Pharmacology Biochemistry and Behavior, Vol. 1, pp. 651–656. ANKHO International Inc., 1973. Printed in the U.S.A.

Effects of Puromycin on Memory for Shuttle Box Extinction in Goldfish and Barpress Extinction in Rats[1]

WILLIAM G. BRAUD AND WILLIAM J. BROUSSARD

Psychology Department, University of Houston, Houston, Texas 77004

(Received 7 August 1973)

BRAUD, W. G. AND W. J. BROUSSARD. *Effects of puromycin on memory for shuttle box extinction in goldfish and barpress extinction in rats.* PHARMAC. BIOCHEM. BEHAV. 1(6) 651–656, 1973.–Five experiments were conducted to investigate the generality of puromycin's reported effect on disruption on memory of a recently learned task. The first experiment replicated previous work on acquisition to determine the effectiveness of the procedures used. The second investigated the role of puromycin's low pH in memory disruption. The third experiment used short training and extinction sessions to determine if puromycin retarded retention of extinction. The fourth experiment used longer training and extinction sessions and multiple and delayed injections of puromycin, and the fifth experiment attempted to extend puromycin's effect on avoidance extinction to extinction produced in an appetitive operant task. Puromycin disrupted retention of extinction of both shuttle box avoidance in fish and barpressing in rats. The role of puromycin's pH was negligible.

Consolidation Puromycin Extinction Memory disruption Short-term memory/Long-term memory

THE INTRACRANIAL injection of mice, goldfish, and Japanese quail with puromycin immediately following a training session has been shown to interfere with subsequent retention of the acquired behavior in a variety of learning situations including escape learning [5], simple avoidance [2], discrimination avoidance and passive avoidance [16], simple classical conditioning [14], and appetitive color-discrimination learning [15]. Since delayed injections typically do not result in interference, and since the injections usually do not impair initial learning itself, puromycin's effect generally is believed to be upon a hypothetical "memory consolidation" process in which information is transferred from a short-term to a long-term store. Along with physiological and biochemical research on the drug's mechanism of action, considerable behavioral research is needed to accurately delineate the generality of puromycin's effect and to specify exactly the various conditions upon which the interference phenomenon depends. Illustrations of the need for further behavioral research are the recent findings [14] that neither injection time nor overtraining altered puromycin's interfering effect on retention of a classical conditioning task and the question [16] of just which component processes of a complex task is puromycin actually affecting.

The present work is concerned with the process generality of puromycin's effect. All research to date has dealt with puromycin's effect upon retention of an acquisition process; the complementary extinction process has been ignored. It was hypothesized that puromycin would inhibit memory for extinction, whether extinction was viewed as a passive decay process or alternatively as an active inhibitory process. Agranoff has provided evidence that repeated injections of acetoxycycloheximide, a drug having behavioral and biochemical effects similar to those of puromycin, could prevent the decay of short-term memory in goldfish [1]. Experimental extinction (if conceptualized as decay) might similarly be affected by puromycin. On the other hand, puromycin should also depress retention of extinction if the latter is viewed as an active inhibitory learning process, a view strongly supported by recent bioassay experiments by Braud [6] and by pharmacological studies by Deutsch and Wiener [9]. The finding that puromycin could interfere with the retention of extinction as well as acquisition would greatly extend the generality of the puromycin phenomenon. An additional question of interest was whether puromycin's effect upon extinction might be time-dependent, as it is in acquisition paradigms. A third question concerned the role of puromycin's pH, and a fourth attempted to extend the generality of the effect to extinction produced in an operant task using positive reinforcement.

