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Effects of Cycloheximide and Puromycin on Learning and Retention in the Cockroach, *P. americana*

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BARRACO, D. A., K. L. LOVELL AND E. M. EISENSTEIN. *Effects of cycloheximide and puromycin on learning and retention in the cockroach,* P. americana. PHARMAC. BIOCHEM. BEHAV. 15(3) 489–494, 1981.—A new one-session T-maze training procedure for cockroaches, in which animals were trained to turn right or left to avoid shock, is described. This paradigm was utilized to investigate effects of protein synthesis inhibiting drugs on learning and retention. Cycloheximide (CXM), which inhibited protein synthesis by over 90% during the training period, did not impair acquisition and did not produce retention deficits at any interval up to 1 day after training. Puromycin (PURO), which inhibited protein synthesis by about 70% during the training period, produced amnesia 5 hr after training, while acquisition was not affected. Thus invertebrates, as well as vertebrates, are susceptible to amnesic effects of puromycin. Although PURO-injected animals showed retention deficits as measured by the number of correct turns, no retention deficit occurred for the behavioral modification consisting of an increase in runway time during the training period. Therefore, PURO appears to show specificity for the different types of longer-term memories that are formed in a training situation.

Cycloheximide Learning Cockroach T-maze training Puromycin Retention Protein synthesis inhibition

PROTEIN synthesis inhibiting drugs have been used extensively to study the molecular basis of learning and retention. These experiments have been performed to test the hypothesis that protein synthesis is necessary for the normal operation of learning and/or memory processes. Antibiotic drugs which inhibit protein synthesis, such as puromycin (PURO), cycloheximide (CXM), and anisomycin, have been shown to produce retention deficits (without affecting acquisition) in rodents, goldfish, and birds when administered before training (for reviews, see [1,4]). The impaired retention is often interpreted as support for the hypothesis that brain protein synthesis is required for some aspect of longer-term memory consolidation. However, additional factors have been proposed to explain some memory deficits since (a) the amount of amnesia seen is not always directly correlated with the degree of protein synthesis inhibition when different drugs or species are compared, (b) the drugs have many physiological effects other than protein synthesis inhibition, and (c) CXM and PURO may affect retrieval as well as consolidation processes.

Very few experiments have investigated effects of drugs on learning and memory in invertebrates. Phylogenetic comparisons in this area can be important for two reasons: (a) to help distinguish between mechanisms which are common to all animals with a nervous system and those which depend on specific circuitry or other specific characteristics of an animal's nervous system, and (b) to shed light on when in the course of evolution a particular mechanism(s) may have evolved. Thus, if it can be shown that both insects and mammals display many of the same behavioral and pharmacological characteristics of shorter and longer-term memory then it is reasonable to suggest that common mechanisms underlie these phenomena in insects and mammals and further that such mechanisms probably evolved prior to the time when these two classes of animals diverged. Such comparisons allow one to make inferences about the evolutionary course of various mechanisms underlying learning and memory.

The research reported here investigated the effects of the antibiotics CXM and PURO on intact cockroaches trained in a T-maze. The apparatus and procedure, developed in our laboratory, comprise the first T-maze training situation in which invertebrates have been reliably trained in one brief (1 hr) training session. The object of these experiments was to determine if the antibiotics, when injected before training, have disruptive effects on learning or retention in cockroaches. The two types of behavioral changes analyzed were turning behavior (animals learned to turn left or right to avoid shock) and habituation behavior (animals showed an increase in time spent in the maze). In addition, the degree of

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LIST OF ABBREVIATIONS

CXM: cycloheximide
PURO: puromycin

protein synthesis inhibition produced by the drugs during training and during retention tests was measured in order to correlate inhibition levels with behavioral results.

