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Temporal and Pharmacological Parameters of Puromycin-Induced Amnesia

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BARRACO, R. A. AND K. E. FRANK. *Temporal and pharmacological parameters of puromycin-induced amnesia.* PHARMACOL BIOCHEM BEHAV 18(5) 809–815, 1983.—Mice were trained in step-down passive avoidance behavior. Bitemporal injections of puromycin (PM) were given either immediately or delayed until 24 hrs after training. PM produced a marked amnesia in both cases during retention testing 3 days later. The amnesia persisted during a second retention test 6 days after training. Of all the antibiotics, only PM is effective as an amnestic agent when injections are delayed 24 or more hours after training. Cycloheximide (CXM) was also injected bitemporally immediately after training. However, CXM produced a weaker amnestic effect even though it produced a much greater inhibition of cerebral protein synthesis, more rapidly, and of longer duration. In an effort to attenuate the amnesia produced by PM, in separate experiments, the mice were injected with combined injections of PM and CXM (bitemporally); mice were also given combined injections of PM (bitemporally) and amphetamine (subcutaneously). The amnesia produced by immediate injections of PM was not attenuated by either CXM or amphetamine. However, the amnesia produced by delayed injections of PM was attenuated by both CXM and amphetamine. These results suggest that delayed injections of PM (24 hours after training) block the expression or retrieval of memory. This study also supports the contention that puromycin has two separate effects on memory with different temporal parameters depending on when the drug is injected relative to initial training.

Memory Puromycin Temporal gradients Passive avoidance Mice Cycloheximide

IT has been almost two decades since the Flexners published their germinal report describing the amnestic effects of puromycin on memory [10]. This began a large series of studies by many investigators using antibiotics and other antimetabolic agents to investigate the neuromolecular mechanisms involved in memory and learning. The initial interest in antibiotics as amnestic agents stemmed from their effects on protein synthesis; they were viewed as an avenue for exploring various hypotheses concerning the role of protein synthesis in memory formation. As a result, many other inhibitors of protein synthesis in addition to puromycin have been subsequently used to impair memory [2, 5, 22].

Three classes of inhibitors of protein synthesis have been widely used in memory-disruption experiments: puromycin (PM), the glutarimides (cycloheximide (CXM) and acetoxycycloheximide (AXM), and, more recently, anisomycin (ANI). Each of these agents has been found to be effective in producing experimental amnesia within dose ranges that are not significantly toxic to the animal. Although a factor common to each is the ability to block protein synthesis, the biochemical mechanisms for protein synthesis inhibition differ for these drugs in a consequential manner. For example, the glutarimides and anisomycin are inhibitors of chain elongation; puromycin interferes with mechanisms involved in translation. Each of these agents may have other biochemical effects as well. In fact, it has been proposed that the behavioral consequences of the various antibiotics result from the unique and separate mode of action of a particular drug on overall cellular metabolism and that protein synthesis inhibition per se is not the crucial correlate of their behavioral effects [2].

In memory disruption experiments, the temporal variation in effectiveness of an amnestic treatment is interrelated in an important manner with the interpretation (both psychological and physiological) of its effects on memory processes. The word amnesia implies that memory processes have been disrupted. To account for deficits during retention performance, two types of interpretation have been enunciated: either the memory no longer exists or the memory cannot be adequately retrieved [22,27]. For the consolidation-interference explanation, the view is taken that an amnestic treatment such as electroconvulsive shock (ECS), affects some time-dependent associative process since the effectiveness of this treatment in altering retention performance decreases as the training-treatment interval increases [22]. In this case, the amnestic treatment must occur in close temporal contiguity to acquisition training to be effective in disrupting memory. For the retrieval-interference explanation, the view is taken that an amnestic treatment impairs some retrieval process involved in recall; the memory is present but not accessible during retention testing. Impairment of retrieval processes can be substantiated when a given memory is recovered, either spontaneously or through various recovery and/or amnesia-attenuation treatments.