GENERAL METHOD

Animals

The fish used in Experiments 1 through 4 were 110

[1]We thank J. McMahan, D. Wong, R. Wetherford, D. Gordon, S. Satin and B. Lacey for assistance during various stages of these investigations.

common goldfish, 7.5 10.0 cm long, obtained from Ozark Fisheries, Stoutland, Missouri. Initially the fish were maintained in groups of ten in constantly aerated and filtered 10-gallon aquaria; however, during the experiments the fish were kept in groups of five in nonaerated and nonfiltered plastic tanks (42 x 23.5 x 10 cm). The fish were kept in darkness from 11:00 p.m. until 8:00 p.m. daily; during the rest of the time they were kept under artificial room illumination. The fish were not fed while the experiments were in progress.

The animals used in Experiment 5 were 16 male Sprague-Dawley rats, 80–100 days old, with a free-feeding weight of approximately 250–280 g. In all animals except those used in a control group, a small hole was drilled bilaterally through the skull just above the angle between the caudal sutures of the parietal bones and the origin of the temporal muscles [9].

Apparatus

In Experiments 1 through 4, the training apparatus consisted of two semi-automated aquatic shuttle boxes similar to those used by Agranoff and Klinger [2]. They were identical clear plastic boxes, 30 x 10.5 x 18 cm, covered on sides and ends with opaque white paper. Centered across the bottom of each box was a 10.5 x 3.5 cm plywood barrier. The water level in the boxes was maintained at a depth of 5.4 cm which allowed 1.9 cm of water over the top of the barrier. A clear stimulus light was taped to the outside of either end of a box and centered near the bottom. Stainless steel mesh electrodes, 11.5 x 3.5 cm were situated near either end and on either side of both boxes. Electric shock consisted of 0.2 sec duration, 9 V d.c. pulses, delivered at a rate of 40 per min.

In Experiment 5, two standard operant conditioning chambers were used. A micro-switch bar was positioned on the left side of the front wall, and 45 mg Noyes pellets were delivered to a feeder trough located to the right of the bar. Each chamber was enclosed in a sound-attenuating chest and located in a room adjacent to that housing the electromechanical equipment used to control it.

EXPERIMENT 1

Effect of Puromycin on Acquisition

Before conducting the extinction study, it was necessary to replicate Agranoff's acquisition work in order to be certain that our behavioral work and biochemical procedures were effective.

Method

Four groups of ten goldfish were given twenty light-signalled avoidance acquisition trials in the shuttle box. A trial consisted of 20 sec of light (illumination of the compartment occupied by the fish), followed by 20 sec of light with pulsed shock, followed by 20 sec of darkness and no shock. There was a five minute interval of darkness before the first trial and following each block of five trials. If a fish crossed the barrier during the first 20 sec of light, an avoidance response was recorded. In order to insure a homogeneous sample, fish that avoided on more than 80 percent or less than 30 percent of the first 20 trials were eliminated and replaced by other fish. Fifteen of the forty fish were replaced in this manner. Immediately following the 20 training trials, one group was injected intracranially with 90 μg puromycin dihydrochloride in saline; another group was injected with saline; and a third group received no injection. A fourth group was injected with puromycin after a 24-hr delay. In all cases, 10 μl of material was injected according to the method of Agranoff and Klinger. Seventy-two hr after initial training, all animals were tested for retention by being given ten avoidance trials without shock.

Fish were not aerated during their stay in the training/testing apparatus. Use of an unaerated shuttle box has been standard operating procedure in our lab. We have never found that lack of aeration during training affected the learning process and have consistently obtained training results comparable to those of other investigators.

Since goldfish learning apparently varies with temperature and season of the year, we report that these studies were conducted during the months of November through January; water temperature was maintained at 21 22°C during training and testing.

Results

Immediate injections of puromycin interfered with retention of acquisition learning (Mann Whitney U = 10, n = 10, $p = 0.001$), while a 24-hr delayed puromycin injection did not (Fig. 1). This finding is consistent with results obtained by Agranoff and his co-workers. However, while Agranoff typically uses large groups of animals, larger doses of puromycin, and measures retention in terms of predicted retention scores derived through the use of regression analysis, we were able to obtain dramatic results in the present study using small groups, a low puromycin dosage, and a direct comparison of raw retention scores. We believe our more sensitive assessment of puromycin's effect is due to the use of a more homogeneous sample of animals and our use of non-shock retention trials.

