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Protein Synthesis Inhibition and Memory for Pole Jump Active Avoidance and Extinction

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FLOOD, J. F., M. E. JARVIK, E. L. BENNETT, A. E. ORME AND M. R. ROSENZWEIG. *Protein synthesis inhibition and memory for pole jump active avoidance and extinction.* PHARMAC. BIOCHEM. BEHAV. 7(1) 71–77, 1977. This study utilizes a pole jump active avoidance task to investigate the effects of protein synthesis on memory formation. An extinction training procedure for this task is also described. Amnesia for extinction is produced by inhibition of protein synthesis and is also demonstrated by active responding, so it is clear that there is no general impairment sufficient to disrupt motor skill, motivation, or retrieval of stored memories. It was found that while inhibition of protein synthesis in brain for 2 hr did not produce amnesia, inhibition for 6 to 8 hr did. These results demonstrate that for both shock-motivated learning and non-shock motivated extinction learning, the duration of inhibition of protein synthesis is important in determining whether amnesia occurs. We conclude that inhibition of cerebral protein synthesis can best account for amnesia induced by anisomycin, cycloheximide, and acetoxycycloheximide.

Active avoidance test Amnesia Anisomycin Protein synthesis Protein synthesis inhibition

INHIBITION of protein synthesis during and after training has been found in many cases to lead to amnesia that appears to be permanent [1–5, 7–13, 16, 18–22, 24]. Control over the parameters of acquisition is needed, since it has been shown that failure to do so can reduce or obliterate the amnesic effect [7, 9, 10, 17]. Recently we reported that as the duration of inhibition of brain protein synthesis increased after passive avoidance training, the percentages of animals classed as amnesic increased [8,9]. This was also found for T-maze footshock avoidance training but the parameters controlling acquisition of T-maze avoidance conditioning were too numerous and the duration of inhibition required for strong amnesic effects was too long (14 hr) for this task to be used regularly in studies of memory trace formation [10]. We report here the effects of protein synthesis inhibition on retention for an avoidance task that is learned more easily than T-maze avoidance. In addition, extinction is treated as an acquisition session, and amnesia for learning not-to-respond is reported.

GENERAL PROCEDURES

Animals and Drugs

The animals for the behavioral experiments were Swiss Webster (CD-1) male albino mice, 60–80 days of age at the time of the experiment. They were obtained from Charles River Breeding Laboratories at 6 weeks of age. Mice used for the behavioral experiments were housed singly 24 hr prior to training and remained so housed until tested for retention one week later.

Anisomycin (2-p-methoxyphenyl-3-acetoxy-4-hydroxypyrrolidine) was a gift from Pfizer Co., Groton, CT through the generosity of Dr. N. Belcher or was obtained from Pfizer Diagnostics, Clifton, NJ. In order to dissolve Ani, an approximately equal molar amount of 3N HCl was added, and the pH was finally adjusted to 6–7. The final solution was 2.0 mg/ml in 0.9% saline; Ani was injected at 20 mg/kg subcutaneously just posterior to the back of the neck. The first injection of Ani or saline was administered 15 min before training. Cycloheximide (Cyclo) obtained as Acti-