METHOD

Measurement of Protein Synthesis Inhibition

The incorporation of ^{14}C(U)-leucine into protein of the central nervous system was measured to estimate the degree of protein synthesis inhibition produced by CXM or PURO. Adult male animals weighing about 1 g were injected with either 250 μg CXM or 300 μg PURO in 20 μl insect Ringer solution, or with corresponding amounts of Ringer solution, into the hemolymph under the ventral cuticle of the abdomen. Relatively high non-lethal doses were chosen empirically to produce high levels of protein synthesis inhibition. These doses produced no incidence of mortality or obviously abnormal behavior up to 1 week following injection. At various intervals after the injection of the drug or vehicle, 10 μl of ^{14}C(U)-leucine (New England Nuclear Corp.; 270 mCi/mM in 0.01 N HCl) containing 1 μCi was injected. The leucine injection times were chosen to measure the amount of inhibition at times corresponding to training and testing periods. One hr after the leucine injection, the brain and three thoracic ganglia were dissected out and immediately placed in ice cold insect Ringer solution. Incorporation of labelled leucine into ganglionic and brain protein was determined by the method of Kerkut *et al.* [8]. The tissue of five animals was used in each group. After dissection, the tissue was homogenized (using a hand operated glass homogenizer) in 1 ml cold Ringer solution. The homogenate was transferred to a centrifuge tube and the homogenizer was rinsed with 1 ml of Ringer solution. After 3 ml of 10% trichloracetic acid (TCA) solution was added to the tube, the mixture was thoroughly agitated for 30 sec and centrifuged at 3000 G for 10 min. One ml of the supernatant was removed, mixed with 15 ml PCS scintillation fluid (Amersham/Searle Corp.), and counted for 20 min on a standard Nuclear Chicago scintillation counter. The pellet was resuspended in 2 ml of chloroform/methanol (1:1) and centrifuged at 3000 G for 10 min. To the resulting pellet, 1 ml of 1 N NaOH was added and the mixture was put in a waterbath at 100°C for 10 min. The resulting solution was cooled and centrifuged to remove undissolved material. The supernatant was removed and counted for 20 min. Inhibition of protein synthesis was calculated according to the method of Barondes and Cohen [3]. The percentage inhibition was calculated as $(1-R_{CXM}/R_{Sal})\times 100$, where R is the ratio of counts per minute (cpm) of the TCA precipitable fraction to cpm of the TCA soluble fraction.

T-Maze Training

Adult male cockroaches were trained to turn either left or right in a T-maze using electric shock as punishment for an incorrect response. The apparatus was a T-maze consisting of a start box (5.0 cm×3.2 cm×3.8 cm), a runway (15.3 cm×3.2 cm×3.8 cm), two choice arms (7.5 cm×3.6 cm×3.8 cm each) with shock grids on each floor, and two goal boxes (5.5 cm×3.6 cm×3.8 cm) which could be moved from the start box entrance to the choice arm exit (Fig. 1). The two goal boxes, fixed at 90° angles to the ends of the choice arms, were totally opaque and provided small dark enclosures, which attract cockroaches. The entire maze was made of Plexiglas. Manually operated guillotine doors were located at the entrance to each goal box, at the entrance and exit of the start box, at the end of the runway, and at the entrance to each arm. The source of the shock was 60 cycle AC current reduced to approximately 8 V with a variable transformer. The amplitude was set so that the shock caused immediate escape from the incorrect arm, but did not produce convulsions or other obviously erratic behavior.

