sensation, even when the attention of the subject is directed to the stimulus. This fact may be taken to indicate that a possible physiological basis could exist for so-called "subliminal perception," at least under the conditions of stimulation employed (in contrast to the conclusion of Schwartz and Shagass, *13*), but this inference requires some clarification. Stimuli which were subthreshold-c, but could still elicit some evoked potential, could be made adequate for conscious sensation by simple repetition at a suitable frequency. It remains to be seen, then, whether responses evoked by subthreshold-c stimuli which are nonrepetitive or of low frequency can play any role in unconscious experience or in behavioral responses to sensory stimuli of which the subject is not aware.

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Actinomycin D Blocks Formation of Memory of Shock-Avoidance in Goldfish

Abstract. *When 2 micrograms of antinomycin D was injected intracranially into goldfish immediately after a training session, the formation of long-term memory of a shock-avoidance was blocked. The results are discussed in relation to similar findings with acetoxycycloheximide and puromycin in the goldfish and with apparently conflicting results in the mouse.*

Goldfish can be trained to swim over a barrier upon a light signal to avoid mild electrical shock administered through the water in a shuttlebox (*1*). They show a significantly increasing probability of avoidance-responding during 20 training trials administered over a 40-minute session, and, on ten retraining trials 3 days later, they demonstrate further improvement in avoidance scores, indicating memory of the training (*2*). Using large numbers of fish and observing responses in six shuttleboxes, we found this paradigm useful in studies on formation of memory. Either of two inhibitors of protein synthesis, puromycin dihydrochloride (170 μg) or acetoxycycloheximide (0.1 to 0.2 μg), blocked memory-formation when injected intracranially immediately after training, but not when injected 1 or more hours later (*3*). We also learned that the decrease of susceptibility to these agents during the period after training (fixation) could be suppressed by manipulating the external environment (*4, 5*). Puromycin injected before training did not affect acquisition and short-term memory but nevertheless blocked formation of long-term memory (*6*). By retraining groups of fish at various times after an immediate injection of puromycin following the trials, short-term memory of the avoidance task was observed to decay over a 2-day interval (*4*). The results suggest that there are two stages of memory in the goldfish: a short-term variety which is not susceptible to puromycin, and a long-term form which also is not susceptible, but whose formation is susceptible to the drug. A relation of the block in memory-formation to the block in protein synthesis is supported by a number of findings. Both puromycin and acetoxycycloheximide block incorporation of leucine-^{3}H into protein in goldfish brain (*7*) at doses that do not depress uridine-^{3}H incorporation into RNA (*8*). Puromycin aminonucleoside and methyl tyrosine, two moieties of puromycin that are not known to block protein synthesis, have no effect on memory-formation (*3*). Also, the vast differences in the doses of puromycin and of acetoxycycloheximide required to block protein synthesis in the goldfish brain extensively are the approximate doses required to block memory-formation. While we believe that these two agents block memory-formation in the goldfish by their roles as inhibitors of protein synthesis, an important question is whether metabolic blocks, other than that of protein synthesis, also block memory. Since we had previously observed that 2 μg of actinomycin D does not markedly block protein synthesis for hours after intracranial injection (*7*), and since actinomycin D is known to block RNA formation selectively (*9*), the effects of

Table 1. Effect of acetoxycycloheximide, injected at various times after training, on the performance on day 4. Acetoxycycloheximide (0.2 μg) in 10 μl of 0.15*M* NaCl was injected intracranially at various intervals after training on day 1. *N*, number of fish; A–P, achieved minus predicted.

Day 4 score	*N*	Hours between training session and injection		
		0	1	3
Achieved	26	1.88	3.54	4.56
Predicted*	27	4.87	5.19	5.18
Retention (A–P)	28	−2.99†	−1.65†	−0.62

* See reference *11*. † $P < .01$.

Table 2. Effect of actinomycin D injected at various times after training on the performance on day 4. Actinomycin D (2 μg) in 10 μl of 0.15*M* NaCl injected at various times after training on day 1. Groups of fish were retrained on day 4 and memory loss was determined as for Table 1. *N*, number of fish; A–P, achieved minus predicted.

Day 4 score	*N*	Hours between training session and injection		
		0	1	3
Achieved	24	2.31	4.07	4.82
Predicted*	26	4.31	4.83	4.67
Retention (A–P)	23	−2.00†	−0.76‡	+0.15

* See reference *11*. † $P < .01$. ‡ $P < .05$.

this agent were studied in the goldfish.