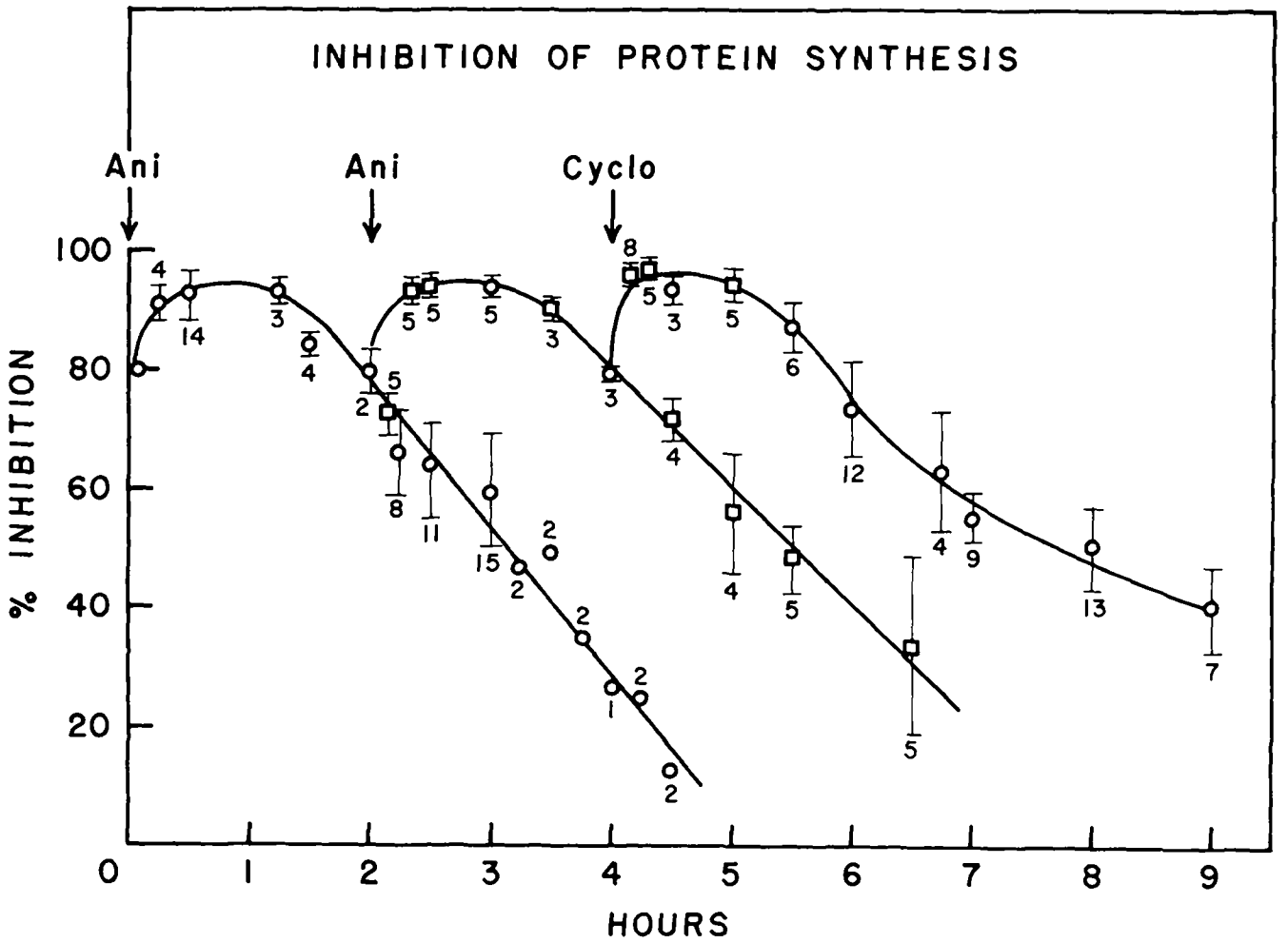

FIG. 1. Inhibition of cerebral protein synthesis in Swiss Webster male mice obtained by subcutaneous injections of Ani, Ani + Ani, and Ani + Ani + Cyclo. The successive curves show the inhibition produced by a single dose of 0.5 mg of Ani, by two successive doses of Ani, and by 2 successive doses of Ani followed by a 2.5 mg dose of Cyclo, each at 2 hr intervals. The number of mice and the standard deviation are shown for each data point where more than two mice were used. The doses (0.5 mg Ani, 2.5 mg cycloheximide) and the injection schedule were the same as used for the behavioral experiments. Two major series of experiments done two years apart are represented by and . The curve for Ani + Ani is a composite curve incorporating data from C, B1/Jf and Swiss male mice. We have found that C, B1/Jf and Swiss mice have essentially identical inhibition resulting from a single dose of Ani and similar degrees of inhibition at 4 hr and 5 hr Ani + Ani data points.

dione from the Upjohn Co., was dissolved in 0.9% saline and administered subcutaneously at a dose of 100 mg/kg. [^{14}C(U)]-L-valine was obtained from the New England Nuclear Corp. The mice for the biochemical experiments were bred in our laboratories from Swiss Webster Charles River mice or C, B1/Jf mice.