A large number of studies indicate that the behavioral effects of CXM, AXM and ANI on memory conform to a consolidation-interference explanation of memory disruption

(even though it is known that the amnestic effects of these drugs are attenuated by a variety of pharmacological and/or behavioral manipulations). Thus the memory-impairing effects of these drugs are generally correlated with the temporal parameters of protein synthesis inhibition and the training-treatment interval [2]. CXM and AXM are usually effective as amnestic agents when injected five minutes to five hours before training [2, 4, 12, 20, 24], marginally effective or ineffective when injected immediately after training [2, 3, 14, 24, 29], and completely ineffective when injected one hour or more after training [2, 28, 31]. Further, the duration of cerebral protein synthesis inhibition produced by anisomycin has also been found to be highly correlated with the magnitude of its amnesic effects [16]. Data from our laboratory are also consistent with these observations since CXM injected immediately after one session of visual discrimination training in pigeons produces a partial amnesic effect [29], whereas CXM was ineffective as an amnesic agent when injected immediately following swim escape training in 30 day old rats [3].

In contrast to the clear time-dependent effects of these antibiotics on memory, the situation for puromycin is very different. With the exception of goldfish, the data available thus far provide no support whatsoever for such a critical temporal period (i.e., training-treatment interval) for memory impairment by PM injections [2]. Thus the temporal parameters of protein synthesis inhibition are not correlated with PM-induced retention deficits. Puromycin injections impair retention when they are made before training, immediately after training, and when they are delayed until 24 or more hours after training. This 24-hour delayed effect has been found repeatedly [15, 18, 25, 26, 30]. Flexner *et al.* [15] even found evidence of amnesia when injections were made as long as several weeks after discriminated shock-avoidance training if injections were dispersed over a wide locus of intracranial sites.

These studies suggest that PM exerts its effects on memory in a manner unlike any other amnestic treatment. The effectiveness of PM injections can be dissociated from the temporal parameters of protein synthesis inhibition and the training-treatment interval. Our studies with pigeons also show a lack of correlation between the overall pattern of cerebral protein synthesis inhibition and the effects of PM injections on behavior. For example, PM produces a much greater amnestic effect than CXM on visual discrimination learning when injected immediately after the initial training session, even though protein synthesis inhibition is more rapid and severe after CXM administration. Moreover, PM produces an effect on subsequent acquisition training to criterion, whereas there is no tendency whatsoever for CXM to produce such an effect [29]. This effect on subsequent acquisition training persists even when PM injections are delayed until 24 hrs after initial training [20].

Thus, it is possible that PM not only exerts a consolidation-type effect on memory but it also may block the subsequent expression of memory. The question arises whether PM has two separate behavioral effects with different temporal parameters (i.e., immediate vs. delayed injections) working through two distinct physiological mechanisms. For example, when PM injections are delayed (i.e., 24 hrs or more after training), the subsequent amnesia can be reversed by a variety of pharmacologic agents, whereas amnesia from immediate injections cannot [2]. It has been repeatedly demonstrated that when PM injections are delayed, memory can be restored wtih bitemporal injections of saline [6, 7, 13, 15, 26], by a variety of stimulant drugs, by neuropeptides such as ACTH, vasopressin, and their analogs when these agents are administered prior to training [2, 8, 9, 11, 18, 25], and, more interestingly, by concurrent administration of cycloheximide [12]. Taken together, these studies indicate that PM, if injected long after training, when presumably consolidation has occurred, prevents the retrieval or expression of memory.

In contrast to the findings concerning delayed PM injections, there is no evidence of memory restoration after immediate PM injections [2]. For example, the amnesia produced by injections of PM immediately after training is not reversed by saline injections [13]. Moreover, in goldfish, concurrent injection of CXM with PM immediately after training also does not block PM-induced amnesia. The same holds true in pigeons where a combined injection of CXM and PM immediately after training attenuated neither the amnesia nor the continued acquisition deficit produced by PM alone [29]. Unfortunately, there is a paucity of studies investigating the attenuation of amnesia produced by injections of PM immediately after training.

Consequently, the purpose of this study was to further evaluate the hypothesis that PM has separate effects on memory with different temporal parameters. We selected a shock-avoidance training paradigm with mice in an effort to correlate our results with the extensive previous literature in this area. The specific objectives of this study were: (1) contrast the amnesic effects of PM and CXM in the same passive avoidance paradigm; (2) to compare the behavioral consequences of immediate and delayed injections of PM; (3) to evaluate the temporal parameters of PM-induced retention deficits through attenuation treatments; and (4) to further explore the role of peptidyl-puromycin in PM's effects on memory by antagonism with concurrent injections of CXM.