EXPERIMENT 2

Since the puromycin solution was not neutralized (puromycin's memory impairing property is dependent upon the particular neutralizing agent used) [10], a second experiment was done to determine the role, if any, of its low pH. It was possible that at least part of the retention deficit observed in the first experiment might have been due to the acidity of the material injected.

Method

Two groups of ten fish each were given twenty avoidance acquisition trials as described in Experiment 1. The fish were immediately injected with 10 μl of either saline or 0.01 N hydrochloric acid, the pH of which equalled that of the puromycin used in the previous experiment (pH = 2.0). Seventy-two hours later, all animals received 20 relearning trials to measure retention.

Results

As may be seen in Fig. 2, the immediate injection of HCl had no effect on retention; in fact, the retention score of the HCl group was almost identical to that of the saline-injected control. Thus, in this experiment, the contribution of puromycin's acidity is negligible.

EXPERIMENT 3

Having determined that our procedures were effective in

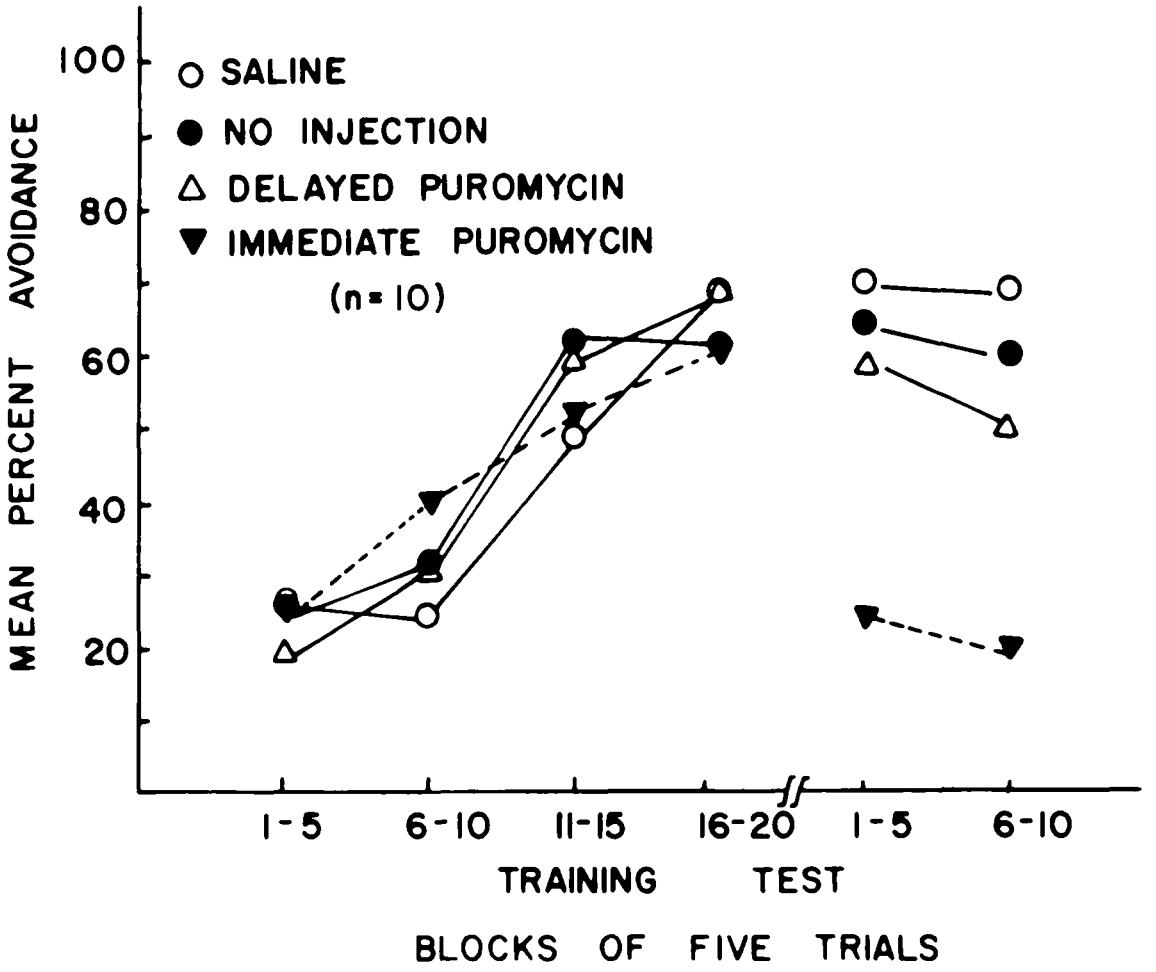

FIG. 1. Acquisition and retention test performance of groups of fish given posttraining intracranial injections of saline or puromycin (immediate and 24-hr delayed) or no injection.

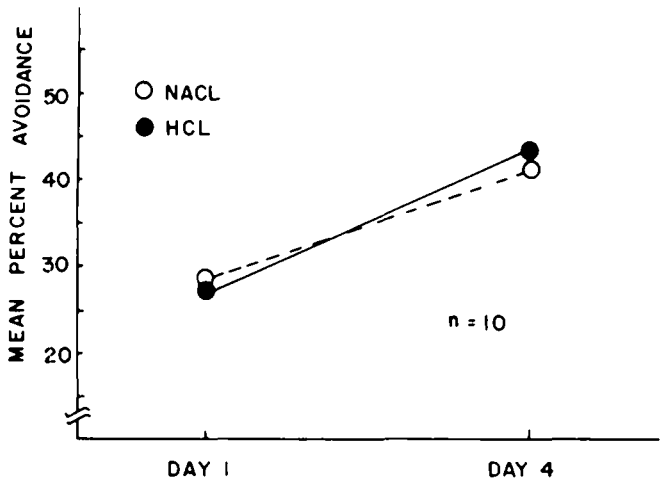

FIG. 2. Avoidance behavior of goldfish given posttraining injection of either saline or hydrochloric acid (as a control for acidity of puromycin).

interfering with memory of an acquisition task, a third experiment was conducted to assess puromycin's effect on memory of experimental extinction. We continue to term this experiment "extinction" since operationally (nominally) it was exactly that; functionally, the animals did not demonstrate the expected extinction behavior.

Method

