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Authors

Flood, James F.
Bennett, Edward L.
Orme, Ann E.

Publication Date

1973-11-01

Submitted to Physiology and Behavior

LBL-2458
Preprint

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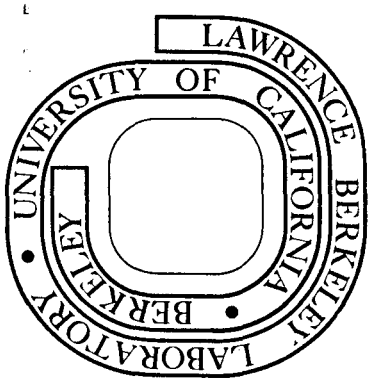
James F. Flood, Edward L. Bennett, and Ann E. Orme

November 1973

Prepared for the U. S. Atomic Energy Commission
under Contract W-7405-ENG-48



DISCLAIMER

Effects of Protein Synthesis Inhibition on Memory for Active Avoidance Training

James F. Flood,[†] Edward L. Bennett and Ann E. Orme

Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720

Mark R. Rosenzweig

Department of Psychology, University of California, Berkeley, California 94720

Mailing address for proofs: Dr. Edward L. Bennett
Lawrence Berkeley Laboratory
University of California
Berkeley, California 94720

Running Title: Amnestic Effects in Mice Trained on Active Avoidance

[†]Present address: Department of Psychiatry and Pharmacology, University of California, Los Angeles, California 90024

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ABSTRACT

Inhibition of brain protein synthesis by anisomycin and acetoxycycloheximide was studied in mice for its biochemical and behavioral effects. At low doses acetoxycycloheximide (100 µg) and anisomycin (500 µg) could be given more than once in succession. By employing both drugs in a series of injections, we were able to inhibit protein synthesis for up to 14 hr at 80% or greater without it causing any detectable permanent impairment to the mice.

The drugs were employed as amnestic agents in mice trained to avoid footshock in a T-maze. It was found that as the duration of inhibition increased the percent mice classed as amnestic increased. This amnesia could be reduced by (a) increasing the rate of acquisition, or (b) practice at avoiding shock. Anisomycin was also shown to cause a significant degree of amnesia for escape learning.

In all drug groups, anisomycin was given 15 min prior to training. This single pre-training injection did not cause significant changes in the acquisition or retention of avoidance conditioning when compared with saline-injected controls. Only the additional injections given after training to prolong inhibition caused high incidences of amnesia. Thus, those injections critical in obtaining amnesia were given at a time at which interference with acquisition could not have occurred.

Key words: Memory Mice Anisomycin Acetoxycycloheximide Active avoidance Inhibition of protein synthesis

Introduction

Active avoidance has been used infrequently in studies of memory formation using protein synthesis inhibition as the amnestic treatment. Flexner and his co-workers have reported that puromycin will block memory for a left-right shock avoidance habit in a Y-maze (5). However, the amnesia seems to be the result of a disruption of retrieval processes rather than a disruption of long-term memory formation (2,3). Flexner, Flexner, and Roberts (4) reported that acetoxycycloheximide so impaired learning of a left-right shock avoidance task that the effects on memory could not be assessed. Reversal training was used and acetoxycycloheximide successfully blocked memory without disrupting learning. It should be noted that the drug was administered intracerebrally several hours prior to training. More recent work has shown that the subcutaneous route of administration establishes high levels of inhibition within a short period of time and thus obviates the necessity of insult to the brain (1).

In our previous studies (6-8), we have employed only passive avoidance training to evaluate the role of brain protein synthesis on memory formation. In this study, we extend this research to active avoidance. The effects of anisomycin (Ani) on retention for passive avoidance and active avoidance conditioning are compared in the discussion section.

GENERAL DESCRIPTION - BIOCHEMISTRY

Procedures

Anisomycin (Ani) was a gift from Charles Pfizer Co., Connecticut, through the generosity of Dr. N. Belcher. The acetoxycycloheximide (AXM) used in these experiments was obtained several years ago, and we do not

know of any current source of this drug. Both drugs were prepared in saline (0.9% NaCl), Ani at either 2.0 mg/ml or 10.0 mg/ml; AXM concentration in the behavioral studies was 0.4 mg/ml. In the behavioral studies the volume of the injection was always 0.25 ml; thus Ani was injected at either 500 µg or 2500 µg and AXM at 100 µg/mouse. The drugs were administered subcutaneously.

Inhibition of protein synthesis was determined by comparing the incorporation of valine-U-^{14}C into the trichloroacetic acid insoluble fraction in drug- and saline-injected mice. Experimental procedures have been given in detail previously (6-8).

Results

Extensive inhibition data for Ani was published previously for C57 Bl/Jf (7) and for the Swiss strain (8); therefore, the inhibition data for Ani was not redetermined. Only the principal findings relevant for this paper will be presented. Ani is relatively non-toxic; at doses 20 times greater than needed to produce significant inhibition of brain protein synthesis, Ani was still not found to be toxic (10 mg/mouse in a single injection). Inhibition of protein synthesis by Ani shows very little dose dependence in the range of 0.5-3.0 mg/mouse. At higher doses the duration of inhibition is only slightly longer and the peak of inhibition is only 1-2% higher. Thus in one of the experiments that follows mice were given an injection of either 500 µg or 2500 µg of Ani. Between these two groups, there is only a very slight difference in the duration and peak of inhibition. A third finding was that inhibition of brain protein synthesis could be prolonged by giving injections of Ani every 2 hr. In the experiments that follow,

up to 6 successive injections of Ani were given; this produced about 12 hr of inhibition of protein synthesis of 80% or greater. The drug does not seem to have significantly different effects upon different strains of mice. Using a fixed dose (disregarding body weight differences across strains), it was found that only small differences existed in the effect Ani had on the duration or peak of inhibition in 7 strains of mice (8).

