*MEMORY FIXATION IN THE GOLDFISH**

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Memory can be disrupted by various physical or chemical agents administered shortly after training, but within minutes to hours it becomes insusceptible to these agents.[1–3] This change in susceptibility to disruption suggests that memory becomes fixed after training. Correlations between changes in brain metabolism and deficits in fixed memory produced by various agents may provide insights into the biochemical basis of memory formation. Puromycin, an antibiotic compound which inhibits protein synthesis, blocks memory in mice[4] and in goldfish[5, 6] when given after training. The extent and duration of inhibition of protein synthesis in goldfish brain produced by intracranial injections of different amounts of puromycin has been studied.[7] In the present investigations we measured the memory deficits obtained by injecting different amounts of puromycin at different times after training. We have also found evidence which suggests that puromycin specifically disrupts the fixation of memory.

Materials and Methods.—The training procedure and apparatus have been described.[5] Goldfish were placed in individual shuttle boxes in which light was paired with repetitive electrical shock (0.2 sec of shock of 3 vac, 0.1 ma, at the rate of 40/min). To avoid the shock, fish had to swim over a hurdle from the light to the dark end of the box. The trial cycle was 20 sec of light alone, 20 sec of light paired with the shock, followed by 20 sec of darkness. A correct response was scored when the fish swam over the hurdle before the onset of the shock. All fish were given 20 trials in a 40-min session on day 1 of an experiment and 10 trials in a 20-min session on day 4. Intracranial (IC) injections of 10 μl of saline or of puromycin dihydrochloride (Nutritional Biochemicals Corp.) in 10 μl of saline were made with a 30-gauge needle. The solutions were injected into the cranial cavity over the tectum at specified times on day 1. On both days 1 and 4, fish were placed in the shuttle boxes in darkness 5 min before the first trial. The trials were given in blocks of 5 separated by 5 min rest in darkness. Six fish were run simultaneously in individual shuttle boxes, and responses were recorded by direct observation.

Measurement of memory: Memory is inferred from an increase in correct responses between blocks of 10 trials. In the development of procedures in earlier work, we evaluated differences in memory between groups of fish by comparing the mean day-4 scores. We subsequently found an alternate method based on a regression analysis of day-1 scores on day-4 scores for uninjected (control) fish. The *retention score* of a fish is obtained by subtracting the score it "achieved" (A) on day 4 from the score which was "predicted" (P) for that fish from its day-1 score. The *retention score* of a group of fish is evaluated by a t-test of the achieved and predicted (A vs. P) scores. Discrepancies in the results of the two methods have been minor, but the *retention scores* of groups of fish have tended to give the most consistent measures of memory.

Results.—Effect of different amounts of puromycin given immediately after the trials on day 1: Groups of fish were given IC injections of 10–210 μg of puromycin within

5 min after the 20 trials on day 1 (Table 1). On day 1, all the groups showed typical, significant increases in responses between trials 1–10 and 11–20. Analysis of variance indicated, however, that the groups differed in their responses in the two blocks of trials. These differences presumably reflect variations in levels of responding between fish, which could contribute to differences between the groups on day 4. The *retention score* (A minus P), which compares the change in responses from day 1 to day 4 of fish to the change found for control fish, is used to measure memory on day 4.

The decrease in *retention scores* with increasing doses of puromycin (Fig. 1) is confirmed by analysis of variance ($F = 8.71$, $df = 5/194$, $p < 0.01$). The t-tests of A vs. P (Table 1) indicate that memory was not disrupted by 10 to 50 μg of puromycin, while 90–210 μg produced memory deficits. The groups given 90 or 130 μg scored significantly higher ($p < 0.01$, $p < 0.05$, respectively) in the trials on day 4 than they did in their first 10 trials on day 1. They therefore retained some avoidance learning. The groups given 170 or 210 μg, however, achieved scores on day 4 which were statistically equivalent to their scores in the first 10 trials on day 1: they appeared to have complete memory deficits.