Number of acquisition trials was increased in this experiment to obtain higher performance and hence enhance detection of a behavioral decrement during extinction.

Two groups of ten fish each were given three days (60 total trials) of avoidance acquisition training followed by one day (20 trials) of extinction training in which shock was omitted. Immediately following the single extinction session, fish were injected with 10 μl of either puromycin or saline. Seventy-two hours later, animals were tested for retention of extinction learning by being given 20 trials without shock.

Results

Retention scores for the puromycin and saline groups

did not differ significantly. Neither group, however, demonstrated extinction behavior following a single extinction session. During extinction, the animals performed so well that they experienced very few actual extinction trials, i.e., trials in which they failed to avoid but received no shock. What was nominally and operationally an extinction session for the experimenter was not functionally an extinction session for the animals. It is likely that puromycin had no effect since proper extinction did not occur; on the other hand, acquisition training was probably already sufficiently consolidated so as to be insusceptible to the action of puromycin.

EXPERIMENT 4

Since the effect of the single puromycin injection and the procedural definition of extinction in Experiment 3 were unclear, a fourth experiment was conducted. In this experiment, the amount of acquisition training was extended and combined with repeated extinction sessions and multiple injections of puromycin. The amount of acquisition training was further increased to better observe an extinction decrement and because unpublished pilot studies have indicated that extinction may be enhanced by giving additional overtraining trials (i.e., an overlearning extinction effect seems to occur in goldfish avoidance learning).