AXM was used in one of the following experiments to lengthen the duration of inhibition. In this experiment, Ani was always given 15 min prior to training and additional Ani or AXM injections were given after training according to the schedule in Table 3. AXM shows a dose dependence such that the greater the amount of AXM administered subcutaneously, the greater the duration of inhibition. However, 100 µg/injection seemed to offer a relatively long inhibition at a relatively low dose (Table 1). Barondes and Cohen (1) have also published data for inhibition of brain protein synthesis with AXM, and insofar as they can be compared our results agree well with their results. We feel that the use of the lowest possible effective dose is important because this reduces problems of systemic side effect as the cause of amnesia. AXM is the more potent of the two inhibitors on a gram for gram basis. The 100 µg injection of AXM inhibits protein synthesis for about 5 hr at 80% or greater, while the 500 µg injection of Ani inhibits for only about 2 hr at the same level. The combination of Ani followed by a 100-µg dose of AXM (2 hr later) extends inhibition by AXM to 6 hr (total inhibition 8 hr:Ani = 2 hr + AXM = 6 hr); thus the drugs together show some significant synergistic action. In some of the groups that are employed in the

behavioral experiments, two AXM injections were given 6 hr apart. Under these conditions the Ani + AXM^2 injections were found to inhibit protein synthesis for 13-1/2 to 14 hr at about 80% or greater.

In the biochemical and behavioral studies, it was observed that no subject appeared to be seriously ill except for diarrhea, which is to be expected after administering such large amounts of antibiotics.

GENERAL DESCRIPTION - BEHAVIORAL

Subjects

The subjects used in these experiments (N=438) were randomly bred male Swiss (CD-1) albino mice reared at our colony in Lawrence Berkeley Laboratory. The breeding stock was originally purchased from Charles Rivers Breeding Laboratory, Inc., Wilmington, Mass. The mice used in these experiments were offspring from the original stock. Subjects were housed 48 hr prior to training in individual metal cages. Food and water were available at all times. The mice were maintained on an 8-hr dark and 16-hr light cycle as previously described. The mice were between 60 and 75 days of age when trained and weighed about 40 g.

Apparatus

The training apparatus consisted of a black Plexiglas T-maze (12.5 cm high, 9.8 cm wide alleys, the start alley being 46 cm long, and the goal boxes 17.5 cm deep). Shock (0.40 ma) was administered through brass floor rods by an 18-pole shock scrambler. Each goal box was fitted with a clear Plexiglas liner, the bottom of which went below the shock grid. This liner was used to remove the subject from the goal box. A small start box was separated from the rest of the start alley by a black Plexiglas guillotine door which prevented the subject from moving down

the start alley until the trial started. Subjects were not permitted to explore the maze prior to training.

Training Procedure

The mouse was placed in the start box on the first training trial. The guillotine door on this, and only this, trial was left in place until 0.01 min prior to shock onset. On all subsequent trials, including the retention trials, the guillotine door was removed 5 sec before shock onset. A trial began when a loud door bell type buzzer sounded; 5 sec later shock (0.40 ma) began, and both continued until the desired response was made. On the first trial the mouse ran into one of the two goal boxes; in all cases this first choice was treated as incorrect and the subject was forced by continuing the shock to move into the other goal box. On subsequent trials the non-preferred side (as determined on the first trial) was correct. As training proceeded, a mouse could make one of two responses--(a) an escape response, running into the goal box while the shock was on, or (b) an avoidance response, running into the goal box before the shock came on (i.e., responses during the 5-sec warning period). When the mouse entered the correct goal box the buzzer alone (avoidance) or buzzer and shock (escape) were terminated. The goal box entrance was blocked off and the mouse removed carefully from the goal box by lifting the liner out. The liner was placed in the mouse's home cage and gently tilted, thus encouraging the mouse to return to its home cage. After about 30 sec, the mouse was picked up by the tail and placed into the start box for the next trial. Care in removing the mouse from the goal box is particularly important in obtaining rapid acquisition and response measures that will best reflect learning.

Injections

Fifteen min prior to training, the mice were given either a saline or Ani injection (volume 0.25 ml) at a dose of 500 µg (except in Experiment 3); subsequent injections of Ani or AXM were given at 2-hr intervals. All injections, prior to or after training, were administered under very light ether anesthesia. All injections were given subcutaneously on the back. Injection schedules will be described in each experiment.

Retention Test

The retention test consisted of retraining the subject until it made one conditioned response (CR). As will be shown, with our training procedure, once a mouse makes one avoidance response, it will continue to do so until extinction begins to occur. Thus, little more information could be gained by retraining the mice to a 9 out of 10 response criterion.

BEHAVIORAL EFFECTS

Acquisition of the Avoidance Task

It has been our contention throughout our research that one can best use the inhibitors to test their effects on memory when one knows to what extent the mice are trained. Thus we will first present some data on acquisition of this habit by the Swiss mice.

Most mice learned the avoidance habit quickly, making their first avoidance response by the 5th or 6th training trial (Figure 1). Thus, mice making their first avoidance response in fewer than 6 trials would be learning faster than the average, and those making their first avoidance response in 7 or more trials would be learning slower than

the average. We will refer to these two groups respectively as mice with fast or slow rates of learning. Also from Figure 1, it is clear that no significant differences in acquisition occurred between the saline- and Ani-injected mice.