Determination of Protein Synthesis

Protein synthesis was determined by the ratio of radioactivity resulting from the incorporation of subcutaneously administered [^{14}C(U)]-L-valine into the trichloracetic acid insoluble fraction to the total amount of activity in the brain sample. The radioactive amino acid was injected 20 min prior to sacrifice. The percent inhibition was determined by a comparison of this ratio in the experimental and control mice. The procedures have been described in detail [7]. Duplicate fractionations and determinations of radioactivity were made for each mouse brain.

BIOCHEMICAL EXPERIMENTS

Results

The purpose of the biochemical studies was to determine the inhibition of protein synthesis achieved by several injection schedules of anisomycin and cycloheximide used in the behavioral studies. After an injection of Ani, the inhibition of protein synthesis rises rapidly to 90% and then falls to 80% after 2 hr (Fig. 1). A subsequent injection of Ani results in an inhibition curve similar to the first one. Thus each injection of Ani maintains inhibition of 80% or more for an additional 2 hr (also see [8]). The inhibition obtained by an injection of Cyclo falls to 80% somewhat more quickly than does the inhibition obtained with Ani, but the subsequent decay is less rapid (Fig. 1). Similar differences in the time courses of inhibition were previously observed after a single injection of the individual inhibitors [8]. Since training of the mice occurred in the behavioral experiments 15 min after the first injection of Ani or saline,

inhibition for Ani-injected subjects was at a high level at the time of training. The important advantage in using Ani over other available protein synthesis inhibitors is that the lethal single dose of Ani in mice is at least 40 times greater than the dose used in the behavioral studies. In other experiments, we have found that up to 7 injections of Ani can be given at 2 hr intervals without marked ill effects and the time course of the inhibition from the seventh injection was essentially superimposable over that obtained from the first ([10] and unpublished). As a result, it is possible to control the duration of inhibition by giving successive injections of Ani at 2 hr intervals. At the time we did these experiments, we had only a limited supply of Ani available; therefore we used Cyclo as the final inhibitor to obtain extended inhibition. We used a sequential series of one to four injections in order to vary the duration of inhibition after training from 1 3/4 hr to about 7-1/2 hr at 80% or greater. Data was obtained for the combination Ani^2 + Cyclo (Fig. 1). The assumption that inhibition for Ani^3 + Cyclo is 80% at 8 hr is a reasonable extrapolation since Ani does not appear to have cumulative effects.

BEHAVIORAL EXPERIMENTS

EXPERIMENT 1: THE EFFECT OF DURATION OF PROTEIN SYNTHESIS INHIBITION ON RETENTION FOR POLE JUMP AVOIDANCE TASK

Apparatus and Training Procedures

The training apparatus for the pole jump task consists of an alley 30 cm long, 11.5 cm wide and 18 cm high divided into two compartments by a guillotine door. A brass grid floor is used to deliver footshock (0.35 mA) in both compartments. The smaller compartment (9 cm long) is a start box. The other compartment (21 cm long) contains a vertical plastic pole in the center. The pole (2.5 cm diameter) is covered with 1/2 inch wire mesh which starts just above the shock grid; the mesh makes it easy for a mouse to climb the pole and to cling to it. The pole can be removed easily with the mouse on it. The apparatus is built of black plastic except for the pole, which is white. A loud door bell buzzer is used as the conditioned stimulus (CS). The training room is dark except for a bright Tensor lamp illuminating the apparatus.

The training procedure consists of the following steps: The mouse is placed in the small compartment and after approximately 15 sec the guillotine door is lifted to give access to the pole compartment. Simultaneously with removal of the guillotine door, the buzzer begins to sound, and 5 sec later footshock is administered if the mouse has not climbed onto the pole. The buzzer and shock are manually terminated as soon as the mouse climbs onto the pole. An avoidance response is scored if the mouse climbs onto the pole within the 5 sec safe period.

After each trial the mouse is returned to its home cage by carefully removing the pole (with the mouse on it) and placing the pole in the home cage. Most mice quickly climb off the pole, but occasionally a light touch to the hind quarters is used to encourage the mouse to dismount. Subsequent trials (training or testing) are run in the same manner. The intertrial interval is about 45 sec. Animals receive only 2 training trials because pilot work showed that saline-injected controls performed equally well on the retention test whether given 2, 4, or 6 training trials.