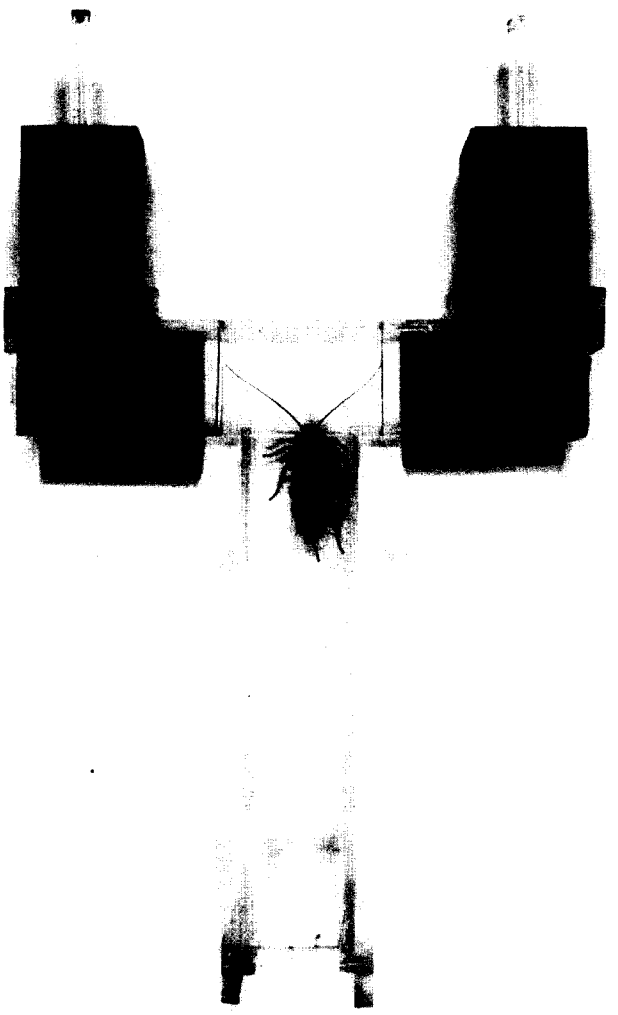

FIG. 1. Top view of the T-maze used for training cockroaches. The floors of the arms of the maze are covered with shock grids. The animal is located at the choice point. The time taken for an animal to proceed from the beginning of the runway to the choice point is the runway time. Details of the training procedure and measurements of the maze are described in the text.

Adult male cockroaches weighing approximately 1 g were obtained from the U.S. Department of Agriculture and housed together with access to dog food and water until the day of the experiment. An animal was then removed from the colony and injected under the abdominal cuticle with 250

TABLE 1

PERCENTAGE INHIBITION OF PROTEIN SYNTHESIS FOLLOWING INJECTION OF PURO OR CXM

Treatment	Dose (μg)	Hours After Injection	Percentage Inhibition
CXM	250	1	98
CXM	250	3	96
CXM	250	7	93
PURO	300	1	49
PURO	300	3	69
PURO	300	9	47

Each group consists of a pooled sample of 20 brain and thoracic ganglia extracted from 5 animals.

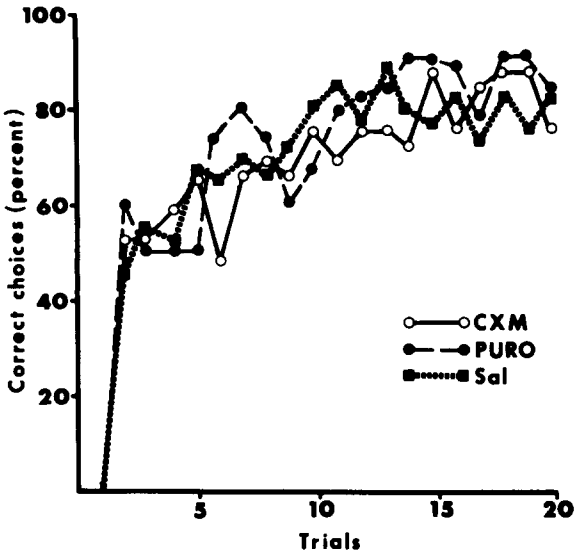

FIG. 2. Learning curve for animals injected with PURO (31 animals, tested at 5 hrs), CXM (32 animals, tested either at 5 min, 1 hr, 5 hr, 22 hr) or saline (55 animals). The percentage of animals making correct choices on each trial is shown. The separate control groups in the PURO and CXM experiments were combined to form the saline group.

μg CXM or 300 μg PURO in 20 μl Ringer solution or with corresponding amounts of the vehicle. Training was started 1 hr after injection for CXM-injected animals and corresponding control animals or 3 hr after injection for PURO-injected animals and corresponding controls (training was delayed for PURO animals since biochemical experiments suggested that PURO-induced protein synthesis inhibition was higher at this time interval). In addition another PURO group was injected 2 hrs after training (3 hrs before testing). During training and testing sessions, the experimenter was unaware of which injection the animal had received.