EXPERIMENT 1

Design

The purposes of this experiment were to test if Ani would cause amnesia for weak active avoidance training (only 5 trials) and how long the inhibition might have to be maintained before amnesia, if any, could be detected. The groups used were: NaCl (saline), in which one group received a single injection of NaCl, $NaCl^3$ which received three successive injections of saline 2 hr apart, and $NaCl^5$ which received 5 successive injections of saline 2 hr apart. The experimental groups received either 1, 2, 3, 4, or 5 injections of Ani (Ani, Ani^2, ... Ani^5), each series starting 15 min prior to training and subsequent injections at 2-hr intervals. Injections of 500 μg of Ani at 2-hr intervals were previously reported to maintain inhibition at 80% or greater (7,8). In addition, two comparison groups were used. Iso indicates a group that was isolated during the retention period and trained for the first time when other mice were being given the retention test. This group establishes the naive-subject baseline. The other comparison group was $Na+Ani^5$ in which saline was administered prior to training and, starting 2 hr later 5 successive injections of Ani were given. This group should not differ from the saline controls if (a) Ani^5 has no permanent debilitating effects and (b) the necessary protein(s) for long term memory can be synthesized during the 1-3/4 hr after training when inhibition is not present.

Procedures

All subjects were given 5 training trials. On the retention test (given 1 week after training), each subject was trained until it made one avoidance response; an avoidance response to the correct side of the T-maze is the conditioned response (CR). Twenty subjects were run for each group. Amnesia for this task will be defined as taking 5 or more trials to make the first CR during retraining (retention test).

Results

Comparing the saline versus the Ani-injected subjects in Figure 2, it will be seen that at least 3 successive injections of Ani (6 hr of inhibition) were required to cause a significant percent of the mice to become amnestic. However, even after 5 successive injections of Ani (10 hr of inhibition) the percentage of amnestic subjects is significantly below the naive-baseline (the Iso group). A clear trend for increasing amnesia with increasing duration of inhibition is evident; the increase runs from 5% amnesia with a single injection of Ani to 60% amnesia with Ani^5. A 15% difference in amnesia exists between Ani^2 and Ani^3, Ani^3 and Ani^4, and also between Ani^4 and Ani^5 (Figure 2).

The distribution of the retention scores (Figure 3) shows that as one moves from Ani to Ani^5, subjects take more and more trials to make their first CR on the retention test. In these graphs, it is clear that the combined NaCl groups, NaCl+Ani^5 and Ani do not differ significantly in distribution of scores, yet all differ markedly from the Iso group. There is almost no overlap in the distributions. Ani^5 is clearly closer to Iso than to the combined NaCl groups.

The Ani injections also had a significant effect upon the escape behavior (Table 2). In the Ani^4 and Ani^5 groups significant numbers of

subjects made an error by escaping to the wrong side of the T-maze (those mice making an avoidance on the first retention trial were not included in these calculations). Few Ani, NaCl+Ani[5] or NaCl injected subjects made discrimination errors.

EXPERIMENT 2

Design

In Experiment 1, subjects received only marginal training (5 trials). In Experiment 2, we tested the inhibitor, Ani, as an amnestic agent on much better trained mice. Three levels of training were used: 6 trials (T-6), 8 trials (T-8), or 10 trials (T-10). Across each of these groups 5 durations of inhibition were tested: 2, 8, 10, 12, and 14 hr. In addition, subjects were classified as to how many trials it took before they made their first avoidance response (CR). Other conditions of shock and training were as in Experiment 1. Table 3 gives the schedule of injections and method by which each duration of inhibition was obtained. In Experiment 2 amnesia will be defined as a savings score of less than 30% on the number of trials to make the first avoidance response, because in this experiment clear differences in rates of learning were evident.

Results

The main effect of drug versus no drug showed that long durations of inhibition had a significant amnestic effect ($P < .001$) in these better trained subjects (Figure 4). A comparison of the saline and combined 8, 10, 12, and 14 hr inhibition groups showed that none of the saline subjects were amnestic while 59% of those subjects in the long duration of inhibition groups were amnestic. The subjects receiving a single Ani injection

prior to training did not differ significantly from the saline controls in the percent amnesia (Figure 4).

After training and testing the subjects, it was clear that a great deal of uncontrolled variability in training performance existed. Due to the small supply of AXM, it was not possible to determine in additional experiments how this variability affected the amnesia induced by inhibition of protein synthesis. In the following paragraphs some performance variables are described which were factored in order to see if a possible effect on amnesia had occurred. Some of the performance variables are: the number of trials, the rate of acquisition, and the number of escape errors.

Within the drug conditions using long durations of inhibition, the rate of learning (number of training trials to make the first CR) had a significant effect on the effectiveness of inhibition of protein synthesis as an amnestic treatment. The faster the rate of learning, the less effective the amnestic treatment (Table 4).

The number of training trials (6, 8, or 10) seemed to have had some effect upon the amnesia (Table 5). A trend is seen for more training trials to reduce the percent amnesia.

Another factor upon which subjects vary is how many discrimination errors they made during the early training trials. This factor also had a possible effect upon the percent amnesia, as those subjects making no error had 70% amnesia while those making 1 error had 55% amnesia. In Table 6 the interaction between the rate of acquisition and the number of errors shows a weak trend for those mice making no errors and having low rates of learning to be the most amnestic and those subjects making

discrimination errors and having high rates of learning to be the least likely to be amnestic. As the number of errors increases, the amount of shock a subject received increased. It may be that, to some extent, the more shock a subject received at training, the less likely the subject would be amnestic at retraining.

The longer the duration of inhibition of brain protein synthesis, the higher the percentage of amnesia (Table 7). Table 7 also shows that the single pre-training injection of Ani, under these conditions of training, did not cause significant percent amnesia. Thus the major effect of inhibition on memory occurs with injection given after training. The Na+Ani+AXM^2 group demonstrates that the duration of inhibition per se does not apparently cause any permanent damage to the mice such that they were not able to remember the training. Also it indicates that memory protein, sufficient for recall 1 week later, was synthesized within 1-3/4 hr of training.