METHOD

Animals

Adult male white mice (CF-1 strain) weighing 30–40 g were obtained from Charles River Breeding Company. The animals were housed 3–4 to a cage and given food and water ad lib.

Apparatus

The passive avoidance apparatus consisted of a box made of plywood and clear Plexiglas with internal dimensions of 36×28×23 cm high to the grid floor. The grid floor consisted of 22 parallel steel rods 0.84 cm in diameter set 1.27 cm apart. In the center of the box was a 2.5×7.6×5 cm high wooden platform covered with rough white paper; 3.8 cm below the grid floor was a thick black cardboard tray. The paper on the platform was changed and the cardboard tray was emptied as necessary during the experiment, and before each new experiment the entire apparatus was cleaned with alcohol. The grid floor was wired to a Lafayette Instrument Co. AC shock generator (Model A-615 B) and grid scrambler (Model A-620) which were set to deliver a continuous 0.5 mA footshock when the switch was turned to the on-position by the experimenter.

Procedure

Pretraining. Approximately 24 hrs prior to acquisition training, animals were given a two minute pretraining

(familiarization) period in the step down apparatus. A pretraining trial consisted of placing an animal on the platform in the center of the box and allowing it to explore the platform and/or grid floor for a total of two minutes in the absence of the footshock. Step down latency (SDL), which is the time, in seconds, it takes for the animal to step down off the platform with all four paws onto the grid floor, was recorded and at the end of two minutes the animal was removed from the apparatus and returned to its home cage.

Training (Tr). Acquisition training took place approximately 24 hrs after pretraining. As in pretraining, mice were placed gently onto the platform and step down latencies were recorded. Immediately following descent from the platform, a very brief 0.5 mA scrambled footshock was delivered through the grid floor. After it was determined that the animal had received the shock (the animal was observed to jump, freeze and/or squeak), the footshock was terminated and the animal was removed from the apparatus. Injections followed immediately or 24 hrs later. Any animals with training latencies of 60 sec or greater were not shocked but were removed from the apparatus and excluded from the experiment.

Test (T). Retention was measured as latency to step from the platform to the grid floor. The first retention test took place 72 hrs after training. Animals were placed upon the platform and step down latencies recorded. After stepping down, the animals were removed immediately from the apparatus and returned to their home cages. A second footshock was not given. Animals not responding within 2 minutes were given a maximum SDL of 120 sec and returned to their home cage.

Retest (R). A second retention test was given 72 hrs after Test. The procedure was the same as for Test.

Drugs, experimental groups, and injection procedure. Nine experimental and control groups were used in this study (Table 1). A single experiment was conducted over a 9 day period and usually involved 15–25 animals. Puromycin dihydrochloride (PM) (Nutritional Biochemicals) and cycloheximide (CXM) (Sigma Chemical Co.) were dissolved in 0.85% saline. PM was titrated to pH 7 with 0.1 N NaOH and prepared in a concentration of 110 μg/10 μl (for use in groups 3,4,8 and 9—see Table 1). A single solution of CXM (110 μg/10 μl) was prepared for group 5. A combined solution of PM (110 μg/10 μl) and CXM (110 μg/10 μl) was also prepared and titrated to pH 7 (groups 6 + 7). In addition to intracerebral injections of puromycin (110 μg/10 μl), mice in groups 8 and 9 received single subcutaneous injections (0.2 ml) of d-amphetamine (AMP) (10 mg/kg body weight) (Sigma Chemical Co.) immediately following the puromycin treatment. The mice in the control groups (groups 1 and 2) received bitemporal injections of saline (10 μl/site). Since the controls showed no differences in relation to time of injection, all controls were pooled for baseline comparison with individual drug groups. The saline-no shock (SAL/NS) group served as the naive controls while the saline-shocked (SAL) group served as the learning controls.