Method

Three groups of ten fish each were given five days (100 total trials) of avoidance acquisition training, followed by two days (40 total trials) of extinction training. After each 20-trial extinction session, animals were injected immediately with 10 μl of either puromycin or saline. Another group was given puromycin injections after a 16-hr delay. Seventy-two hr after the last extinction session, fish were tested for retention by being given 20 nonshock (light only) trials.

Results

As seen in Fig. 3, puromycin had its predicted effect following multiple extinction sessions. Immediate puromycin injections interfered with retention of extinction ($U = 4$, $n = 10$, $p<0.001$), while delayed puromycin injections had no effect.

EXPERIMENT 5

Since Experiment 4 demonstrated puromycin's effect on extinction of an avoidance task, it was felt that the generality of the phenomenon could be extended by investigating puromycin's effect on retention of extinction in an operant task using positive reinforcement.

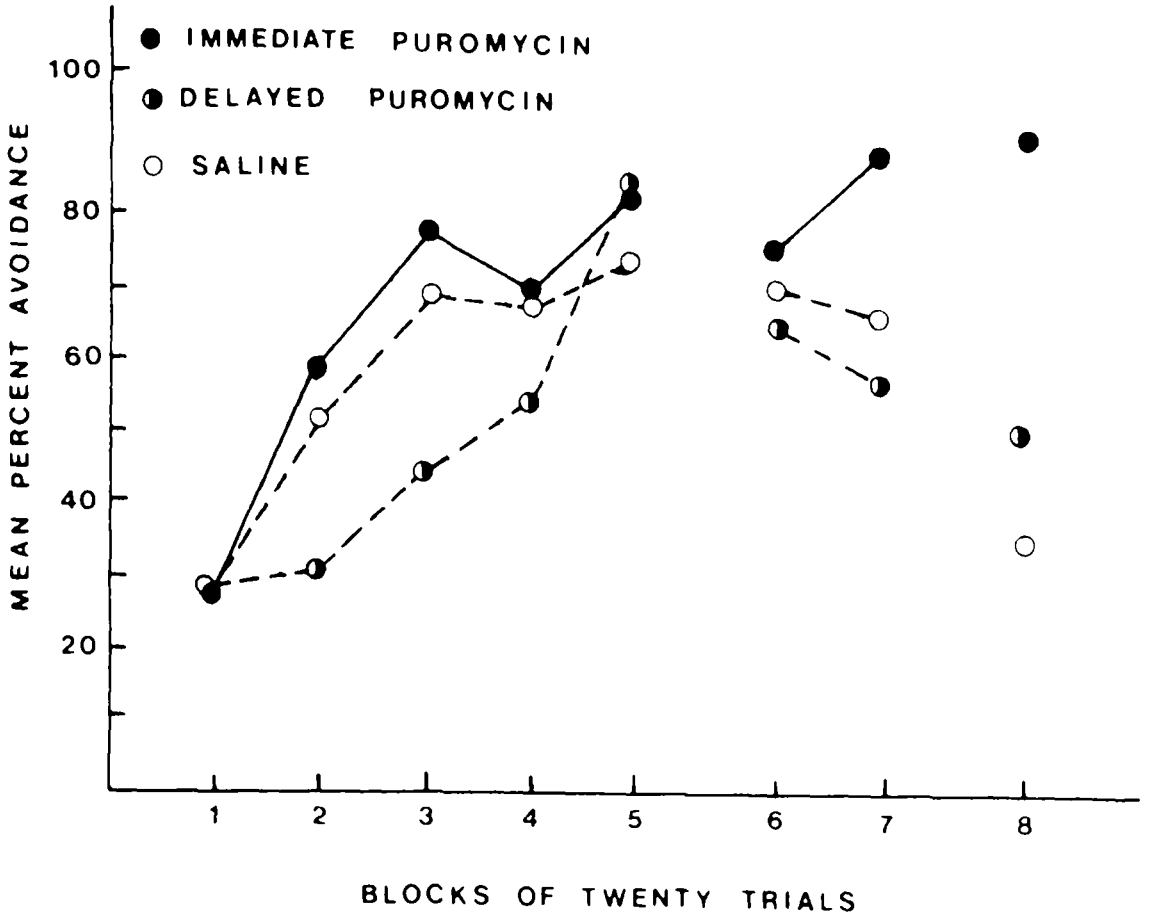

FIG. 3. Acquisition and extinction performance of fish given multiple, postextinction training injections of either puromycin (immediate or 16-hr delayed) or saline.

Method

Sixteen rats were placed on food deprivation and reduced to 80 percent of their free-feeding weight. During training, all animals were given one day of magazine training followed by two days of shaping to a fixed ratio (FR) schedule requiring 30 barpresses for one food presentation consisting of a 45 mg Noyes pellet. The shaping sessions were followed by six alternating days of one-hour sessions on the FR-30 schedule. Alternating training sessions and days in the home cage (off days) on a maintenance food schedule totaled 12 days for response rate to stabilize. On Day 13, all animals were extinguished to a baseline defined as no responses occurring for 15 min. Immediately following extinction procedures, animals were anesthetized and puromycin or saline was intracranially injected. A third group was given sham operations, and a fourth group was returned to the home cages. Day 14 was a usual off day. On Day 15 all animals were placed back in the operant chamber and remained there until