The training procedure consisted of 20 training trials in which shock was administered in one arm of the maze. An animal was trained to turn opposite to the direction it chose on the first trial, so that it always made an incorrect choice and received shock on the first training trial. At the beginning of a trial, the animal was in the goal box which was placed at the entrance to the start box. The animal was pushed gently into the start box with a plunger. It remained in the start box for 15 sec, after which the door to the runway was raised. If the cockroach did not leave the start box within 20 sec, it was gently prodded. If the animal placed two legs more than 2 cm into the incorrect arm, shock was received and a wrong choice was recorded. The animal immediately left that arm and was allowed to enter the correct arm and attached goal box. After entering the goal box, an animal remained there for 2 min, at which time the goal box was placed at the entrance to the start box and another trial began. At the end of the 20 training trials, cockroaches were kept in individual containers in the dark with access to water for 5 hrs, until the testing session began. The retention test consisted of 10 training trials (for the CXM group) or 20 training trials (for the PURO groups); animals were retrained with shock given on the same side as for initial training.

Two behavioral measures were analyzed: the time taken for an animal to proceed down the runway (runway time) and the actual direction turned (turning behavior). In the analysis of data, saline injected animals used as controls in both the CXM and PURO experiments were combined into one control group. A few animals were trained but not tested and this data was included in the training results (Fig. 2). For statistical analysis, the number of correct turns made by animals in each block of 10 training or testing trials was determined (as in Table 2). The distributions of number of correct responses for treated and control groups were compared by means of a 2-tailed Mann-Whitney U test. Similarly, a Mann-Whitney U test was used to compare the mean runway times determined for blocks of 10 trials (as in Fig. 3).

RESULTS

Inhibition of Protein Synthesis

Table 1 shows the amount of protein synthesis inhibition produced by 250 μg CXM or 300 μg PURO at several intervals after injection. These data demonstrate that the dose of CXM used in the T-maze training experiments produced inhibition of greater magnitude than the dose of PURO. CXM produced over 90% inhibition for at least 1–7 hr after injection. In contrast, 300 μg PURO produced a maximum of about 70% inhibition. Since we trained animals 3 hr after PURO injection, the 9 hr interval in Table 1 corresponds to the time of retention testing for PURO-injected animals (i.e., injected 3 hr prior to training + 1 hr of training + 5 hr delay before testing). Similarly, the 7 hr interval for CXM-injected animals in Table 1 also corresponds to the time of retention testing since cockroaches receiving CXM were trained 1 hr following injection.

Effects of CXM and PURO on Turning Behavior Learning and Retention

When trained to turn left or right in the T-maze to avoid shock, cockroaches reached a criterion of about 83% correct in one training session of 20 trials (Fig. 2). Saline-injected animals required a mean of 11.3 trials to reach a criterion of 5 correct out of 6 trials (5/6 correct). Thus, cockroaches learned this task relatively quickly and T-maze training can be reliably used as a one-session learning procedure for cockroaches.

Figure 2 shows a comparison of learning curves for cockroaches injected before training with saline (55 animals),