All intracerebral injections were conducted under light ether anesthesia. Intracerebral injections were delivered bitemporally in volumes of 10 μl/site by hand through a #705 Hamilton microsyringe with 26 ga, 0.95 cm disposable needles to a depth of 2.5 mm beneath the skull and at sites 2 mm posterior to Bregma and 2 mm lateral, left and right, to the midsaggital suture. A stop was mounted on the needle to avoid a too-deep penetration. On the day preceding pretraining, the heads of the animals were shaved and the bitemporal sites for injection were marked. Injections took place either immediately after training (0h) or 24 hours after training (24h). Prior to the injections and prior to training and retention testing, animals were removed form their home cages and individually housed in small plastic cages. The animals were returned to these cages following injections and remained there until they had recovered sufficiently from anesthesia.

TABLE 1

EXPERIMENTAL GROUPS

Group	Treatment	Time of Injection
1	No Shock—Saline* (SAL/NS)	Oh
2	FS—Saline* (SAL)	Oh
3	FS—Puromycin 110 μg/site* (PM Oh)	Oh
4	FS—Puromycin 110 μg/site* (PM 24h)	24h
5	FS—Cycloheximide 110 μg/site* (CXM)	Oh
6	FS—Puromycin 110 μg/site + Cycloheximide 110 μg/site* (PM + CXM Oh)†	Oh
7	FS—Puromycin 110 μg/site + Cycloheximide 110 μg/site* (PM + CXM 24h)†	24h
8	FS—Puromycin 110 μg/site* + Amphetamine 10 mg/kg‡ (PM + AMP Oh)	Oh
9	FS—Puromycin 110 μg/site* + Amphetamine 10 mg/kg‡ (PM + AMP 24h)	24h

FS=footshock; Oh=injections administered immediately following training; 24h=injections administered 24 hrs after training.
*Bitemporal injection.
†Combined bitemporal injection.
‡Subcutaneous injection.

Statistics. Latencies were determined and scores were subjected to an overall analysis of variance for a 2 factor design with repeated measures on one factor and unequal group size. An unweighted means solution was employed. A posthoc comparison of all possible pairs of means on Tr, T and R was made according to the Newman-Keuls procedure with $p<0.05$. In addition, t-tests were run to evaluate step-down latencies at Test and Retest within individual groups and to evaluate step-down latencies between certain experimental groups (e.g., PM + CXM 24h vs. SAL/NS, PM + AMP 24h vs. SAL/NS and vs. PM 24h at R, CXM vs. SAL/NS at R). However, to maintain more conservative statistical estimates, significances determined by t-test are not included in the figures.

Analysis of inhibition of cerebral protein synthesis. Animals were housed, fed and injected intracerebrally as described in previous sections. Solutions of puromycin and cycloheximide were prepared as for the behavioral experiment (110 μg/10 μl, bitemporally). At various times (2, 4, 8, 12 and 24 hours) after intracerebral injection of PM, CXM or saline, animals were injected subcutaneously on the back of the neck with 5 microcuries of L-valine-l-C^{14} (New England Nuclear, specific activity 27.7 mC/mM). Thirty minutes later the animals were sacrificed and the cerebrum was removed

for analysis. Data at each point was calculated as the average of three separate samples.

The methods for extraction of proteins, isotope counting, and calculation of percent inhibition of protein synthesis have already been described in detail [29] except in this study the standard volumes used in the procedure were scaled down for the mouse.

RESULTS

Biochemical Results

Puromycin. For PM 110 μg, the level of cerebral protein synthesis inhibition was 37% at 2 hrs and it reached a maximum level of 77% at 4 hrs; the percent inhibition decreased thereafter. Protein synthesis was returned to normal levels 24 hours after the injection.

Cycloheximide. A stronger and more sustained inhibition of cerebral protein synthesis was observed after bitemporal injections of 110 μg CXM. Inhibition developed rapidly to 96% within 2 hours of the injection and this high level of inhibition was sustained at 4 hrs. Cerebral protein synthesis inhibition at 8 hours was 81% and a significant degree of inhibition was still evident 24 hours later (76%).

Statistical Summary of Behavior

Analysis of variance. An overall analysis of variance revealed significant effects between subjects A=drug treatment (i.e., SAL, PM, CXM, etc.), $F(12,305)=5.95$, $p<0.01$; and within subjects B=trial (Tr, T, R), $F(2,610)=273.03$, $p<0.01$; and for AB $F(24,610)=1816$, $p<0.01$.