Effect of 170 μg of puromycin given at different times after the trials on day 1: Groups of 35 fish were given IC injections of 170 μg of puromycin at either 30, 60, or 90 min after the trials on day 1. Figure 2 includes the data for fish given 170 μg of puromycin 1 min after the trials (Table 1) and data for fish given 90 μg at different times up to 6 hr after the trials.[6] The 170-μg dose was significantly more effective ($p < 0.01$) when injected 1 and 30 min after the trial. Neither dose had a significant effect when injected 60 min or more following trial 20, indicating that the action of puromycin is temporary, or that it does not cause an enduring disablement which might prevent fish from performing the avoidance response on day 4.

Effect of puromycin given prior to the trials on day 1: Memory of the avoidance response during the 20 trials on day 1 was inferred from the increased number of correct responses between the first and second 10 trials. Two experiments were performed to determine whether puromycin given before the trials interferes with this memory. Such experiments seemed feasible because we had observed that fish showed no sluggishness or locomotor disability following an IC injection of puromycin. In the first experiment, three groups of fish were given injections of saline, 90 μg of puromycin, or 170 μg of puromycin 1 min before the 40-min per-

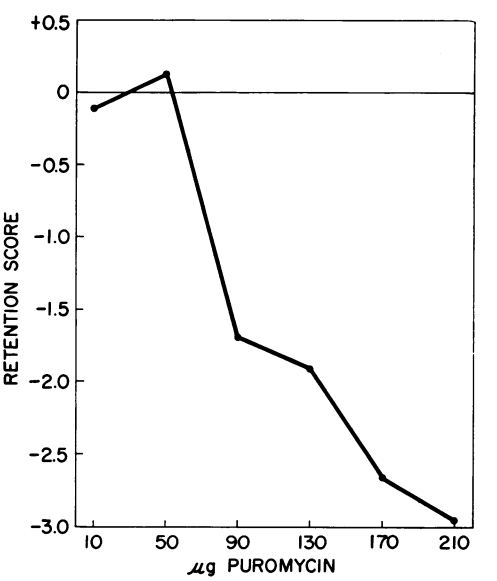

FIG. 1.—The effect on memory measured on day 4 of different amounts of puromycin administered immediately following the trials on day 1. The *retention scores* obtained with 90 μg or more of puromycin represent significant memory deficits; 170 and 210 μg produced complete memory deficits.

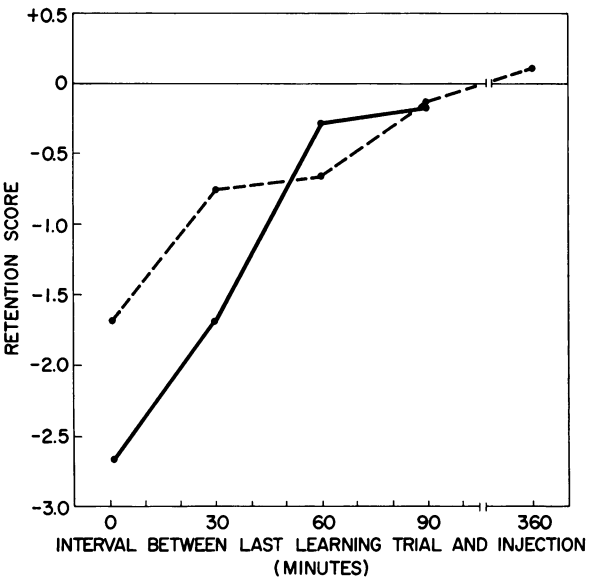

FIG. 2.—The effect on memory measured on day 4 of 90 μg (dashed line; $F = 4.52, df = 4/178, p<0.01$) and 170 μg (solid line; $F = 11.77, df = 3/137, p<0.01$) of puromycin injected at different times following the trials on day 1. The *retention scores* obtained with injections immediately or 30 min after the trials, of 90 or 170 μg of puromycin, represent significant memory deficits; injections 60 min or more following the trials did not produce significant memory deficits.