The duration of inhibition and the number of training trials both affect amnesia (Table 8), such that those subjects with the most training and the shortest duration of inhibition are the least likely to be amnestic when retested and that those subjects given the fewest number of trials and the longest duration of inhibition are most likely to be amnestic when retested.

Thus it seems probable that several of these factors affect the memory processes. These factors are: (a) the number of training trials, (b) the rate of acquisition, (c) the number of discrimination errors prior to avoiding shock, and (d) the duration of inhibition.

EXPERIMENT 3

Design

One may ask, why did we not use one large injection of Ani rather than giving several small doses of Ani. The answer is in two parts: (a) larger doses of Ani do not greatly prolong inhibition (7)--thus, if an increase in amnesia were shown to be related to an increase in dose, it would have to be due to some side effect since the inhibition would be relatively unchanged--and (b) large doses of Ani given prior to training could impair acquisition, reduce sensitivity to shock, etc. The effects of a 500 µg dose was compared with that of a 2500 µg dose. The groups used were: 5Ani+Ani--in this group the subjects received a 2500 µg dose 15 min prior to training and 2 hr later received the standard 500 µg dose. The second group received Ani+5Ani (500 µg dose followed 2 hr later by the 2500 µg dose). The third group, Ani^3, received three successive 500 µg injections of Ani at 2-hr intervals. The last group, Ani^6, received six successive injections of Ani at the 500 µg dose. In all these groups the first injection was given 15 min prior to training. The following data should make clear why these various groups were employed. The duration of inhibition at 80% or greater is approximately as follows: Ani^6 = 12 hr, Ani^3 = 6 hr, Ani+5Ani = 5 hr, and 5Ani+Ani = 4 hr. In addition, the total amount of drug given to the subjects in the Ani^6, Ani+5Ani, and 5Ani+Ani groups was 3000 µg.

The subjects in this experiment were given 8 training trials, and only those subjects making their first avoidance response on trials 5, 6, or 7 were included. Other conditions of training and testing are as for the previous two experiments. Amnesia is defined as a savings score on the retention test of less than 30%.

Results

The results of this experiment can be compared in two ways: (a) the total inhibition time and (b) the total amount of drug received. Ani^3, Ani+5Ani and 5Ani+Ani caused about the same duration of inhibition of protein synthesis. Ani^3 caused 10% amnesia, Ani+5Ani caused 0% amnesia, but 5Ani+Ani caused 80% of the subjects to be classed as amnestic. The second comparison is based upon subjects receiving 3000 µg of Ani in total. Ani+5Ani, Ani^6, and 5Ani+Ani all received the same amount of drug. Ani+5Ani caused 0% amnesia, Ani^6 caused 40% amnesia, but 5Ani+Ani caused 80% of the subjects to become amnestic. By each comparison the 5Ani+Ani group does not reflect the expected outcome. With this level of training the short durations of inhibition (4 to 6 hr) should not have had a significant amnestic effect judging from the results of Experiments 1 and 2. Of the groups with short durations of inhibition, only the 5Ani+Ani group showed significant amnesia. By considering the total amount of drug given, one can only conclude from Ani^6 and Ani+5Ani that total drug received does not necessarily account for amnesia. In a similar experiment using passive avoidance, we concluded that duration of inhibition, not the quantity of drug *per se*, influenced amnesia (7). For 5Ani to cause a high percentage of the subjects to become amnestic, it had to be given prior to training, as the 5Ani+Ani and Ani+5Ani comparison shows. If 5Ani does not achieve its amnestic power by either duration of inhibition or by virtue of the total amount of drug administered, then how does 5Ani cause amnesia?

DISCUSSION

The main finding of these studies is that there appears, in principle, to be little difference between the effect of brain protein synthesis inhibition on memory for passive avoidance and active avoidance.

Training Strength

If we consider training strength as any parameter of training that influences retention, then increases in training strength, in both passive (6-8) and active avoidance, reduce the amnestic effect of a given duration of protein synthesis inhibition (Exp. 1 vs. Exp. 2, Table 5). However, increasing the duration of the inhibition was observed in both passive (7,8) and active avoidance to counteract the effect of increasing the training strength (Table 8).

Duration of Inhibition

The results with passive and active avoidance training differ with respect to the duration of inhibition that one must work within. In the best trained subjects of passive avoidance, no more than 5 successive Ani injections (10 hr of inhibition) were required to cause 80% to 100% amnesia (7). This same level of amnesia was obtained with active avoidance but only in the most poorly trained subjects and with 14 hr of inhibition (Table 8). The two tasks differ considerably in the (a) total amount of shock received by the subjects (passive avoidance, 0.01-0.08 min; active avoidance, 0.3-0.8 min), and (b) the total time exposed to the training situation (passive avoidance, 30 sec; active avoidance 10-15 min). For the Swiss strain, the shock intensity producing minimal learning in passive avoidance was 0.38 ma and in active avoidance 0.40 ma. Subjects trained on active avoidance experience more shock and have longer exposure to the training situation. These two factors probably account

for the greater duration of inhibition required to achieve amnesia for active avoidance training.

As in passive avoidance (7,8), active avoidance was shown to be sensitive to the duration of inhibition. The longer the duration of inhibition, the more likely the subject was to be amnestic when tested 1 week after original training.