Posthoc comparison of individual group means. A Neuman-Keuls post hoc comparison of all possible pairs of means with $p<0.05$ for the nine groups of animals was made. The significance of comparisons of individual group means against SAL and SAL/NS controls is indicated in Figs. 1–3. No significance for mean SDL at Tr was revealed ($p>0.05$ for comparisons of all possible pairs of means).

Student's t-test. A standard paired *t*-test of SDL's within individual groups at Test and Retest revealed a significant effect (to at least $p<0.05$) in all groups except PM 24h and PM + AMP 0h.

Behavioral Results

Puromycin. Mice injected bitemporally with puromycin in doses of 110 μg, either immediately after or 24 hours after training exhibit substantial retention deficits at both Test and Retest. A statistical comparison of group means reveals significant decreases in step down latencies of PM animals over saline injected controls (PM 0h vs. SAL, T + R: $p<0.05$, Fig. 1; PM 24h vs. SAL, T + R: $p<0.05$, Fig. 1). There was no difference between the PM animals and the naive controls ($p>0.05$ vs. SAL/NS in all instances at both T and R).

Cycloheximide. CXM injected bitemporally immediately after passive avoidance training produced marked retention deficits in mice. Comparisons of Test SDL means reveal that there was a significant decrease in latencies of cycloheximide-injected animals over saline-injected controls (CXM vs. SAL at T: $p<0.05$; Fig. 1). Furthermore, there was no significant difference between CXM and the SAL/NS (naive) control group at Test (CXM vs. SAL/NS at T: $p>0.05$; Fig. 1). These results indicate that substantial anmesia was present at the first retention Test (T). In the ensuing 72 hours between Test and Retest, however, it appears that some recovery of memory may have occurred. At Retest CXM was no longer significantly different from SAL. A standard *t*-test run separately showed that CXM was significantly different from SAL/NS (CXM vs. SAL/NS at R: $p<0.05$).

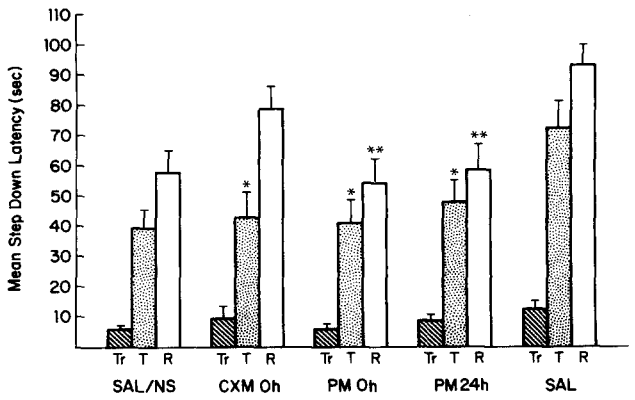

FIG. 1. Effects of cycloheximide injected immediately after training (CXM 0h) and puromycin (PM) injected immediately (PM 0h) after or 24 hours (PM 24h) after training on memory at Test and Retest (*differs from SAL by $p<0.05$ at T; **differs from SAL by $p<0.05$ at R). CXM is significantly different from SAL at Test but not at Retest. CXM is not significantly different from SAL/NS at Test or Retest. Neither PM group is significantly different from SAL/NS at Test or Retest. Furthermore, there is no significant difference between PM 0h and PM 24h at Test and Retest.

Combined injections of puromycin and cycloheximide. Memory of step down training is significantly impaired when puromycin and cycloheximide are injected together immediately after training. Comparisons of group SDL means indicate that significant amnesia is present at both Test and Retest (PM + CXM 0h vs. SAL at T: $p<0.05$; at R: $p<0.05$; PM + CXM 0h vs. SAL/NS at T: $p>0.05$; at R: $p>0.05$; Fig. 2). In contrast, there is evidence of attenuation of amnesia when a combination of the two drugs is given 24 hours after training. The PM + CXM 24h group mean is not significantly different from either the SAL or SAL/NS controls at Test or Retest (Fig. 2), but instead lies somewhere between the two. In both cases (i.e., at Test and Retest) the difference between experimental and SAL/NS group means is just barely below the level of significance. The mean SDL of this group, especially at Retest (Mean SDL=80.83 sec) is exceptionally high and not typical of an amnesic group. Thus, it appears that attenuation of amnesia is substantial if not complete. A standard paired *t*-test conducted separately reveals a significant difference between SAL/NS and PM + CXM 24h group means at Test and a marginal significance at Retest.