iod of trials on day 1. In the second experiment, three groups of fish were given similar injections 20 min before the training period on day 1. In both experiments all groups showed significant increases in correct responses between the two blocks of 10 trials on day 1 (Table 2). Moreover, analysis of variance of the scores on day 1, for the six experimental groups and an uninjected control group, revealed that for both the first and second 10 trials the groups had equivalent levels of responding. A similar over-all analysis of variance of the scores on day 4 indicated significant differences between the groups. There was a significant difference among the groups

TABLE 1

EFFECT OF DIFFERENT AMOUNTS OF PUROMYCIN ON MEMORY WHEN INJECTED 1 MIN AFTER THE TRIALS ON DAY 1

Dose (μg)	N	Trials: Day 1 1–10	Day 1 11–20		Day 4 Achieved (A)	Day 4 Predicted (P)*	A vs. P	Retention score ($A - P$)
10	17	2.6	4.5	$p < 0.01$	5.7	5.8	ns	−0.1
50	24	1.5	2.3	$p < 0.05$	4.7	4.5	ns	+0.2
90†	59	1.6	3.4	$p < 0.01$	3.3	5.0	$p < 0.01$	−1.7
130	29	2.3	3.9	$p < 0.01$	3.4	5.3	$p < 0.01$	−1.9
170	36	2.5	3.8	$p < 0.01$	2.7‡	5.4	$p < 0.01$	−2.7
210	35	3.2	4.4	$p < 0.01$	2.9‡	5.8	$p < 0.01$	−2.9
Control†	72	2.3	3.4	$p < 0.01$	5.3	5.3	ns	0
F ratio (over-all)		$F = 3.21$ $df = 6/265$ $p < 0.01$	$F = 2.16$ $df = 6/265$ $p < 0.05$		$F = 8.18$ $df = 6/625$ $p < 0.01$			

* $P = 3.65 + 0.3\,x$ (total correct responses in 20 trials on day 1).
† Data from previous experiments.[6]
‡ Not significantly different from score for trials 1–10 on day 1.

TABLE 2

EFFECT OF PUROMYCIN ON MEMORY WHEN INJECTED PRIOR TO THE TRIALS ON DAY 1

		Trials						
		Day 1			Day 4			
	N	1–10	11–20		Achieved (A)	Predicted (P)	A vs. P	Retention score (A − P)
Injections 1 min before trials:								
Saline	42	3.3	4.5	$p < 0.01$	6.1	6.0	ns	+0.1
90 μg Puromycin	42	2.7	4.5	$p < 0.01$	4.8	5.7	$p < 0.02$	−0.9
170 μg Puromycin	39	3.0	3.8	$p < 0.05$	3.3*	5.5	$p < 0.01$	−2.2
Injections 20 min before trials:								
Saline	41	2.3	3.9	$p < 0.01$	5.7	5.4	ns	+0.3
90 μg Puromycin	40	2.7	3.4	$p < 0.05$	5.6	5.4	ns	+0.2
170 μg Puromycin	35	2.4	3.3	$p < 0.02$	4.2	5.2	$p < 0.02$	−1.0
Uninjected control								
	72	2.3	3.4	$p < 0.01$	5.3	5.3	ns	0
F ratio (over-all)		$F = 1.15$ $df = 6/304$ ns	$F = 1.74$ $df = 6/304$ ns		$F = 5.84$ $df = 6/304$ $p < 0.01$			

* Not significantly different from score for trials 1–10 on day 1.

injected 1 min before the day-1 trials ($F = 14.25$, $df = 2/120$, $p < 0.01$) and among those injected 20 min before ($F = 3.46$, df 2/113, $p < 0.05$). In the first experiment both groups given puromycin achieved significantly lower scores on day 4 than the group given saline ($p < 0.01$), and the fish given 170 μg of puromycin scored lower than the group given 90 μg ($p < 0.01$). Comparison of the day-4 scores to the scores for the first 10 trials on day 1 indicated that the 90-μg dose caused a partial memory deficit while 170 μg caused a complete deficit. In the second experiment, the groups given saline or 90 μg of puromycin achieved equivalent scores on day 4. The group given 170 μg of puromycin, however, scored significantly lower ($p < 0.05$) than the group given saline, indicating a partial memory deficit.