no responses occurred for 15 min; the animals then were returned to the home cages. The extinction procedure was repeated on Days 17 and 19. The amount of puromycin administered in this experiment was calculated on the basis of the reported effective dose in mice [11] and the wet-brain weight to body weight ratio reported for mice and rats [3]. Such a ratio resulted in a dosage of 0.84 mg/0.012 ml per injection site. For Experiment 5 only, puromycin was neutralized with NaOH to a pH of 7.2.

Results

During training, all animals reached the FR-30 criterion during the first two days following shaping. Response rate was stable during the following six alternating days on the FR-30 schedule. Previous to extinction, animals were assigned to one of the four groups on the basis of response rate so that every group would consist of both low and high responders.

Immediate injections of puromycin interfered with retention of extinction on all three days of testing. Figure 4 shows the average number of responses and Fig. 5, the average time until the last response was recorded for each group. Analysis of variance revealed a difference between groups ($p<0.001$) for both response and time measures.

DISCUSSION

The results of these experiments suggest that puromycin's ability to disrupt memory of extinction is dependent upon (a) the length and effectiveness of extinction sessions, and (b) the postextinction interval between the session and drug administration. In Experiment 3, neither puromycin nor saline-injected animals exhibited a decrease in percent responding following one extinction session. In Experiment 4, however, immediate puromycin injection interfered with retention of extinction while saline or delayed puromycin did not. Results of these two experiments again indicate the importance of functional definitions of the behavioral procedures used in animal research.

The results of Experiments 4 and 5 extend the generality of puromycin's effect to extinction produced in both shuttle box avoidance and operant conditioning. In the latter study, puromycin-injected animals demonstrated a high rate of barpressing on all three days of extinction testing. The data from Experiment 5 suggest that immediate puromycin