Combined injections of puromycin and amphetamine. Puromycin and amphetamine were injected either immediately after or 24 hours after passive avoidance training. Comparisons of Test and Retest SDL means to SAL and SAL/NS control groups indicate that when both drugs are injected 24 hours after training, amphetamine treatment results in an attenuation of the memory loss associated with treatment by puromycin (Fig. 3). In addition, it appears that the attenuation of puromycin-induced amnesia in this case may invovle a time-dependent or a reminder-type process since recovery is not complete until the second retention test (i.e., at Retest). Results at Test were inconclusive since the experimental group at this time was not statistically different

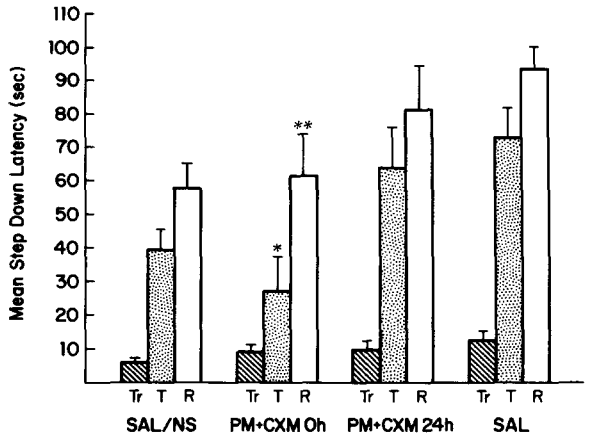

FIG. 2. Effect of combined bitemporal injections of puromycin (PM) and cycloheximide (CXM) when given immediately after (0h) or 24 hours (24h) after training on memory at Test and Retest (*differs from SAL by $p<0.05$ at T; **differs from SAL by $p<0.05$ at R). PM + CXM 0h is significantly different from SAL but not SAL/NS at both Test and Retest. PM + CXM 24 h is not significantly different from SAL/NS or SAL at either Test or Retest.

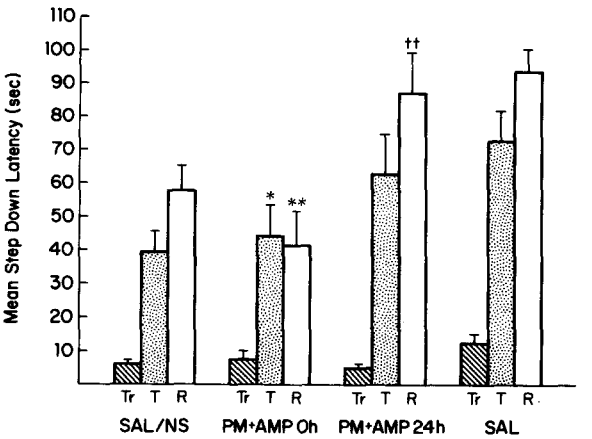

FIG. 3. Effect of combined injections of puromycin (PM) (bitemporal) and amphetamine (AMP) (subcutaneous) when given immediately after (0h) or 24 hours (24h) after training on memory at Test and Retest (*different from SAL by $p<0.05$ at T; **different from SAL by $p<0.05$ at R; ††different from SAL/NS by $p<0.05$ at R). PM + AMP 0h is significantly different from SAL but not SAL/NS at both Test and Retest. PM + AMP 24h is not significantly different from SAL/NS or SAL at T or SAL at R. PM + AMP 24h is significantly different from SAL/NS at Retest.

from either naive or trained controls (PM + AMP 24h vs. SAL or SAL/NS: $p>0.05$). A standard t-test run separately, however, did reveal a significant difference between PM + AMP 24h and SAL/NS at Test. By Retest, however, reversal of amnesia was complete (PM + AMP 24h vs. SAL: $p>0.05$; PM + AMP 24h vs. SAL/NS, $p<0.05$; Fig. 3). Also, a standard t-test run separately showed that PM + AMP 24h was significantly different from PM 24h at R (PM + AMP 24h vs. PM 24h at R: $p<0.05$).