The *retention score* (*A* minus *P*) would not be a valid measure of memory on day 4 in this kind of study if the injections altered the regression of day-1 scores on day-4 scores. The similarity of the day-1 scores of the experimental and control groups suggests, however, that the regression was not altered, and the evaluation of the retention scores (*A* vs. *P;* Table 2) confirms the foregoing analysis of the day-4 scores.

Discussion.—The present studies indicate that the memory which forms during the 20 trials on day 1 is different from the memory detected on day 4. Memory on day 1, which is inferred from the increased responding during the 20 trials, is apparently not disrupted by puromycin (Table 2). Memory on day 4, on the other hand, can be completely or partially blocked by administering puromycin at different times during the hour following trial 20 on day 1 (Fig. 2). The change in susceptibility suggests that memory during the trials on day 1 is temporary, or "short-term," and that it becomes fixed into "long-term" memory, which is detected on day 4. The insusceptibility of the short-term memory to puromycin indicates that the drug acts specifically on the fixation process, in which long-term memory is

formed. While our data indicate that memory fixation becomes insusceptible to puromycin within an hour after training on day 1, it is not evident when fixation begins. The results with puromycin injected before the trials on day 1 suggest that 90 μg of puromycin no longer affects fixation an hour after it is injected, while 170 μg does. Electroconvulsive shock produces deficits in long-term memory when given within 2 hr following trial 20.[6] This could signify that fixation also involves processes not directly affected by puromycin which last longer than an hour.

Most agents which have been found to disrupt memory also temporarily disable the subject. Goldfish given puromycin intracranially maintain normal postures and appear to be as active as uninjected fish. The results obtained with injections before the trials on day 1 substantiate the impression that puromycin does not impair responsiveness. Puromycin has been reported to affect the responsiveness of mice. Drowsiness following subcutaneous injection of puromycin was used as a criterion of an effective administration of the drug.[8]

Puromycin is similar in structure to the amino-acyl end of transfer RNA. It has been shown that puromycin inhibits protein synthesis at the ribosomal site by combining with forming peptides, which causes the premature release of peptidyl-puromycin.[9] It is difficult to correlate the effect of puromycin on protein synthesis in the whole goldfish brain (Fig. 3) with its effect on memory. The behavioral studies suggest that a dose of 90 μg of puromycin can act on memory for less than 60 min and that 50 μg has no effect on memory. These doses, however, suppress the incorporation of H^3-leucine into protein to a similar extent for the first hour after injection. A dose of 170 μg of puromycin may remain effective on memory for slightly more than an hour, yet the H^3-leucine incorporation data indicate that pro-