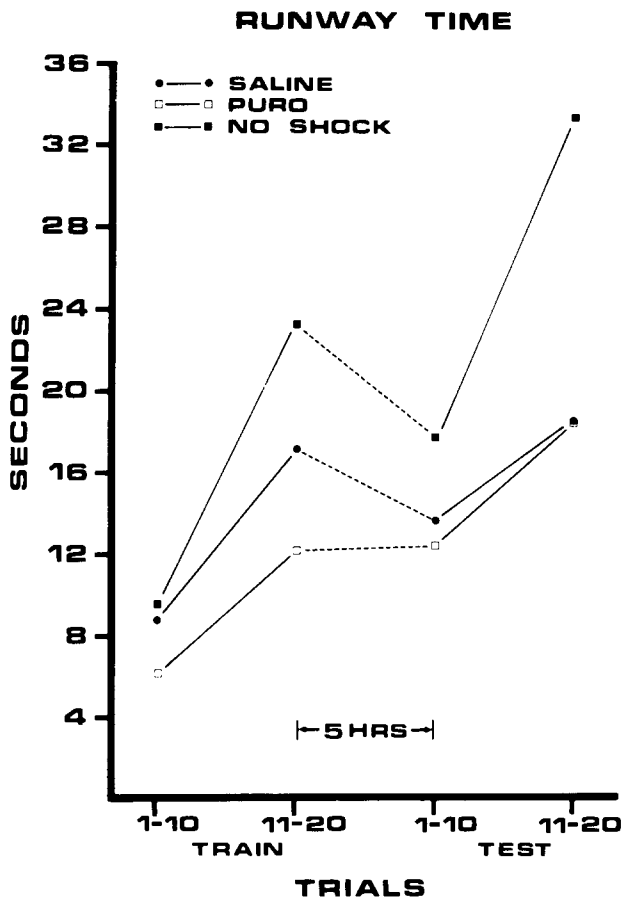

FIG. 3. Mean runway time for the first and second 10 trials of training and testing. There were 31 animals in the PURO group, 23 animals in the saline group, and 6 animals in the no-shock group. The 23 saline animals represent those run as a control for the PURO group. All three groups show a significant increase between training trials 1–10 and 11–20 ($p<0.05$). There is no significant decrement in any group between training trials 11–20 and testing trials 1–10 ($p>0.25$), indicating significant retention of this behavioral modification.

CXM (32 animals) or PURO (31 animals). There was no apparent difference in the characteristics of learning among the three groups. Thus, neither CXM nor PURO cause impairment of T-maze learning. Consequently, neither drug impairs the shorter-term memory processes necessary for continued improvement during the 1 hr training period.

The number of correct turns during training and retention trials for animals tested 5 hr after training are shown in Table 2. There is a significant difference ($p<0.05$), between the first 10 trials of training and the first 10 trials of testing for control animals, indicating that good retention exists at this interval. CXM injection (which caused over 90% inhibition of protein synthesis) did not produce a significant difference between the saline and CXM groups (Table 2). In addition, when retention tests were given at other intervals (5 min, 1 hr, 22 hr) following training of CXM-injected animals (data not shown), the retention of CXM groups was as high or higher than control groups at all intervals. Thus, CXM did not produce amnesia. However, PURO injections, which produced substantially less protein synthesis inhibition than CXM, did cause amnesia in animals tested 5 hr after training (Table 2); there was a significant difference ($p<0.05$) between pre-PURO and saline groups during the first 10 testing trials. Although the difference between post-PURO and saline groups for the first 10 testing trials was not significant at the 0.05 level, the values for pre-PURO and post-PURO groups were similar, suggesting that PURO injected after training may also produce retention deficits in cockroaches. The values for testing trials 11–20 indicate that PURO-injected animals reached control levels during the last half of testing, suggesting no impairment in learning ability or performance as a result of drug treatment.

Effects of PURO on Habituation Learning and Retention

Figure 3 shows the time taken by cockroaches to move from the start box to the choice point (runway time). These results indicate that animals injected with PURO were not physically impaired since they ran the maze as fast as control animals. Both groups of animals show the same change in behavior during training, with a progressive increase in runway time with succeeding trials. In order to determine if the increase in runway time resulted from habituation or reflected an association of the runway with the possibility of receiving shock, a group of non-injected animals was given trials in the maze with no shock administered. In all other respects, the "training" and "testing" procedures were identical to those for animals given shock. The "no-shock" group also showed a marked increase in runway time during training (Fig. 3). These results suggest that the increase in runway time observed in the groups receiving shock is due to habituation in the maze and cannot be attributed to an association of the runway with the possibility of receiving shock.

Testing results show retention of this habituation behavior for no-shock, saline and PURO-injected groups (i.e., comparison of training trials 11–20 with testing trials 1–10 does not show a statistically significant decrease in any group). Thus, since PURO produced a retention deficit in the correct turning behavior (Table 2) but did not alter retention of the runway habituation, PURO apparently does not obliterate all the different memories which occur in a given shock avoidance training situation.