The retention test follows 1 week after training, and consists of retraining a mouse until it makes one avoidance response. Training and testing are always done between 8 a.m. and 2 p.m. The number of trials prior to making the first avoidance response is taken as a measure of retention. In this experiment, amnesia is defined as taking 3 or more test trials to make an avoidance response. This criterion is valid since it classifies 79% of naive mice as amnesic (Fig. 2, Panel A).

Ten of the saline-injected subjects were given 10 test trials each in order to test whether an animal continues to avoid after making its first avoidance response. The mean percent avoidance responses after each mouse made its first avoidance response was 97.5% across the 10 animals. Only 2 mice received additional shock – one a shock on the 6th trial and the other on the 7th trial. Thus training the mice to a 9 out of 10 criterion on the retention test would have provided little additional information. Also, more retention trials can confuse the distinction between retention of a habit vs maintenance of a habit.

Design

The purpose of this experiment was to study the effect of duration of inhibition of protein synthesis on retention for pole jump training. Three drug groups were used. These were Ani (single pretraining injection 15 min prior to training), Ani^2 + Cyclo (a single pretraining injection of Ani followed 1-3/4 hr after training by another injection of Ani and then an injection of Cyclo 3-3/4 hr after training), Ani^3 + Cyclo (single pretraining injection of Ani, two Ani injections after training at 1-3/4 hr, and 3-3/4 hr and Cyclo at 5-3/4 hr after training). Three saline control groups were run; they received saline injections at the time the comparable drug groups received their injections. A seventh group was used to establish the performance of naive subjects. This naive baseline group was isolated at the time when the other groups were trained and received no injections. The naive group was first trained when the other groups were being tested for retention. The N's are given in Fig. 2.

Results

The saline control animals combined (Fig. 2B) and the group given a single 20 mg/kg Ani injection (Fig. 2C) showed good retention. Only 8% and 9% of these groups, respectively, were classed as amnesic. Both groups differ clearly from the naive baseline group (Fig. 2A) in which 79% of the animals would have been scored as amnesic. Because some of the naive mice learned the task in 1 or 2 training trials, the percent amnesia was not 100%. The Ani^2 + Cyclo group (Fig. 2D), which had 6 hr of protein synthesis inhibition at 80% or greater, yielded 38% amnesic animals; this was significantly different from both the saline controls and the group that received only a single pretraining injection of Ani ($p<0.001$, χ^2 test). Sixty-six percent the Ani^3 + Cyclo group (Fig. 2E), which had 8 hr of inhibition of protein synthesis, were amnesic; this percentage of amnesic mice not only differed significantly from the saline controls and the single Ani group but it also showed a greater percentage of amnesia than did the Ani^2 + Cyclo group ($p<0.025$). In fact, the performance of the Ani^3 + Cyclo group did not differ significantly from the naive baseline group ($p<0.25$). Thus, increased durations of

