the length of period of the clock became markedly lengthened but very constant and apparently independent of all external and internal disturbances.

Heavy water (D\textsubscript{2}O) has proved its usefulness as a tool for study of functions of the 24-hr clock in a variety of animals—hamsters, rats, white and laboratory monkeys, and squirrel monkeys (1–5, and C. F. Richter, manuscript in preparation).

This study deals with the control of length of period of the 24-hr clock of the “dark” active hamster.

Spontaneous running activity in a revolving drum served as the activity “hand” telling the clock is running and at what rate. It has been widely used through the last 20 years for study of the 24-hr clock of a variety of animals in this laboratory.

This study was started and completed in 1970. A preliminary account appeared in 1970 (5). In part it parallels and confirms 1969 and 1972 observations reported by Palmer and Dowse (3, 4). Differences in methods of recording functioning of the 24-hr clock probably account for differences in observations and interpretations.

METHODS

Previous papers have given a full description of cages and general technique used in these experiments (6, 7). A cage composed of a revolving drum and a living compartment housed each hamster. The living compartment contained a nonspillable food cup containing McCollum finely ground diet (graham flour, 72.5%; skimmed milk powder, 10%; casein, 10%; butter, 5%; calcium carbonate, 1.5%; sodium chloride, 1%) and a graduated inverted 100 ml water bottle. This compartment connected with a revolving drum through a metal partition; each revolution of the drum registered on a cyclometer. The animal was in no way forced to run. Each revolution of the drum in either direction registered on a cyclometer and in an adjoining room on an operation recorder. Records were made daily of total food intake, water intake, and running activity; continuous records of the distribution of running activity were obtained on operation recorders throughout the 24 hr of each day.

Room temperature ranged from 22–24°. Light from four fluorescent Sylvania (F40D) bulbs illuminated the laboratory.

The hamsters were housed in a laboratory room that contained 5 activity stands (each with 16 cages) and 40 holding cages for rats and other animals. Hamsters used in these experiments were distributed at random among the cages of the five stands. They were subjected to all the disturbances and noises involved in taking records, talking of assistants, cleaning of cages, weighing of animals, etc. Experience had shown that these disturbances have little or no effect on the clock.

Base lines were obtained for 15–20 days of the various functions before start of the heavy water.

Heavy water (1, 5, 10, 20, 35, and 50%) replaced plain water usually periods of 40–60 days. The bottles were filled daily with enough heavy water to make it readily available to the hamsters at all times.

For the understanding and evaluating of results of these studies, a short account of basic information about functioning of the hamster’s 24-hr clock is needed.

Entrainment Pattern of Normal Hamster. The hamster is a strictly “dark” active animal. When kept in alternating 12-hr periods of light and darkness it is active in the dark and totally inactive in the light. Most hamsters become active at the start of the dark period, as may be seen in Fig. 1A; a few become

![Fig. 1. Activity-distribution records of two normal hamsters kept in alternating 12-hour periods of light and darkness. (A) Common pattern; (B) pattern found in some normal hamsters.](image)
active at a fixed interval, up to 1–3 hr after the start of the dark period, as seen in Fig. 1B.

Blinding or Constant Darkness. Such conditions free the hamster’s clock from the effect of entrainment allowing it to run either earlier or later. Fig. 2A shows a record of a blinded [optic nerves cut (B)] hamster whose clock had a period of 24 hr and 18 min. Such observations establish existence of the 24-hr clock.

Constant Light and Heavy Water. At the present time, only two agents besides blinding are known to affect the 24-hr clock—(i) constant light and (ii) heavy water.

When kept in constant light, hamsters become active later each day with some regularity as may be seen in Fig. 2B. Here the clock had a period of 25 hr and 20 min. In general, the length of period is definitely longer than that of blinded animals, as may be seen by comparing lengths of period in Fig. 2A and B.

Metabolic Effects of Ingestion of Heavy Water in Various Concentrations. The records in Fig. 3 summarize observations made on metabolic effects of five concentrations of heavy water on hamsters—20, 35, 50, 75, and 99.8%. The bottom panels record the average daily fluid intake in milliliters; the top row shows average daily food intake in grams. The chart shows the number of hamsters used on each concentration. The fluid and food intake records show average daily intake for each one of the 10 days before starting the heavy water regimen and for each one of the 20 days on heavy water.

The records show that the hamster drank 20, 35, and 50% concentrations quite as freely as plain water; they drank progressively less of the 75% and 99.8% concentrations. On the first day of exposure, hamsters, like rats, drink the purest heavy water (99.8%) quite as freely as plain water (B).