Active Avoidance as a Research Tool

Active avoidance seems to involve learning two tasks: (a) where to direct the escape response and (b) to anticipate the shock onset. Where the subject directs its response is learned within the first few trials; many subjects never made a discrimination error (left-right choice) except on the first training trial in which the first choice was treated as an incorrect response for all mice. Thus most mice received a considerable amount of practice on learning where to direct their avoidance response before they actually learned to avoid the footshock. Learning to anticipate the onset of shock is necessary if a subject is to learn to avoid being shocked. Most subjects learned this portion of the task by the 5th or 6th training trial. Thus, those subjects in the 10-trial group received considerable practice at avoiding the footshock (4 or 5 CR's on the average). This may not seem like much practice; yet the change in behavior over the 4-5 CR's is dramatic. The first CR is usually of a long duration (4-4.9 sec, the shock coming on at 5.1 sec). The 2nd and 3rd avoidance responses tend to show latencies of about 2.5-3 sec duration. The 4th and 5th CR's are usually less than 2 sec duration and many responses of only 0.6 sec duration. The subject making the fast latency CR's has no time to ponder the situation; the response appears to be almost automatic and will show no further improvement with additional training.

Active avoidance generates a great deal of variability. Some trends were reported which suggest that variability in the number of training trials, number of discrimination errors, rate of acquisition, and probably the total amount of shock influence the degree of learning. In order to obtain control over the amnestic effect, one needs control over the amount of learning. This control requires factoring the training data into many groups, thus making even a small experiment a major project.

The measures of learning (avoidance and escape responses) are not always reliable indicators of what and when a subject has learned; this seems particularly true of the avoidance response. Numerous mice in the control groups showed no signs of having learned to avoid shock; yet, at the retention test they required only 1 or 2 trials to make the avoidance response. While our procedure improved the reliability of the learning measures, there are still obvious discrepancies between what the training record indicated was the level of learning and what the retention test showed to be the level of learning. Thus, one can easily overtrain a subject and not be able to detect it, thus adding variance to the amnestic effect.

It appears that active avoidance is not particularly useful for a careful study of the processes underlying memory formation because (a) reliability of the learning measure is questionable, (b) too much variability is generated, and (c) the task involves learning at least 3 problems (i.e., habituation, escape, avoidance). While one is training the subject on the avoidance component, you are overtraining the subject on the escape component and even more overtraining on the habituation

that is likely to have occurred. Indeed, 20 subjects given 5 escape training trials showed a mean of 3.5 trials to make their first avoidance response when tested on the avoidance training 1 week after the escape training. Naive subjects took only 5.6 trials to make their first avoidance responses. Thus the escape training provided some savings when it came to learning to avoid the footshock. By comparison, passive avoidance remains the more useful research tool.

Amount of Drug versus Duration of Inhibition

The results of Experiment 3 raise a problem of how one can interpret the findings. The 5Ani+Ani injection caused no significant detectable impairment of acquisition. The mean trials to make the first avoidance response, the percent simple versus complex responses (Figure 5), and the duration of shock were within normal limits. Yet, 5Ani+Ani caused highly significant percent of the subjects to become amnestic, while Ani+5Ani did not. It is not at all clear how this was accomplished. The 2500 μg dose of Ani does not significantly alter the duration or extent of inhibition caused by the 500 μg dose of Ani (Flood et al., 1973). Why would the 2500 μg dose of Ani only have this greater amnestic effect when given prior to training as the first but not as the second injection? Thus the principal problem of interpreting how the large dose of Ani caused amnesia, is that no known mechanism can be related to this amnestic effect. Therefore, the amnesia caused by the large dose of Ani provides us with little information as to the mechanisms underlying long-term memory formation. The large dose of Ani could conceivably cause amnesia in many ways such as by some subtle impairment of learning, interference with electrophysiological activity or by disrupting other biochemical processes besides protein synthesis.

Possible Drug Effect on Acquisition

In the early training trials subjects escaped from shock by a simple or complex pathway. Simple pathways are those that get the subject to the goal box with a minimum of retracting of its previous run through the box for a given trial. In Experiment 1, the NaCl- and Ani-injected subjects showed no significant differences in the percent of simple versus complex responses. However, in Experiment 2, Ani-injected subjects made significantly more complex escape responses than the NaCl-injected subjects ($P < 0.01$). However, it was the NaCl group that changed between Experiments 1 and 2 (Figure 5). In spite of the very large N's in each experiment, the tendency for NaCl-injected subjects to make fewer complex escape responses does not seem reliable. In addition, when the Ani-injected subjects are compared on the complex vs. simple response measure the percent amnesia was not significantly different (44% amnesia for complex, 58% amnesia for simple). The general pattern seems to indicate that the pretraining injection of anisomycin had no systematic effect on acquisition (Figure 1). In addition, subjects given only a single pretraining injection of Ani did not show significant levels of amnesia (Figures 2 and 4).

Memory Loss with 3 Inhibitors

We have shown in this study that Ani and AXM could be administered hours after training, as part of an injection series, and cause significant amnesia, where a single pre-training injection of Ani had no detectable amnestic effect (Figure 2, Table 7). Cyclo had previously been shown to be an effective amnestic agent when administered as the second injection of a series of injections in a passive avoidance experiment (7).

Thus, Ani, Cyclo, and AXM have been demonstrated to cause amnesia at a time when they could not have impaired learning.

The results of these experiments extend the previous findings with passive avoidance to active avoidance. This extension adds additional support to the hypothesis that protein synthesis is required for long term memory formation.

Acknowledgments

This work was supported, in part, by the U. S. Atomic Energy Commission, and National Science Foundation Grant GB-30368X. We would like to express our appreciation to Mr. Robert Sheets for his careful assistance in running the behavioral experiments.

REFERENCES

1. Barondes, S. H. and H. D. Cohen. Memory impairment after subcutaneous injection of acetoxycycloheximide. Science 160: 556-557, 1968.