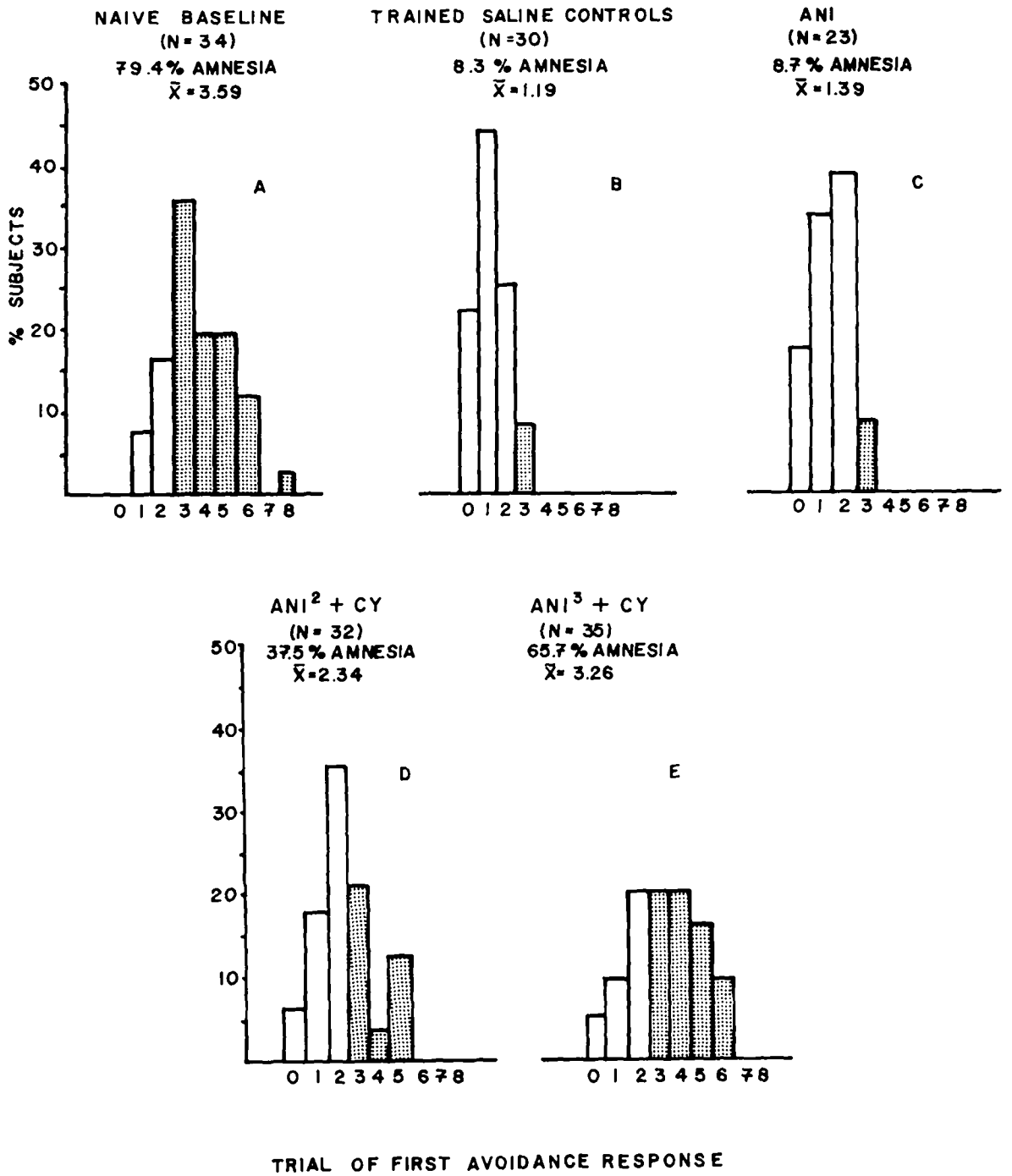

FIG. 2. Effects of duration of inhibition of protein synthesis on retention for pole jump training (Experiment 1). The series of figures show the frequency distribution of animals versus trial of first avoidance response for 5 experimental groups. Animals requiring 3 or more trials to make an avoidance response were scored as amnesic (shaded bars). As the number of injections of inhibitor increased from one to four (Ani, Ani^2 + CY, Ani^3 + CY), increasing the duration of protein synthesis inhibition, the percentage of mice showing amnesia increased. Thus over 90% of the saline control group (Panel B) and the Ani group (Panel C) made the first avoidance in 2 trials or less, while only 34% of the Ani^3 + CY group (Panel E) made an avoidance during the first 2 trials. Correspondingly, the mean number ($\bar{X}$) of trials to the first avoidance response increased from 1.19 to 3.26. Because some of the naive mice learned the test in 1 or 2 trials, the percent amnesia as defined was not 100%.

inhibition of protein synthesis (6 or 8 hr) led to increased percentage of amnesia.

EXPERIMENT 2: EFFECT OF DURATION OF INHIBITION OF PROTEIN SYNTHESIS ON EXTINCTION TRAINING

Materials and Procedures

The apparatus was the same as in Experiment 1, and similar subjects were used. In order to build up an avoidance habit that would be extremely resistant to extinction or forgetting, the following procedures were used: Training days were spaced. The mice were given 20 training trials per day on Monday, Wednesday and Friday of the first week and on Monday and Wednesday of the second week. Subjects were permitted to make two errors of omission (i.e., no avoidance response) without being shocked, on the third consecutive failure to respond shock was given. This type of training schedule is referred to as partial reinforcement. An occasional animal that began to form a pattern of two failures to respond followed by a response, was shocked on every error until it responded with three consecutive avoidances. This was done to discourage learning a response pattern that would result in avoiding shock with a relatively low percentage of avoidance responses. Lastly, animals failing to make an avoidance on either the first or the last trial of a day were shocked for not making the avoidance.

