Reports

Timing Behavior in Rats with Water Drinking as a Mediator

Abstract. *Rats, reinforced for spacing their responses 20 seconds apart, used water-drinking as a means of "pacing" their response rate. "Timing" may be based on mediating response sequences such as drinking.*

Reinforcement schedules where the delivery of food is contingent on the passage of time offer an opportunity to study the extent to which, and the mechanisms by which, organisms "tell time." One form of time-contingent reinforcement schedule is a spaced-response schedule where subjects are reinforced only for those responses spaced at least x seconds apart. The resulting response rate is low, and fairly steady, with a large proportion of responses spaced just far enough apart to secure reinforcement (*1*). What is the nature of the behavioral processes underlying timing? Does timing "go on in the head" or are there peripheral stimuli present during timing performances that could plausibly serve as the cues for the timed response? Successful timing on a spaced-response schedule is often ascribed (*2, 3*) to the subject's acquiring, in the course of adjusting to the schedule, an orderly sequence of mediating (collateral) behavior that fills up the necessary time interval. The last response in the sequence may provide unique stimulation that can serve as the cue for performance of the critical conditioned response. One problem in this explanation is that the presumed mediating behavior is often unobserved and unrecorded. More direct demonstrations of the role of mediating responses in timing are needed.

In an experiment on spaced responding our rats drank inordinate amounts of water. Excessive drinking occurred on another time-contingent variable-interval reinforcement schedule (*4, 5*). Mediation of timing has been observed in a monkey, based on its licking the plastic holder of its water bottle (*3*). Water-oriented behavior, therefore, seems a natural "filler" on dry food reinforcement schedules. Simultaneous recording of drinking and conditioned responding revealed an orderly relation between the two behaviors which suggested that drinking was mediating the conditioned response. Rats reinforced with dry food would appear to tend naturally toward drinking as a mediating response. This fact, together with the ease of recording drinking, may make it a useful variable for the study of peripheral factors in timing.

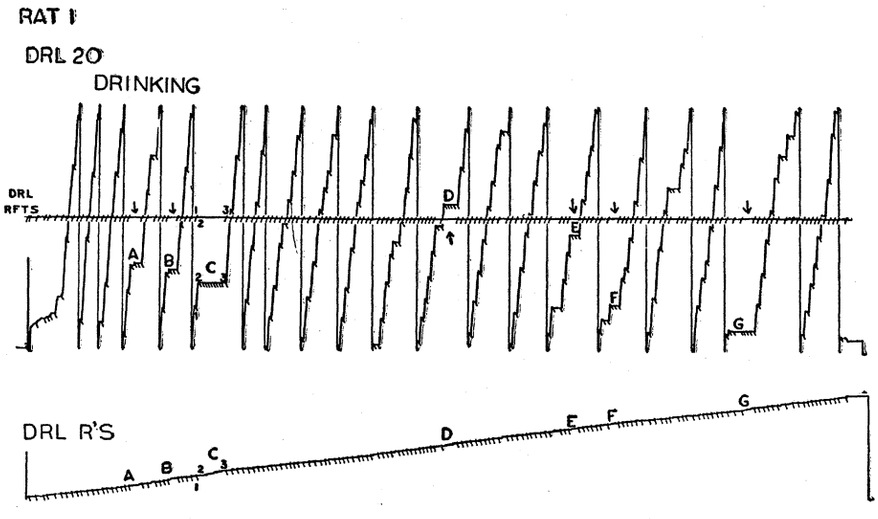

Fig. 1. Correlation of drinking and spaced-responding.

Three adult female albino rats were placed on a 23-hour food-deprivation schedule (water was always available) and reinforced during daily experimental sessions for key-pressing responses spaced at least 20 seconds apart. Responses spaced less than 20 seconds apart reset the reinforcement timer but were not reinforced. Within a few sessions all rats acquired the pattern of behavior illustrated in Fig. 1. The curve labeled DRL R's is a cumulative record of key presses over time. Downward deflections represent dry food reinforcements. The curve labeled drinking is a cumulative record of water-licking responses. The downward deflections in the drinking record represent key presses. The horizontal line superimposed on the drinking record shows reinforcements, and makes it easy to follow the time-course of drinking, key pressing, and reinforcement. The pattern of behavior may be summarized as follows: a long, uninterrupted period of drinking was followed by a key press which, if reinforced, was followed by ingestion of the food pellet and then another uninterrupted period of drinking. The points marked *A, B, C, D, E, F,* and *G* represent the rare interruptions of this orderly sequence.