Charts in Fig. 3 (upper) show that heavy water in concentrations of 20 and 35% had no effect on food intake; that food intake decreased slightly on the 50% concentration; and markedly on the 75 and 99.8% concentrations. Body weight showed only a slight decrease on the 75% concentration, but a definite decrease on the 99.8% concentration (not shown).

Slowing Effect of Heavy Water on the Clocks of Blinded Hamsters. The pioneer studies of Suter and Rawson (1) showed that heavy water (5–30%) given in drinking water slowed the clock of white mice (Peromyscus leucopus noveboracensis) kept in constant darkness in proportion to concentration of heavy water. I confirmed these observations on blinded rats. Fig. 4A shows the slowing effect of a 10% concentration of heavy water on the clock of a blinded hamster. Length of period was 24 hr and 40 min; Fig. 4B shows the slowing effect of a 20% concentration. Length of period was 25 hr and 4 min; and Fig. 4C shows the slowing effect of a 35% concentration. Here the length of period was 25 hr and 48 min.

In all three instances, lines of onsets of active phases followed a straight line, free from all fluctuations. Each group comprises three blinded hamsters.

Preliminary Observations on This Slowing Effect of Heavy Water in Hamsters Kept in Alternating 12-Hr Periods of Light and Darkness. In 1965 at the start of our experiments on heavy water, it was known that heavy water slows the 24-hr clock of animals kept in constant darkness or after blinding, but it was not known whether it slows the clock of animals kept in alternating 12 hr of light and darkness—that is, whether heavy water can overcome the strong effects of entrainment to light and darkness. Believing that heavy water cannot have any effect on animals whose clocks are entrained by light and darkness Suter, Rawson, and I, in our respective experiments, eliminated effects of entrainment either by constant darkness or blinding.

In further experiments, I kept hamsters and rats in alternating 12 hr of light and darkness and gave them, at first, a 10% con-
12-hr periods were chosen for these studies. There were onsets of activity in three hamsters at different times of the day, showing a consistent 24-hr pattern of activity fluctuations. The clocks became entrained to the 24-hr period of the hamster when they were blinded with no light to help them orient themselves to the clock. This experiment demonstrated that the hamsters could entrain to the clock without light if they were kept in a constant environment.

**Effects of Heavy Water (1-50%) on the 24-Hr Clocks of Hamsters in Alternating 12-Hr Periods of Light and Darkness.** Results produced by heavy water on the 24-hr clock of hamsters can be divided into three groups according to concentrations of the heavy water: Group I (1, 5, and 10%); Group II (20%); Group III (35 and 50%).

**Group I (1, 5, and 10%).** Fig. 5 shows the activity-distribution records of three hamsters that received a 10% concentration of heavy water to drink. The clock of the first hamster (Fig. 5A) became entrained with a period of 24 hr and 1 min. When onsets of the active phases reached 6:45 p.m., they became entrained to a 24-hr period. The other two hamsters (Fig. 5B and C) showed similar records. For 10-15 days, their clocks had periods of 24 hr and 2 min and 24 hr and 4 min, respectively. Then their clocks became entrained again to a 24-hr period—the clock of the hamster in Fig. 5B to 8 p.m., the clock of the hamster in Fig. 5C to 9:30 p.m. In both instances, the clocks showed definite fluctuations in lengths of period from time to time, but overall maintained a consistent 24-hr entrainment.

A comparison of these three records with the typical record in Fig. 4A of a blinded hamster, also on 10% heavy water, clearly established the strong effect of entrainment on the clock.

The summary chart in Fig. 6 shows the lines of onsets of the three hamsters on the 10% concentration and of four hamsters on 5% and of the one hamster on 1%. It shows these lines of onsets for the successive 12-hr dark periods. It will be seen that the fluctuations were more pronounced in the animals on a 5% concentration than in the animals on a 10% concentration. There were marked differences in the location of the 24-hr entrainment lines with relation to start of the dark period; they ranged from 6 p.m. to 9:30 p.m.

A pulling back and forth between the slowing force of heavy water and the opposing force of entrainment may explain the marked fluctuations in lengths of period.

**Group II (20%).** Fig. 7 shows the activity-distribution record of a hamster that received the 20% concentration of heavy water—a concentration that has no effect on metabolism. See Fig. 3. For 15 days, the clock ran slower with a period of 24 hr and 16 min. Then, as was true for the hamsters on the 10% concentration, as shown in Fig. 5, the clock of this animal became entrained to a 24-hr period. This entrainment, however, lasted only 9 days before the clock definitely slowed again. Then, in the following 12-hr period of constant light, as would be expected from Fig. 2B, it became markedly slowed to 26 hr and 12 min. Shortly after onsets reached the following dark period, they started again to become entrained to the 24-hr period.