2. Flexner, J. B. and L. B. Flexner. Restoration of expression of memory lost after treatment with puromycin. Proc. Nat. Acad. Sci. U.S. 57: 1651-1654, 1967.

3. Flexner, L. B. and J. B. Flexner. Intracerebral saline: Effect on memory of trained mice treated with puromycin. Science 159: 330-331, 1968.

4. Flexner, L. B., J. B. Flexner and R. B. Roberts. Stages of memory in mice treated with acetoxycycloheximide before or immediately after learning. Proc. Nat. Acad. Sci. U.S. 56: 730-735, 1966.

5. Flexner, L. B., J. B. Flexner and R. B. Roberts. Memory in mice analyzed with antibiotics. Science 155: 1277-1283, 1967.

6. Flood, J. F., E. L. Bennett, M. R. Rosenzweig and A. E. Orme. Influence of training strength on amnesia induced by pretraining injections of cycloheximide. Physiol. Behav. 9: 589-600, 1972.

7. Flood, J. F., E. L. Bennett, M. R. Rosenzweig and A. E. Orme. The influence of duration of protein synthesis inhibition on memory. Physiol. Behav. 10: 555-562, 1973.

8. Flood, J. F., E. L. Bennett, M. R. Rosenzweig and A. E. Orme. Comparison of the effects of anisomycin on memory across six strains of mice. Behav. Biol., in press.

Table 1

Percent Inhibition of Brain Protein Synthesis

by AXM and by Ani and AXM

Dose of AXM µg	AXM % Inhibition 4 hr	AXM % Inhibition 6 hr	Ani^2+AXM* % Inhibition 4 hr	Ani^2+AXM* % Inhibition 6 hr	Ani^2+AXM* % Inhibition 8 hr	Ani^2+AXM* % Inhibition 10 hr
50			72	66	51	28
75			84	70	63	43
100	80	69	87	80	63	53
150	85	77	87	81		
200	84	82	90	80		
250	86	85	91	83		

*Hours means the time after the AXM injection; for total time one should add 4 hr. Thus Ani^2+AXM at the 100 µg dose has a total inhibition time of 10 hr at 80% inhibition or greater (4 hr by Ani^2 and 6 hr by AXM).

Table 2

Effects of Ani on Retention for the Left or Right Escape Response

Treatment	NaCl	NaCl+Ani^5	Ani	Ani^2	Ani^3	Ani^4	Ani^5
% Error	11.7	15	15	20	20	35	55

Naive subjects showed no left or right side preference (54% went to the right side on the first training trial); thus 50% errors could be considered complete amnesia. One assumption being made is that if one could repeatedly test a single subject to see what its first choice would be, it would show no preference. We can say that a group has no side preference. However, it cannot be determined if an individual mouse has a side preference. In the groups receiving 4 or 5 Ani injections, significant numbers of the subjects forgot which side was correct. The Ani^5 group may be completely amnestic for the escape response portion of this training task. All the groups have N's = 20 except NaCl (N = 60).

Table 3

Groups for Experiment 2

Injection Group	Time of Injection(s)	Duration of Inhibition >80%
NaCl	4 injections at times 0, 2, 4, 6 hr	0 hr
Ani	1 injection at time 0	2 hr
Ani+AXM	Ani at 0, AXM at 2 hr	8 hr
Ani^2+AXM	Ani at 0 and 2 hr, AXM at 4 hr	10 hr
Ani^3+AXM	Ani at 0, 2, and 4 hr, AXM at 6 hr	12 hr
Ani+AXM^2	Ani at 0 hr, AXM at 2 and 8 hr	14 hr

The groups used in Experiment 2, the types and times of injection and the duration of inhibition. All injections were given subcutaneously. Training is always 15 min after the first injection (first injection given at time 0).

Table 4

Rate of Acquisition and Percent Amnesia

Made 1st CR on trial No.	Percent Mice Amnestic*
7	73% (N=37)
6	63% (N=43)
5	54% (N=41)
4	10% (N=21)

*Amnesia defined as a savings score of less than 30%.

Table 5

Training Trials and Percent Amnesia

Number of Training Trials	Percent Mice Amnestic*
6	77% (N=44)
8	60% (N=35)
10	50% (N=42)

*Amnesia is defined as a savings score of less than 30%.

Table 6

Rate of Acquisition, Discrimination Errors and Percent Amnesia*

Made 1st CR on trial No.	Number of Discrimination Errors Made at Training		
	0	1	2
5	60% (N=15)	50% (N=12)	too few scores
6	60% (N=15)	60% (N=15)	60% (N=10)
7	81% (N=16)	55% (N=11)	44% (N=9)

*The percent amnesia is defined by a savings score of less than 30%. It appears as if those subjects that made more errors at the training session were less likely to be amnestic when tested 1 week after training. Across the subjects making no errors, 70% were amnestic, while 55% of the subjects making 1 error were amnestic. None of the comparisons were significant; however, large N's might confirm a weak trend.

Table 7

Duration of Inhibition and Percent Amnesia*

Treatment	Duration of Inhibition at 80% or greater	Percent Mice Amnestic
Na^4	0 hr	0% (N=39)
$Na+Ani+AXM^2$ (T-6 only)	14 hr but delayed until 1-3/4 hr post training	0% (N=10)
Ani	2 hr	7% (N=30)
Ani+AXM	8 hr	55% (N=33)
Ani^2+AXM	10 hr	55% (N=29)
Ani^3+AXM	12 hr	67% (N=27)
$Ani+AXM^2$	13-1/2 - 14 hr	73% (N=30)

*As the duration of inhibition increases, the probability increases that a subject will be amnestic at retraining. Within the groups given 8-14 hr of inhibition, the trend does not quite reach significance; however, it is generally consistent with trends in other experiments that we have reported.