At *C* the events occurring during such interruptions are noted in detail. At *C*1 a key press occurred and was reinforced, and this was followed, after a pause just sufficient to permit the ingestion of a pellet, by drinking (*C*1 to *C*2). At *C*2 a key press occurred prematurely and was not reinforced. Drinking did not resume; rather, between *C*2 and *C*3, other, unrecorded, behavior occurred, punctuated by occasional premature key presses. It was not until *C*3 that a key press finally occurred spaced far enough from the preceding response to secure reinforcement. Immediately, drinking was resumed and the pattern—drinking, key-pressing, eating, drinking—prevailed until *D*. From this record it is evident that the cue for drinking was ingestion of a dry food pellet; nonreinforced key presses never led to drinking. It is also evident that drinking mediated successful timing, for whenever drinking was absent, key presses were rarely spaced far enough apart to be reinforced. The volume of water ingested during this 1-hour session was about 100 ml (*6*).

An unresolved question is, what causes the rats to stop drinking and perform the reinforced response? Interoceptive stiumulation from the ingestion of water may provide the necessary discriminative cues, but this remains to be determined (*7*).

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References and Notes

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3. W. Hodos, G. S. Ross, J. V. Brady, *J. Exptl. Anal. Behav.* **5**, 473 (1962).
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5. J. L. Falk, *Science* **133**, 195 (1961).
6. We have not measured daily water intake of individual rats, but we find that 100 ml per day is ordinarily sufficient for a group of three rats caged together and allowed free access to food. Average water intake for rats of 1 ml per hour is reported in the *Handbook of Biological Data*, W. S. Spector, Ed. (Saunders, Philadelphia, 1956), p. 355. Falk (*5*) reports intakes of about 25 ml per day per rat on free-feeding, and intakes of about 100 ml for food-deprived rats during 3-hour experimental sessions of variable-interval dry-food reinforcement.
7. Supported by grant G 18132 from the National Science Foundation (E.F.S.) and by an NSF undergraduate summer research training grant (S.M.H.).

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Tumors Induced in Hamsters by Simian Virus 40: Persistent Subviral Infection

Abstract. *The cells of two hamster ependymomas, originally induced by intracerebral inoculation with simian virus 40 in newborn animals, have been serially cultured in vitro. None of the virus was detected in cell-free culture fluids or cell lysates. All cells retained their neoplastic potential when newborn hamsters were inoculated. Data suggest that viral nucleic acid is permanently present in some tumor cells.*

In extracts of ependymomas induced in newborn hamsters by simian virus 40 (SV_{40}) we were consistently unable to detect the virus, but we could detect it in intact tumor cells seeded onto sensitive indicator cells (*1*), thus the virus may exist in an altered state in some tumor cells. Subsequent work has supported this hypothesis.

Two cell lines, EP_A and EP_H established from two primary ependymomas induced in hamsters with SV_{40} (*1*) were cultivated in vitro for 55 and 35 passages during a period of 14 months. The EP cells were grown in Eagle's basal medium (*2*), 5 percent calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). This medium was free of any detectable inhibitors of SV_{40}. Primary cultures of African green monkey kidneys and rabbit kidneys were grown in a medium consisting of 0.5 percent lactalbumin hydrolyzate, 5 percent calf serum, penicillin, and streptomycin (as above) in Hanks' balanced salt solution. All cell cultures were maintained with Eagle's medium and 2 percent calf serum.