On the other hand, amphetamine does not attenuate puromycin's ability to produce amnesia when both drugs are injected immediately after training. Comparisons of SDL means indicate that amnesia is well established at Test (PM + AMP 0h vs. SAL: $p<0.05$; PM + AMP 0h vs. SAL/NS: $p>0.05$; Fig. 3), and that no tendency toward recovery can be observed upon Retest (PM + AMP 0h vs. SAL: $p<0.05$; PM + AMP 0h vs. SAL/NS: $p>0.05$; Fig. 3).

DISCUSSION

The pattern of results obtained in this study and from numerous previous studies provides compelling evidence consistent with the hypothesis that although PM and CXM both produce amnesia, the mechanisms mediating their effects differ in a consequential manner [2]. Further, the nature of the differences is not consistent with the differential effects produced by these substances on protein synthesis per se. In this study with mice, as in earlier work [1, 3, 17, 21, 29], CXM produced a higher level of inhibition of protein synthesis, more rapidly, and with a longer duration of peak inhibition; however, it produced considerably less of an amnesic effect. Thus we can infer that the amnesic effects of PM must be associated with some relatively unique effects of this substance not reflected directly in the general level of protein synthesis inhibition.

Insofar as drug-induced debilitation would make the animal lethargic or unable to perform, the passive avoidance paradigm we used mitigates against drug-induced debilitation as the cause of amnesia since to be labeled amnesic an animal would have to be physically active and alert. We also chose to provide a second session of retention testing (Retest) with the same temporal parameters as the Train-Test interval to evaluate the stability of antibiotic-induced retention deficits over time and within the context of situational cueing provided by the first retention test (Test). Thus, this task provided an excellent paradigm for the study of the temporal parameters of puromycin-induced amnesia without confounding effects from toxicity.

CXM produced a retention deficit in this study for a specific avoidance response when injected immediately after passive avoidance training. However, the amnesia did not persist. There have been numerous reports of cycloheximide-induced transient amnesia and, therefore, these results were not surprising. However, studies reporting recovery are important; they suggest that the massive inhibition of protein synthesis present during training (when glutarimides have been injected shortly before) does not necessarily block the memory consolidation and storage process. Moreover, inspection of the raw data in this experiment reveals that only 22% of the animals are still amnesic at Retest compared with 74% at Test. It is therefore possible that recovery processes in some animals occur at a slower rate or require stronger reminder cueing.

In contrast to CXM, however, PM administered both immediately after and 24 hrs after passive avoidance training, produced a significant amnesia. Moreover, there was no evidence of test-induced or spontaneous recovery of memory over time for either treatment condition. Of all the antibiotics used in memory disruption experiments, only PM is effective as an amnestic agent when injections are delayed long after training (24 or more hours) after when presumably consolidation has occurred.

The results with delayed injections suggest that PM may have separate effects on memory with different temporal pa-

rameters. It may be that when PM is administered long after training occurs (delayed injections), amnesia is the result of interference with the retrieval or expression of memory since the amnesia is susceptible to a variety of attenuation treatments [2]. However, few experiments have evaluated the attenuation of amnesia when PM is given immediately after training. For example, intracerebral saline did not attenuate the amnesia produced by immediate injections of PM [13]. Similarly concurrent injections of CXM with PM immediately after training did not attenuate PM's effects on retention [29].

We attempted to replicate these findings in the present study. In separate experiments, combined bitemporal injections of puromycin and cycloheximide were given either immediately after or 24 hours after passive avoidance training. In agreement with previous results with intracerebral saline and combined PM-CXM injections, we were unable to demonstrate that CXM had any protective effect against puromycin-induced amnesia when the drugs were administered immediately after training. Amnesia was evident at Test and Retest. In contrast, when puromycin and cycloheximide were administered concurrently 24 hours after training, puromycin-induced amnesia was attenuated.