DISCUSSION

In the T-maze training procedure described here, an invertebrate species achieved good retention following a single training session. Most previous maze-running invertebrate training paradigms utilized multiple training sessions spaced over several days and were not as suitable for investigation of the effects of drugs on learning and retention. The procedures used here are similar to those used in T-maze training paradigms for vertebrates and allow a more direct phylogenetic comparison of learning and memory characteristics.

The present experiments demonstrated that neither CXM nor PURO impaired shock avoidance learning of cockroaches trained in a T-maze. Previous research utilizing intact cockroaches trained to lift a leg to avoid shock also showed that CXM did not produce an acquisition impairment [10]. Similarly, the absence of an antibiotic-induced learning impairment has been observed in many experiments with vertebrates.

During a training session of more than one trial, operation of a shorter-term memory component is necessary for con-

TABLE 2

NUMBER OF CORRECT TRIALS DURING TRAINING AND TESTING OF ANIMALS INJECTED WITH SALINE, WITH CYCLOHEXIMIDE 1 HR BEFORE TRAINING (CXM), WITH PURO 3 HR BEFORE TRAINING (Pre-PURO) OR WITH PURO 2 HR AFTER SHOCK AVOIDANCE TRAINING (Post-PURO)

	Number of Correct Trials			
	Training Trials		Testing Trials	
Treatment (n)	1–10	11–20	1–10	11–20
Saline (23)‡	5.5 ± 0.3*	7.6 ± 0.4	7.3 ± 0.2	8.2 ± 0.2
Pre-PURO (31)	5.5 ± 0.2	7.7 ± 0.2	6.3 ± 0.3†	8.1 ± 0.3
Post-PURO (10)	5.1 ± 0.4	7.3 ± 0.4	6.4 ± 0.4¶	8.0 ± 0.3
Saline (8)§	5.2 ± 0.4	7.6 ± 0.5	7.8 ± 0.4	—#
CXM (8)	5.4 ± 0.3	7.8 ± 0.4	8.0 ± 0.4	—#

*Mean ± S.E.M.
†Significantly different from corresponding control group ($p<0.05$).
‡This represents the 23 animals run as controls for the PURO experiments.
§This represents the 8 animals run as controls for the CXM experiments.
¶The pre- and post-PURO groups do not differ from each other.
#The CXM group and corresponding control group were given only 10 testing trials.

tinued improvement. Since PURO does not affect improvement during the entire 1 hr training period, the drug apparently does not impair shorter-term memory, although it causes a retention deficit 5 hr later. This suggests that there are separate shorter-term and longer-term memory components in cockroaches with different susceptibilities to disruption, a phenomenon which has been repeatedly observed in vertebrates (see [4]). Other experiments also have supported the existence of separate shorter- and longer-term memory components in insects and cephalopods [2, 5, 9, 11] with the transition between shorter- and longer-term phases occurring within a few hours after training when one training session was used.

Previous reports of sickness induced in PURO-injected rodents may suggest the possibility that the PURO-injected cockroaches performed poorly on retention tests due to sickness. However, several lines of evidence indicate that sickness did not contribute to the retention deficit of the PURO-injected animals: (a) in toxicity studies, the dose of PURO used caused no deaths or obvious behavioral or locomotor abnormalities up to 7 days after injection, when observations were discontinued; (b) activity levels in the runway were similar for both saline and PURO groups (Fig. 3); (c) during training the PURO-injected animals learned as well as saline-injected animals (Fig. 2); (d) during testing the PURO-injected animals showed the same improvement during the 20 testing trials as saline animals did during initial training. If the PURO animals had performed at chance levels during the first 10 testing trials due to sickness, the performance level should not have improved during the second half of the testing period to the same level as the saline control animals (Table 2). Also, in view of the fairly high level of protein synthesis inhibition maintained throughout training and testing, it is unlikely that state dependent changes could account for the retention deficit observed in the PURO group.