Table 8

No. of Training Trials, Duration of Inhibition and Percent Amnesia*

Number of Training Trials	Duration of Inhibition					
	0	2	8	10	12	14
6	0% (N=13)	10% (N=10)	77% (N=13)	73% (N=11)	70% (N=10)	90% (N=10)
8	0% (N=10)	10% (N=10)	44% (N=0)	50% (N=10)	71% (N=7)	77% (N=9)
10	0% (N=16)	0% (N=10)	36% (N=11)	38% (N=8)	60% (N=10)	62% (N=13)

*An interaction exists such that the more trials a subject is given and the lower the level of inhibition, the lower the probability that such subjects will be amnestic at retraining. On the other hand, subjects that receive the fewest number of trials and the greatest duration of inhibition of protein synthesis are most likely to become amnestic.

Figure Captions

Figure 1. The figures that follow show the acquisition curves for two groups of subjects being trained to avoid foot shock. In Figure 1A, the cumulative distribution for the trial on which subjects made their first avoidance response during training is plotted. Each additional trial contains the percent of the preceding trials. Thus by trial 6, 70% of the subjects have made at least 1 CR. If we were to plot the percent subjects making an avoidance on each trial the curve would be almost identical because with this training procedure, once a subject starts making avoidance responses it continues to do so. Few subjects required additional shock. This curve is based on the subjects run in Experiment 2. The N's for trials 1-6: NaCl = 46, Ani = 169; trials 7 and 8: NaCl = 26, Ani = 116; trials 9 and 10: NaCl = 16, Ani = 63. In Figure 1B, the 1st avoidance response is plotted in terms of what percent of the sbujects made their 1st CR's on which trial (non-cumulative). From this nearly normal distribution, we can see that the majority of subjects have made a 1st CR on trials 5, 6, or 7.

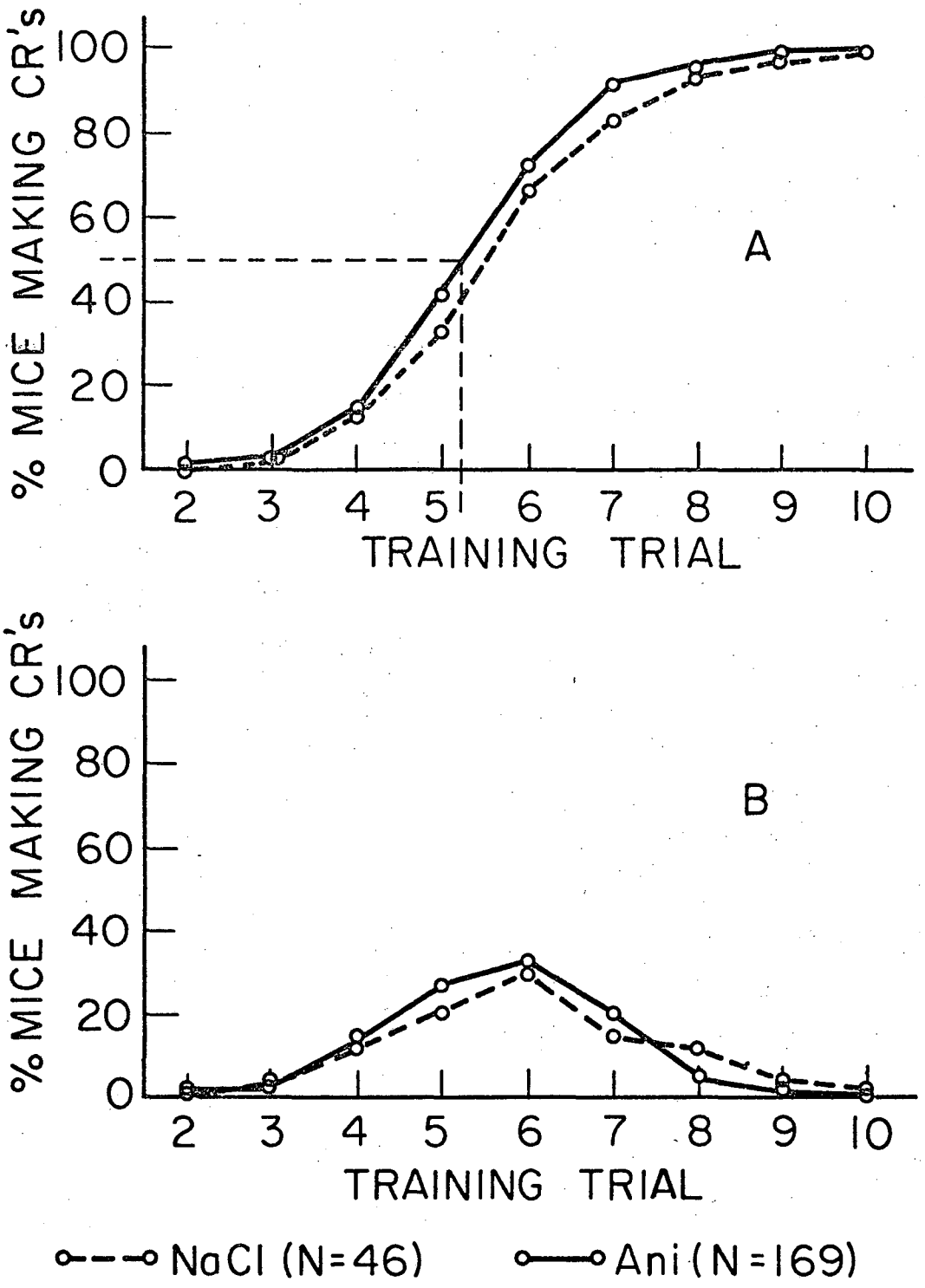
With these meausre of acquisition, NaCl and Ani did not differ significantly. The pre-training injection of Ani apparently has no adverse effect upon acquisition of avoidance training.