The fact that CXM antagonizes the amnesia-producing effects of puromycin when both drugs are administered 24 hours after but not immediately after training, indicates that the biochemical mechanisms for amnesia in each situation may not be the same. Although the amnestic effects of delayed PM injections on the expression of memory may result from the formation of peptidyl-puromycin fragments [2], the effects of immediate injections of puromycin may not. Specifically, cycloheximide, which prevents the formation of puromycin peptides, does not attenuate the amnesia produced by immediate PM injections. The results of final experiment with amphetamine tend to support our contention that PM has separate effects on memory with different temporal parameters.

Various stimulants have been shown to attenuate amnesia produced by delayed injections of PM including amphetamine, pentylenetetrazol, and caffeine [8, 18, 25]. However, the effect of stimulant drugs such as amphetamine on amnesia produced by PM injections administered immediately after training has not been evaluated. Thus, in our final experiment, we injected puromycin (bitemporally) and amphetamine (subcutaneously) either immediately after or 24 hours after passive avoidance training. Amphetamine did not appear to have any effect on puromycin's ability to induce amnesia when both drugs were administered immediately after training. There was significant memory impairment at both Test and Retest. Amphetamine, however, attenuates puromycin-induced amnesia when both drugs were administered 24 hours after training. Recovery of memory occurred gradually, over time. At first the mice appeared to be neither clearly retentive nor amnestic, since at the initial retention test, latency scores indicated that animals were not significantly different from both naive and trained controls. By Retest, however, recovery of memory was complete, since these animals were signifciantly different from SAL/NS and also from PM 24h (PM + AMP 24h vs. PM 24h at R; $p<0.05$).

The evidence from this last experiment and the preceding one, supports the contention that puromycin has two separate mechanisms with different temporal parameters for the production of amnesia. This is inferred from the fact that when puromycin injections are delayed (24 or more hours after training), the amnesia produced is labile and susceptible to various amnesia-attenuating treatments such as CXM, amphetamine, caffeine, saline injections and certain neuroactive peptides (retrieval-type effect). However, when puromycin is injected prior to or immediately after training, these agents are ineffective and the amnesic effects persist (consolidation-type effect). Nevertheless, the fact that PM has separate behavioral effects working through different physiological mechanisms does not preclude the possibility that the physiological substrate of memory may itself progress through various temporal stages which makes it susceptible to puromycin in distinct and separate ways.

Whether immediate injections of PM disrupt some time-dependent associative process (consolidation) is not clear at this time. On the other hand, it is apparent that delayed injections of PM block the expression or retrieval of memory. Following puromycin injections, the brain is inundated with peptidyl-puromycin complexes, some of which persist in nerve endings for weeks. Since CXM blocks the formation of puromycin peptides, the amnesia-attenuating effects of CXM (and AXM) strongly implicate the involvement of these puromycin-peptide fragments in blocking the expression of memory. A possible neuromolecular mechanism is suggested by the fact that puromycin is a structural analog of adenosine, which has potent depressant effects on neurons at many levels of the neural axis. Since puromycin has recently been shown to be a potent inhibitor of adenosine uptake in the brain ($IC_{20}=5.6\times10^{-9}$) [23] (thereby elevating extracellular levels of adenosine), it is possible some of these persistent puromycin-peptide moieties may block memory expression by enhancing the activation of physiologically crucial adenosine receptors in the brain, resulting in the suppression of neuronal activity in critical neural networks.

In summary, the major points of this report are three-fold: (1) the crucial correlate of puromycin's effects on retention is not necessarily the overall level of inhibition of cerebral protein synthesis (as suggested when its amnestic effects are compared to those of cycloheximide); (2) of all the antibiotics, only puromycin is effective as an amnesic agent when injections are delayed 24 or more hours after training; and (3) puromycin may have separate effects on memory with different temporal parameters depending on when the drug is injected relative to the initial training.

In closing, we feel this work serves to draw attention to the prolific and interesting body of literature, primarily reported by the Flexner group in the last decade, concerning the effects of delayed injections of puromycin on the retrieval or expression of memory and, collaterally, the characteristics of amnesia-attenuation treatments on this retrieval-type blockage produced by puromycin. Indeed this report attempts to put some distance between the rather large body of literature exploring the effects of protein synthesis inhibitors on memory and a divergent area of research concerned with the unique effects of puromycin on retention.

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