Thus the experiments with PURO indicate that memory processes in cockroaches are susceptible to manipulation by an antibiotic, i.e., retention deficits can be produced in cockroaches by pretraining injections of PURO, as has been observed in vertebrates. In view of this phylogenetic similarity, it is not clear why CXM had no effect on retention. Since overtraining can prevent the occurrence of CXM-induced amnesia in vertebrates [6,13], the apparent stability of memory in the cockroach during inhibition of protein synthesis by CXM also may be due to overtraining. This hypothesis is supported by preliminary results obtained in our laboratory. Cockroaches given CO_2 following 20 training trials of shock avoidance in a T-maze failed to show any retention decrement 5 hr after training. However, when CO_2 was administered after 12 training trials, no evidence of retention was observed 5 hr later even though excellent retention was observed in a control group given 12 training trials without CO_2 administration. One interpretation of these results is that increasing the training schedule from 12 to 20 trials permits the establishment of longer-term memory which is not susceptible to disruption by CO_2 or CXM.

The possibility that overtraining may have prevented the production of amnesia in CXM-treated animals does not explain why an agent such as PURO, producing substantially less protein synthesis inhibition than CXM, is capable of producing retention deficits while CXM does not. These results suggest that some other physiological effect of PURO other than overall protein synthesis inhibition would appear to be responsible for impairment of longer-term memory. Possibilities include (a) specific proteins which are affected differently by CXM and PURO; (b) peptidyl-PURO fragments (PURO attached to an incomplete prematurely released peptide chain); (c) other biochemical or physiological effects of PURO, e.g., effects on neural conduction or synaptic transmission processes.

The most likely explanation for the increased runway time in all groups is general habituation. It is a well-known phenomenon that animals will exhibit exploratory behavior when placed in a novel environment. As the animal becomes acclimated to the situation exploration declines and activity levels decrease resulting, in this case, in longer runway times with succeding trials. In these experiments, PURO did not

affect the occurrence of habituation nor the retention of the habituation behavior 5 hr after training. This inability of PURO to affect cockroach maze habituation has been supported by results showing that neither acquisition nor retention of habituation of arm entries in a Y-maze by cockroaches (N=6) are influenced by the same dose of PURO used in the present experiments (Lovell and Eisenstein, unpublished). Thus, PURO causes a retention deficit in correct turning behavior but no retention deficit in runway habituation behavior.

Differential effects of PURO on the different memories that accompany a training situation have also been observed in goldfish [14] and in rats [7]. In goldfish, PURO injected just before or immediately after a training session in a shuttle box did not block retention of conditioned cardiac deceleration although it produced a retention deficit of the shock-avoidance response. Schoel and Agranoff [14] hypothesized that the more neurally complex a learned task is (i.e., more synapses involved), the more susceptible it is to interference by amnesic agents, such as PURO. They suggested that avoidance conditioning is more susceptible to an amnesic agent than is conditioning of an autonomic response simply because of its more complex neural circuitry. Thus, it is reasonable to suggest that in the cockroach the act of making a choice to turn right or left may be mediated at a higher level in the central nervous system and involve a more complex neural circuit than that mediating runway habituation behavior. Such a difference may underlie differences in susceptibility to the action of PURO.

PURO has been shown to cause amnesia of at least some longer-term memories in insects, fish, birds and mammals despite differences in neural organization. In addition, very similar maze behaviors have been observed in insects and mammals. For example, the phrase "vicarious trial and error (VTE)" was first used by Meunzinger [12] to describe the behavior of the white rat at a choice point in a T-maze in which the rat stopped and turned its head back and forth several times before making its turning choice. He related this behavior to the decision-making process. Such behavior may have a behavioral analogue in the cockroach which also stopped at the choice point and turned its antennae and head back and forth before making a choice. Thus, our experimental results and observed behavioral similarities in the insect and mammal raise the possibility that their underlying learning and memory processes share many common features and suggest that the fundamental mechanisms of memory storage may have evolved several hundred million years ago, before divergence of the phyla that these classes represent.

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