On Friday of the second week, we used 10 training trials to select subjects with strong avoidance habits. To be retained, subjects had to make an avoidance on the first trial and receive no more than one shock in the ten training trials. Animals (about 90%) meeting this criterion were shifted on Trial 11 to either the conventional extinction procedure or to the more effective Katzev extinction procedure [15]. The following rules are used for conventional extinction: (1) if the animal responds in 5 sec or less (an avoidance), terminate the CS (buzzer), or (2) if the animal fails to respond in 5 sec, terminate the CS promptly at the end of 5 sec. In either case no shock is given, but with well trained subjects these procedures produce little or no decrement in responding because the mice rarely fail to respond and thus have only experienced additional training (Rule 1) and no true extinction trials (Rule 2). For the groups given Katzev extinction procedure the following rules were used: (1) if the animal responds within 5 sec, leave the CS on for 30 sec following the response, or (2) if the animal fails to respond, terminate the CS promptly at the end of 5 sec. Rule 1 is where the two procedures differ: Katzev Rule 1 breaks the response contingencies of training, showing the animal that continued sounding of the buzzer is no longer associated with shock, and this leads to a rapid decline in avoidance responding [15]. With the Katzev procedure, mice reached the criterion of two successive failures to avoid after a mean of 6.95 extinction trials; 72% of the animals given the Katzev procedure reached extinction in 6, 7, or 8 trials. The Katzev groups were run first, and animals receiving the conventional treatment were matched with Katzev animals; thus they were given 7 or 8 extinction trials even though this did not bring them to the criterion of extinction.

Drug Conditions

To test the effect of duration of inhibition of protein synthesis on retention for extinction training, we used four saline and two Ani groups. Of two conventional extinction groups, one received a single pretraining injection of saline; the other received three successive injections of saline at the following times – 15 min before extinction training and 1-3/4 and 3-3/4 hr after training. These groups serve as controls to monitor any possible indirect effects that might lead to a decreased avoidance rate during the retention test (e.g., effect of one week without training, the injections, additional trials without shock). Two other groups received the same saline injection schedules (one injection or three) but were given Katzev extinction. These groups measure the degree to which mice will recall effective extinction training. The two experimental (Ani) groups received either a single pretraining injection of Ani or three successive injections of Ani. Both of these groups were given Katzev extinction. These groups reveal the effect of two durations of protein synthesis inhibition (2 vs 6 hr of inhibition at 80% or more) on retention for extinction training.

Retention Test

The retention test was given 1 week after the extinction session. All mice were given 20 conventional extinction trials to test the strength of their avoidance habit. No shock was used at this session.

Results

Saline-injected mice given conventional extinction trials retained a very strong avoidance habit over the one week retention period, as they showed 94% and 96% mean avoidances (Saline and Saline^3); that is, the conventional procedure did not yield extinction with the number of trials given. The saline-injected mice given the Katzev procedure had reached extinction during the extinction session, and they still tended to show extinction one week later: they gave only 26% and 27% mean avoidances (Saline and Saline^3) – significantly less than the groups given the conventional extinction training. The group receiving a single injection of Ani and Katzev extinction showed poor responding with 32% mean avoidances, so they retained the extinction they had acquired. But the group receiving three successive injections of Ani and Katzev extinction responded with 94% mean avoidances and clearly did not differ from subjects receiving conventional extinction (Table 1); thus, although the response had been extinguished the week before, the 6 hr of inhibition of protein synthesis had prevented long-term storage of the extinction.

DISCUSSION

The results of these experiments show that for both shock-motivated learning (pole jumb avoidance) and for non-shock motivated learning (extinction) the duration of inhibition of protein synthesis (i.e., the number of Ani injections) is important in determining whether amnesia will occur. With the strength of training used, a single pretraining injection of Ani is not sufficient to disrupt retention and it requires the addition of one or more posttraining injections to obtain amnesia. Since these injections did not impair acquisition, we conclude that Ani blocked long-term memory storage. Old memories are not affected by inhibitors of protein synthesis, since in the extinction experiment the mice given three successive injections of Ani continued to make avoidances – thus revealing their earlier training – while forgetting the