The extraordinary activity-distribution record in Fig. 8 shows the full operation of all three forces: (i) slowing effect of heavy water; (ii) slowing effect of constant light; (iii) counteracting force of entrainment over nearly 6 months. The record at the left shows the activity-distribution record in the form that it is usually presented to save space. The records at the right show this same record but in a form that makes it possible to follow the lines of onsets of daily active periods without interruption from beginning to end.

It will be seen that during each successive 12-hr period of constant light the clock was definitely markedly slowed while during each successive 12-hr period of constant darkness the clock was progressively speeded up, right up to the last dark period, when it had almost been fully reentrained to the 24-hr period.

The summary chart in Fig. 6 shows the wide range of fluctuations in lengths of period of the clock in the eight hamsters on the 20% concentration of heavy water.

Here again the marked fluctuations in length of clock period

![Fig. 4. Activity-distribution chart. Lengths of periods of the clock for: (A) hamster that had for some time been drinking a 10% concentration of heavy water and then was blinded (O.N.); (B) blinded hamster on a 20% concentration of heavy water; (C) blinded hamster on a 35% concentration of heavy water.](image)

![Fig. 5. Activity-distribution charts showing freeing of clock from entrainment for three hamsters kept on 10% heavy water in alternating 12-hr periods of light and darkness.](image)
must reflect the counteraction of the three forces—the strong slowing effects of heavy water and constant light and the strong speeding up effects of entrainment.

The records leave no doubt about the strength of the force that tends to retrain the clock to its original 24-hr period.

**Group III (35% and 50%).** Results with the 35% concentration of heavy water (a concentration that likewise has no effect on metabolism, see Fig. 3) showed that the stronger force of this higher concentration almost completely overcame the entraining force thus eliminating all fluctuations. The successive activity periods were almost continuous. Length of period of the clock was 25 hr and 48 min. Three other hamsters gave similar records as may be seen in the summary chart in Fig. 6. Suter and Rawson's deer mice on a 30% concentration of D₂O had a period of 25 hr and 30 min.

The remarkable record in Fig. 9 shows that lengths of period of the clock of a hamster on the 50% concentration of heavy water (a concentration that has only a slight effect on food intake—see Fig. 3) followed a perfectly straight line. There was not the slightest indication of the presence of any fluctuations.

Of special interest here is the fact that under influence of the 50% concentration of heavy water, the clock of this animal

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**Fig. 6.** Summary chart showing lines of onsets of active periods of individual hamsters on the various concentrations [1–100% (99.8%)] of heavy water during 12-hr period of darkness. Hamsters were kept on various concentrations of heavy water in alternating 12-hr periods of light and darkness.

**Fig. 7.** Activity-distribution chart (spontaneous running) of hamster kept on a 20% concentration of heavy water and in alternating 12-hr periods of light and darkness.

**Fig. 8.** Same as Fig. 7 for another hamster on a 20% concentration.
became independent of all external and internal disturbances. All the many disturbances in the laboratory, handling for weighing, etc., had no effect on the clock. Two other animals on heavy water gave similar records, as may be seen in the summary chart in Fig. 6.

**DISCUSSION**

The results of these experiments demonstrated the value of heavy water as a tool for the study of the phenomenon of entrainment of the 24-hr clock of the hamster.

In lower concentrations of heavy water, the slowing force acting on the clock just sufficed to free the 24-hr clock from entrainment. The force of entrainment and the force of slowing effects just managed to counterbalance one another giving rise to marked fluctuations of lengths of period of the 24-hr clock.

Entrainment forces tend to bring onsets of the active phases of the 24-hr clocks in line with start of the 12-hr dark period. But a more important goal of entrainment may be the 24-hr period—possibly a deeply ingrained demand of the organism, to some extent independent of the 12-hr light and dark periods. This report contained a number of examples of such entrainment: Figs. 5A–C, and Figs. 7 and 8. This entrainment occurred at almost any location in the dark period, often well removed from the start of the dark period.

This form of entrainment may also occur in normal hamsters. Thus, although in most instances the 24-hr clock is rigidly entrained to start of the dark period, in a number of instances active phases of the clock do not occur until an hour or two after start of the dark period (see Fig. 1B) and then continue to occur at this interval with great constancy indefinitely.

These observations in the laboratory would indicate that in their natural habitat—deep burrows in the tropics—some hamsters might not emerge from their burrows promptly at 6 p.m. at start of darkness, but at a fixed interval of one or more hours later.

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