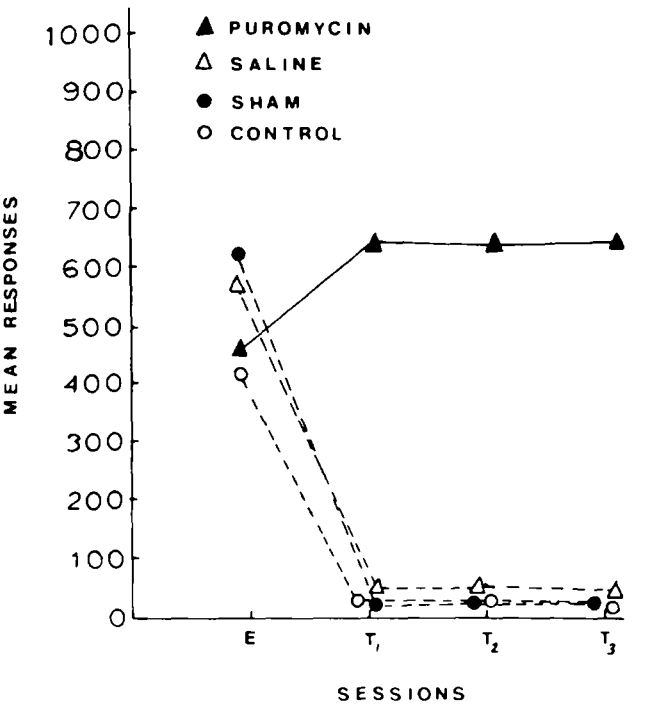

FIG. 4. Average response scores for control and experimental groups during the inital extinction day (E) and subsequent testing days (T_1, T_2, T_3) following puromycin injections.

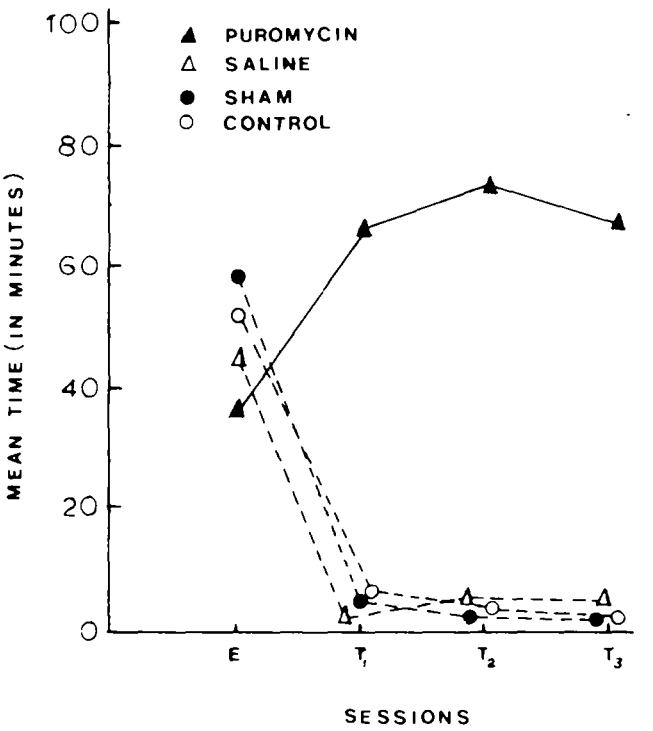

FIG. 5. Average time (in min) for control and experimental groups during the initial extinction day (E) and subsequent testing days (T_1, T_2, T_3) following puromycin injections.

injections not only interfered with retention following the initial extinction session, but continued to block retention for at least the subsequent two testing days.

The combined results of all five experiments suggest the following conclusions: (a) puromycin interferes with retention of acquisition and extinction, (b) puromycin's effect extends to both avoidance and operant behavior, (c) the drug's low pH contributes little to the disruptive effect, and (d) immediate injections are effective while delayed administration is not. We are aware that Experiment 5 did not include a time course study of puromycin's effect and that any statements about time-dependence derive from our goldfish experiments.