TABLE 1

EFFECT OF PROTEIN SYNTHESIS INHIBITION ON RETENTION FOR LEARNED EXTINCTION

Treatment Group	Mean % Avoidances	% Animals Maintaining*
Saline + Conventional Extinction	94.5	100
Saline[a] + Conventional Extinction	96.5	90
Saline + Katzev Extinction†	26.5	0
Saline[a] + Katzev Extinction	27.0	0
Ani + Katzev Extinction	31.0	0
Ani[a] + Katzev Extinction	93.5	100

*Maintaining of a shock avoidance habit is defined as making 80% or more avoidances.
†See reference [15].

substantial extinction training. An interesting feature of the use of extinction as a learning task is that forgetting is revealed by active responding. Usually one associates amnesia with decreased responding. It is not always clear whether this decrement in responding is due to permanent drug-induced damage, to lack of motivation, or to poor memory storage. In the extinction situation the animals that forget are the ones that continue to respond, and thus it is clear that there is no general impairment sufficient to disrupt motor skill, motivation, or retrieval of stored memories. We conclude that subjects given a sufficient number of Ani injections lack stored memories for the specific task for which the drug was employed.

While it is possible that some side effect and not inhibition of cerebral protein synthesis is responsible for the amnesia, we interpret our findings as indicating that as the duration of inhibition of protein synthesis increases the probability becomes greater that long-term memory storage will not occur.

Few studies have been made of the side effects of anisomycin on brain neurochemistry, especially on the neurotransmitter systems. Zech and Domagk have reported that anisomycin is a relatively poor inhibitor of acetylcholinesterase, so it is unlikely that the effects of anisomycin can be attributed to inhibition of AChE [25]. Flexner and Goodman have pointed out that important side effects on the central adrenergic system appear to be common to all inhibitors of protein synthesis and that these side effects may contribute to the amnesia [6,14]. Indeed, they conclude that the behavioral manifestations may not be attributable solely, or at all, to inhibition of protein synthesis. They showed that protein synthesis inhibitors puromycin, cycloheximide, acetoxycycloheximide and anisomycin had the common property of depressing the rate of accumulation of norepinephrine, dopamine, and total catecholamines and at the same time markedly elevating the levels of tyrosine. However, in the case of anisomycin, Flexner and Goodman presented data for only one dosage and one time point after administration (2 hr), and until more complete data are available, it is difficult to evaluate the significance of these results for the interpretation of our behavioral experiments. Squire, Kuczensik, and Barondes have compared the amnesic effects of cycloheximide, anisomycin, and α-methyl-p-tyrosine and their effectiveness as inhibitors of brain tyrosine hydroxylase activity [23]. They found that doses of α-methyl-p-tyrosine which depressed tyrosine hydroxylase activity as much as or more than either cycloheximide or anisomycin did not affect memory, while anisomycin and cycloheximide did. They concluded that the effect of protein synthesis inhibitors on brain tyrosine hydroxylase activity is not sufficient to explain the amnesic effect.

Recently we tried to obtain amnesia for active and passive avoidance with diethyldithiocarbamate (DDC) and α-methyl-p-tyrosine (AMPT), two relatively specific and long lasting inhibitors of catecholamine (CA) neurotransmitter synthesis. In doses that did not interfere with gross behavioral movements, neither agent caused amnesia for active avoidance. Yet under the same condition of training, Ani caused amnesia (Flood *et al.*, in preparation). In the passive avoidance task, DDC and AMPT caused amnesia only under the weakest possible training condition (threshold footshock intensity). As footshock intensity increased, Ani consistently caused amnesia, but the CA synthesis inhibitors showed a rapid decline in ability to induce amnesia. If specific and very long lasting inhibition of CA synthesis cannot cause amnesia, then it seems unlikely that this is the most pertinent mode of action of the protein synthesis inhibitors in blocking memory formation. In addition, the CA inhibitors showed no relationship between the number of injections given and amnesia. In fact, additional posttraining injections of CA inhibitors did not induce any further increases in amnesia. Yet, increasing the number of successive injections of Ani shows a clear effect of increasing the percent amnesic animals, presumably because of longer durations of protein synthesis inhibition. We believe that inhibition of cerebral protein synthesis is best able to account for the amnesia induced in active and passive avoidance and in extinction training by anisomycin, cycloheximide and acetoxycycloheximide.

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