The apparatus and training schedule designated Task III (*10*) differ from previously reported ones in several ways. Light beams to photodetectors flank the barrier and record automatically both escape and avoidance responses. When a fish crosses the barrier, the trial is automatically terminated. A swinging plastic gate, which reduces spurious crossing, is suspended over the barrier and must be deflected by the fish. These modifications do not materially alter the observed raw scores for trials on day 4, but the scores for the first ten trials are reduced so that the ratio of scores for trained fish to those for naive fish in Task III is about 9 : 1 compared to about 2 : 1 in Task I. As in previous studies, loss of memory is estimated by comparing achieved scores with predicted scores obtained by means of a regression equation (*11*). An experiment was performed with acetoxycycloheximide, a drug first demonstrated to block memory in Task I (*3*). The results (Table 1) show that in Task III memory becomes insusceptible (or fixed) to acetoxycycloheximide within 3 hours. A complete memory deficit with this drug has not been seen, although larger doses have not been investigated.

Intracranial injection of over 10 μg of actinomycin D killed our goldfish in several days. Injection of 2 μg of actinomycin D caused a rapid decrease in uridine-^{3}H incorporation into brain RNA (Fig. 1), yet is not lethal for at least 2 weeks. Injection of 2 μg of actinomycin D after training impairs memory-formation, and fixation appears complete within 3 hours (Table 2). Since protein synthesis is not significantly inhibited for several hours after the injection of actinomycin D, we suggest that this drug impairs memory not by blocking protein synthesis but by some other means, presumably by its well-known role in blocking DNA-mediated RNA synthesis. An inhibition in protein synthesis that follows inhibition of RNA synthesis has also been observed in vivo in rat brain following injection of actinomycin D (*12*). It might reflect protein synthesis that is dependent on rapidly turning-over messenger RNA, or a decrease in ribosomal RNA. Preferential inhibition of ribosomal RNA has been reported under conditions of partial inhibition of RNA synthesis by actinomycin D (*13*).

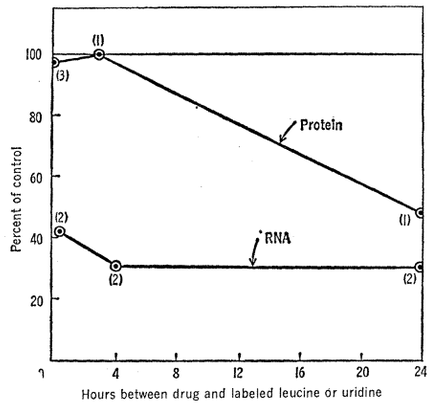

Fig. 1. Effect of intracranial injection of 2 μg of actinomycin D on incorporation of leucine-^{3}H into protein and on incorporation of uridine-^{3}H into RNA. The data on leucine-^{3}H incorporation are similar to a previous study (*7*). For protein synthesis, groups of 30 fish were each injected intraperitoneally with 10 μc of leucine-^{3}H and were killed 30 minutes later. Radioactivity in the trichloroacetic acid supernatant fraction and precipitate were compared with that of control groups. In the RNA studies, 5 μc of uridine-^{3}H was injected intracranially into each of a group of ten fish; 35 minutes later brains were removed and pooled, and a trichloroacetic acid precipitate was obtained, extracted, and then hydrolyzed with 1*N* KOH by use of a magnetic stirrer (*20*). The ratio of radioactivity released by the alkaline treatment to that in the trichloroacetic supernatant fraction was compared with that of control groups. Numbers of groups for each time point are in parentheses.

These results in the goldfish are of interest in view of recent studies on the formation of memory of maze-training in mice. Puromycin is reported to block memory-formation in the mouse (*14*) while acetoxycycloheximide has been variously reported not to affect memory (*15*), to block the effect of puromycin (*15*), and more recently to cause a temporary (*16*) or a sustained (*17*) loss of memory. Interpretation of the effects of antimetabolites in the mouse have been further complicated by a report that intracerebral injections of physiological saline, up to 2 months after the injection of puromycin, restores memory (*18*). Actinomycin D has been reported not to affect either acquisition or formation of memory in the mouse, although a greater inhibition of RNA synthesis was achieved in the mouse than in the goldfish brain (*17, 19*). The lethal effect of actinomycin D in mice, however, precluded testing of memory several days after training. Hence, a loss of long-term memory may have been obscured by residual short-term memory.

Our experiment with actinomycin D in the goldfish suggests that, in addition to RNA-mediated protein synthesis in the cytoplasm, intact nuclear RNA synthesis is also required during long-term memory-formation. We have by no means excluded the participation of still other metabolic processes in the development of memory.

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