An interesting observation from Experiments 4 and 5 is that puromycin-injected animals not only continued to respond during testing days, but demonstrated increased response (and time) scores on testing days compared to initial extinction day levels. If puromycin injections produced a general facilitation effect, then the delayed injection animals of Experiment 4 should also have shown response facilitation on testing days. An alternative explanation is suggested from research concerning puromycin's effect on biochemical [4, 7, 13], electrical [8], and histological [12] mechanisms. Since puromycin injections produce such a variety of changes in neural tissue, it is possible that such injections interfere with short-term memory processes. We are fully aware that an interference with short-term memory by puromycin or other amnesic agents is not usually reported in the literature. This is the very reason we report such an apparent effect here: perhaps STM disruption effects have been present in the findings of others, but have been overlooked. If puromycin did partially interfere with short-term memory, then an increase in response scores as observed in these experiments would be anticipated. The increased response and time scores during later extinction tests in the immediately injected puromycin groups in these experiments do indeed indicate that extinction continues to occur within a session, but it takes longer than it should; i.e, the animals learn extinction much slower than normally, hence there may be an impairment of short-term memory for extinction learning, as measured within sessions. Certainly this aspect of puromycin's effect should be more closely examined. It is interesting to note that Swanson, McGaugh and Cotman [17] have reported a similar disruption of short-term memory in mice by the protein synthesis inhibiting drug, acetoxycycloheximide.

REFERENCES

1. Agranoff, B. W. Recent studies on the stages of memory formation in the goldfish. In: *Molecular Approaches to Learning and Memory*, edited by W. L. Byrne. New York: Academic Press, 1970, 35–39.
2. Agranoff, B. W. and P. D. Klinger. Puromycin effects on memory fixation in the goldfish. *Science* **146:** 952–954, 1964.
3. Altman, P. L. and D. S. Dittmer. *Growth: Including Reproduction and Morphological Development.* Washington: Federation of American Societies for Experimental Biology, 1962.
4. Appleman, M. M. and R. G. Kemp. Puromycin: A potent metabolic effect independent of protein synthesis. *Biochem. Biophys. Res. Commun.* **24:** 564–568, 1966.
5. Barondes, S. H. and H. D. Cohen. Puromycin effects on successive phases of memory storage. *Science* **151:** 594–595, 1966.
6. Braud, W. G. Extinction in goldfish: facilitation by intercranial injection of RNA from brains of extinguished donors. *Science* **8:** 1234–1236, 1970.
7. Burkhalter, A. Effects of puromycin on cholinesterase activity of embryonic chick intestine in organ culture. *Nature* **199:** 598–599, 1963.
8. Cohen, H. D., F. Ervin and S. H. Barondes. Puromycin and cycloheximide: different effects on hippocampa electrical activity. *Science* **154:** 1557–1558, 1966.
9. Deutsch, J. A. and N. I. Wiener. Analysis of extinction through amnesia. *J. comp. physiol. Psychol.* **69:** 179–184, 1969.
10. Flexner, J. B. and L. B. Flexner. Puromycin: effect on memory of mice when injected with various cations. *Science* **165:** 1143–1144, 1969.
11. Flexner, J. B., L. B. Flexner and E. Stellar. Memory in mice as affected by intracerebral puromycin. *Science* **141:** 57–59, 1963.
12. Gambetti, P., N. K. Gonatas and L. B. Flexner. The fine structure of puromycin-induced changes in mouse endorhinal cortex. *J. cell. Biol.* **36:** 379–390, 1968.
13. Gambetti, P., N. K. Gonatas and L. B. Flexner. Puromycin action on neuronal mitochondria. *Science* **161:** 900–902, 1968.
14. Huber, H. and N. Longo. The effect of puromycin on classical conditioning in the goldfish. *Psychon. Sci.* **18:** 279–280, 1970.
15. Mayor, S. J. Memory in the Japanese quail: effects of puromycin and acetoxycycloheximide. *Science* **166:** 1165–1167, 1969.
16. Potts, A. and M. E. Bitterman. Puromycin and retention in the goldfish. *Science* **158:** 1594–1596, 1967.
17. Swanson, R., J. L. McGaugh and C. Cotman. Acetoxycycloheximide effects on one-trial inhibitory and avoidance learning. *Communs. Behav. Biol.* **4:** 239–245, 1969.