Cultures of EP cells which had not been cloned consisted mainly of epithelial cells, occasional fibroblastic cells, and numerous multinucleated giant cells. The cells exhibited a high mitotic activity and had a generation time of approximately 48 hours. The length of time population cultures can be maintained—2 to 4 weeks with periodic changes of medium—depends on the density of the cell. During the past 14 months, cell-free fluids and lysates of EP cell cultures have been tested repeatedly for SV_{40} with a 28-day assay in monkey kidney cells.

We have been unable to detect infectious virus in such samples, nor did fluorescent antibody tests reveal viral antigen in EP cells. These cells retained their neoplastic potential when newborn hamsters were inoculated.

When cells are placed in contact with sensitive indicator cells from monkey kidney characteristic cytopathogenic effects (CPE) appear within 18 to 20 days in these cells which are adjacent to colonies of EP cells. The virus in these mixed cultures was identified as SV_{40} by neutralization tests with specific antiserum. The initial localization of the CPE near the EP cells and the consistent absence of intra- or extracellular virus in EP cell cultures suggested an inheritable cell-to-cell transfer of infectious viral material, presumably viral deoxyribonucleic acid (DNA). To test this hypothesis the following experiments were carried out:

1) Rabbit kidney cell cultures were infected with SV_{40} at a multiplicity of 0.5 $TCID_{50}$ per cell (tissue culture infective dose—50 percent effective). After a 2-hour adsorption period at 37°C, the inoculum was removed and frozen for assay for residual virus. A sample of the inoculum before adsorption was also frozen. The infected rabbit kidney cell sheet was thoroughly washed, incubated for 14 days, and frozen for assay. Duplicate rabbit kidney cell cultures were infected with phenol-extracted DNA obtained from strain 777 of SV_{40} which originally induced these hamster tumors (*3*). After removal of the inoculum by repeated washing the rabbit kidney cells which had been infected with DNA were incubated for 14 days and frozen for assay. The virus titration in monkey kidney cells was observed for 21 days. Rabbit kidney cells failed to support virus growth after infection with intact SV_{40} (Table 1). By contrast, exposure of these cells to infectious viral DNA resulted in synthesis of small amounts of virus. Viral DNA treated with deoxyribonuclease, 5.0 μg/ml for 10 minutes at room temperature destroyed its infectivity.

Table 1. Results of infection of rabbit kidney cell cultures with intact SV_{40} or infectious viral DNA.

Preparation	$Log_{10}TCID_{50}$/0.2 ml
Intact SV_{40} before adsorption	5.2
Intact SV_{40} 2 hours adsorbed to RK	5.1
RK infected with intact SV_{40}	*
Viral DNA, original inoculum	4.3
RK infected with viral DNA	1.5
RK infected with viral DNA pre-treated with 5.0 μg/ml of DNAase	*

* Noninfective when tested undiluted 0.5 ml/tube.

2) Milk dilution bottle cultures of rabbit and monkey kidney, as well as milk dilution bottles without cells (glass), were seeded with approximately 20,000 EP_{A50} or EP_{H29} cells (50th and 29th passage). In addition, 2×10^7 cells of EP culture were lysed by 10 cycles of freezing and thawing, and the cell-free fluids were tested for virus content (Table 2). No virus was detectable in the lysates of EP cells or of the uninoculated controls. In contrast, significant amounts of SV_{40} were recovered from rabbit, as well as monkey kidney cells.

Additional experiments gave the following results: (i) no activity comparable to that of interferon (*4*) could

Table 2. Results of inoculation of intact or lysed tumor cells. Cell-free lysates of 2×10^7 tumor cells inoculated onto both monkey (GMK) and rabbit (RK) kidney cells were negative. Uninoculated cell controls for both monkey and rabbit kidney were also negative.

Tumor cell line	20,000 intact tumor cells seeded onto*		
	GMK	RK	Glass
EP_{A50}	5.17†	1.97†	Negative
EP_{H29}	5.67	2.17	Negative

* Cells or lysates inoculated into milk dilution bottle cultures. At 10 days, dilutions of the cell-free lysates were subcultured to GMK tube cultures which were held for 21 days. †Total virus $log_{10}TCID_{50}$ present in bottle cultures containing 15 ml medium. Virus was identified as SV_{40} by neutralization tests.