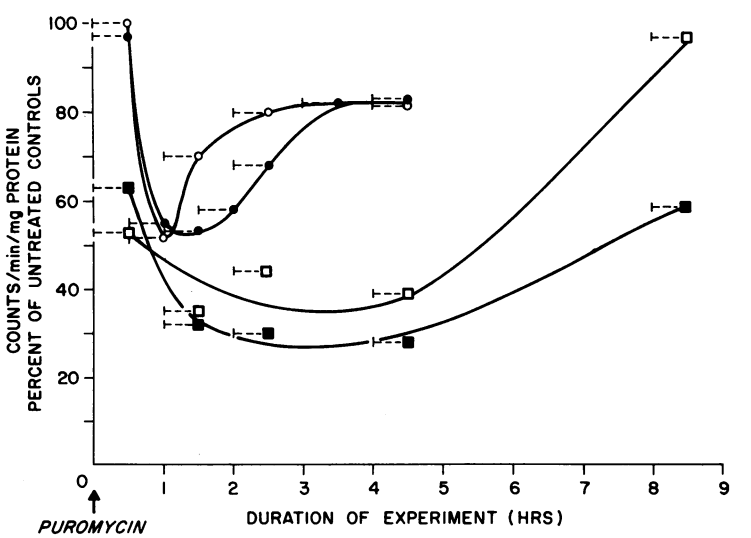

FIG. 3.—Effect of puromycin on incorporation of leucine into protein. In each case puromycin was given at zero time. Groups of five fish each received 20 μc of L-leucine-H^3 intraperitoneally and were sacrificed 30 min later. Brain protein was isolated after successive extractions with cold TCA, hot TCA, ethanol, and ether, and counted. The dotted lines represent the time period during which incorporation of leucine into protein was being measured. Open circles, 50 μg of puromycin; solid circles, 90 μg of puromycin; open boxes, 170 μg of puromycin; solid boxes, 170 μg of puromycin corrected for an apparent increase in specific activity in the soluble fraction.

tein synthesis is substantially depressed much longer. If puromycin disrupts memory fixation by inhibiting protein synthesis, it would appear that synthesis of the relevant proteins escapes inhibition earlier than most protein in the brain. If memory fixation is localized, then the time-course of the inhibition of protein synthesis in the whole brain may not be an adequate indicator of the time-course of puromycin's action on memory fixation. It is noteworthy that the intact puromycin molecule is required to disrupt memory. Puromycin aminonucleoside[5] and methyltyrosine,[10] cleavage products of puromycin, do not produce deficits in long-term memory of the avoidance response.

The concept of short- and long-term memory arose primarily from studies on learning in man.[11] It has been proprosed that short-term memory, like the initial input, is bioelectrical. Our findings in the goldfish are compatible with this premise and, further, they indicate that long-term memory is chemical. Memory fixation may involve protein synthesis and, more generally, growth. If puromycin affects the electrical properties of the brain, the disturbance does not noticeably interfere with the acquisition of the avoidance response on day 1. Other phenomena, such as changes in protein conformation or ionic translocations, may be the mediators of short-term memory.

Summary.—Further investigations on the effect of intracranial injections of puromycin on memory of an avoidance response in goldfish are reported. The magnitude of the deficit in "long-term" memory of the response varies with the amount of puromycin injected; 170 μg effectively obliterates the long-term memory. We propose that puromycin specifically disrupts the formation but not the maintenance of long-term memory, and that temporary, or "short-term," memory is insusceptible to puromycin.

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[1] Gerard, R. W., in *Brain Mechanisms and Learning*, ed. A. Fessard *et al.* (Springfield: C. C Thomas, 1961), p. 21.

[2] Glickman, S. E., *Psychol. Bull.*, **58**, 218 (1961).

[3] Bureš, J., and O. Burešova, *J. Comp. Physiol. Psychol.*, **56**, 268 (1963).

[4] Flexner, J. B., L. B. Flexner, and E. Stellar, *Science*, **141**, 57 (1963).

[5] Agranoff, B. W., and P. D. Klinger, *Science*, **146**, 952 (1964).

[6] Davis, R. E., P. J. Bright, and B. W. Agranoff, *J. Comp. Physiol. Psychol.*, in press.

[7] Brink, J. J., and B. W. Agranoff, submitted for publication.

[8] Flexner, J. B., L. B. Flexner, E. Stellar, G. de la Haba, and R. B. Roberts, *J. Neurochem.*, **9**, 595 (1962).

[9] Nathans, D., these PROCEEDINGS, **51**, 585 (1964).

[10] Unpublished data.

[11] Hebb, D. O., in *Brain Mechanisms and Learning*, ed. A. Fessard *et al.* (Springfield: C. C Thomas, 1961), p. 37.