Figure 2. The effect of the duration of inhibition of protein synthesis by Ani on memory for footshock avoidance training.

Figure 3. The distribution of retention scores (the number of trials to make the 1st CR). The area of the combined saline groups (NaCl) was made equal in area to the other groups because the combined saline controls constitute 60 subjects while the other groups have 20 subjects each. The shaded area represents those subject's scores that have been classified as amnestic (i.e., 1st CR on trial 5 or later). Note that across the Ani groups (Ani to Ani^5) the shaded area is increasing, and the means are shifting toward the amnestic value (those greater than 4 trials). Three naive subjects learned so quickly that they are classed as having remembered the training which they never had. Thus, to some extent, even with a reasonable criterion of what constitutes retention, it is difficult to obtain 100% amnesia for this task.

Figure 4. The distribution of retention scores (trial on which the 1st avoidance response was made) as a function of the drug condition. Across the multiple injection drug groups (o——o), 59% of the subjects were amnestic on a fixed criterion basis (amnesia = 5 or more trials to make the 1st CR on retraining). Those subjects receiving only the single pre-training injection of Ani showed only 7% of the subjects to be amnestic. None of the NaCl subjects were amnestic.

Figure 5. Distribution of complex escape responses for NaCl-, Ani-, and 5Ani-injected subjects.



XBL 739-4067

Fig. 1

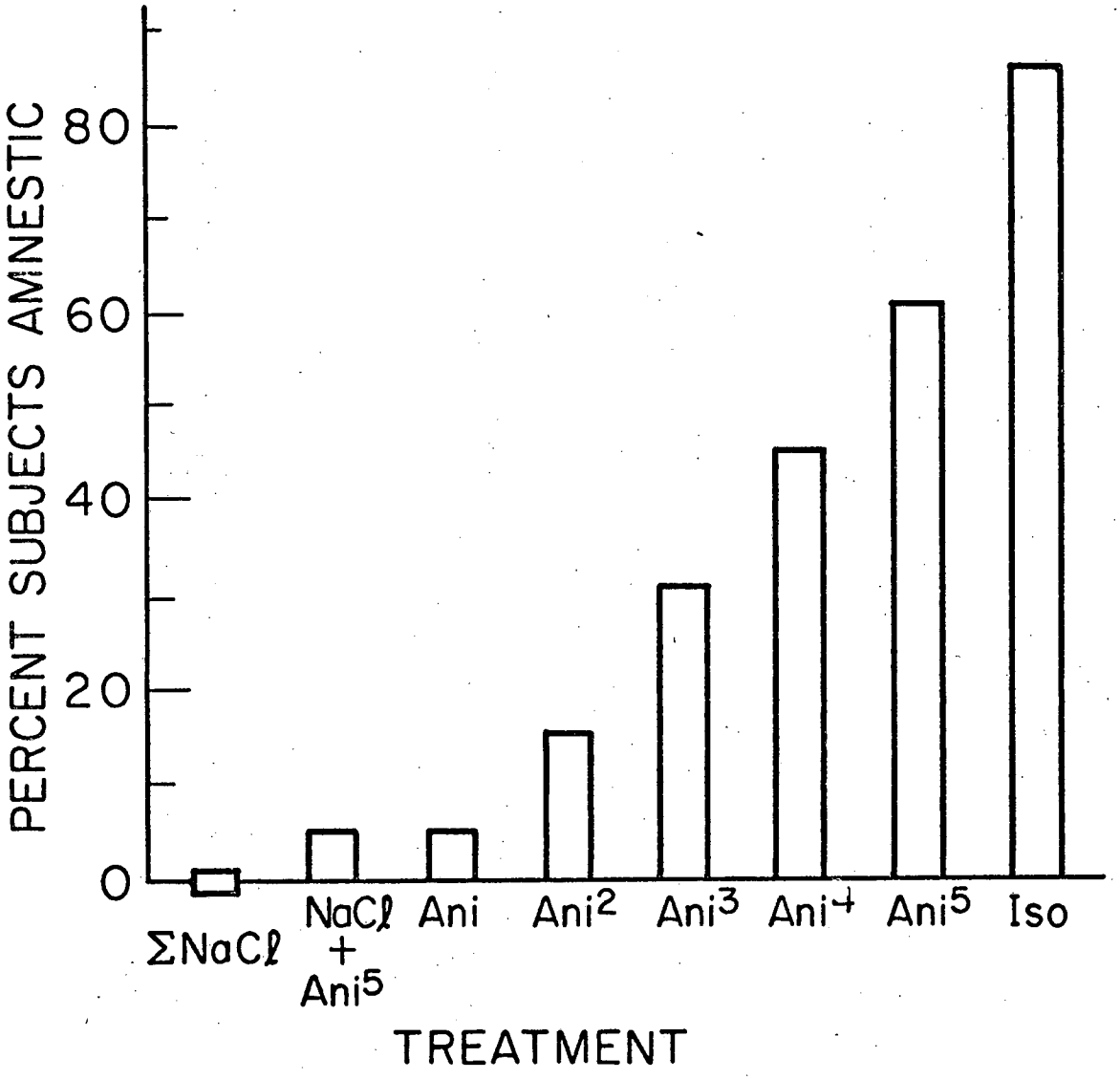

PERCENT SUBJECTS AMNESTIC
0
20
40
60
80
ΣNaCl
NaCl + Ani5
Ani
Ani2
Ani3
Ani4
Ani5
Iso
TREATMENT
XBL739-4049

Fig. 2

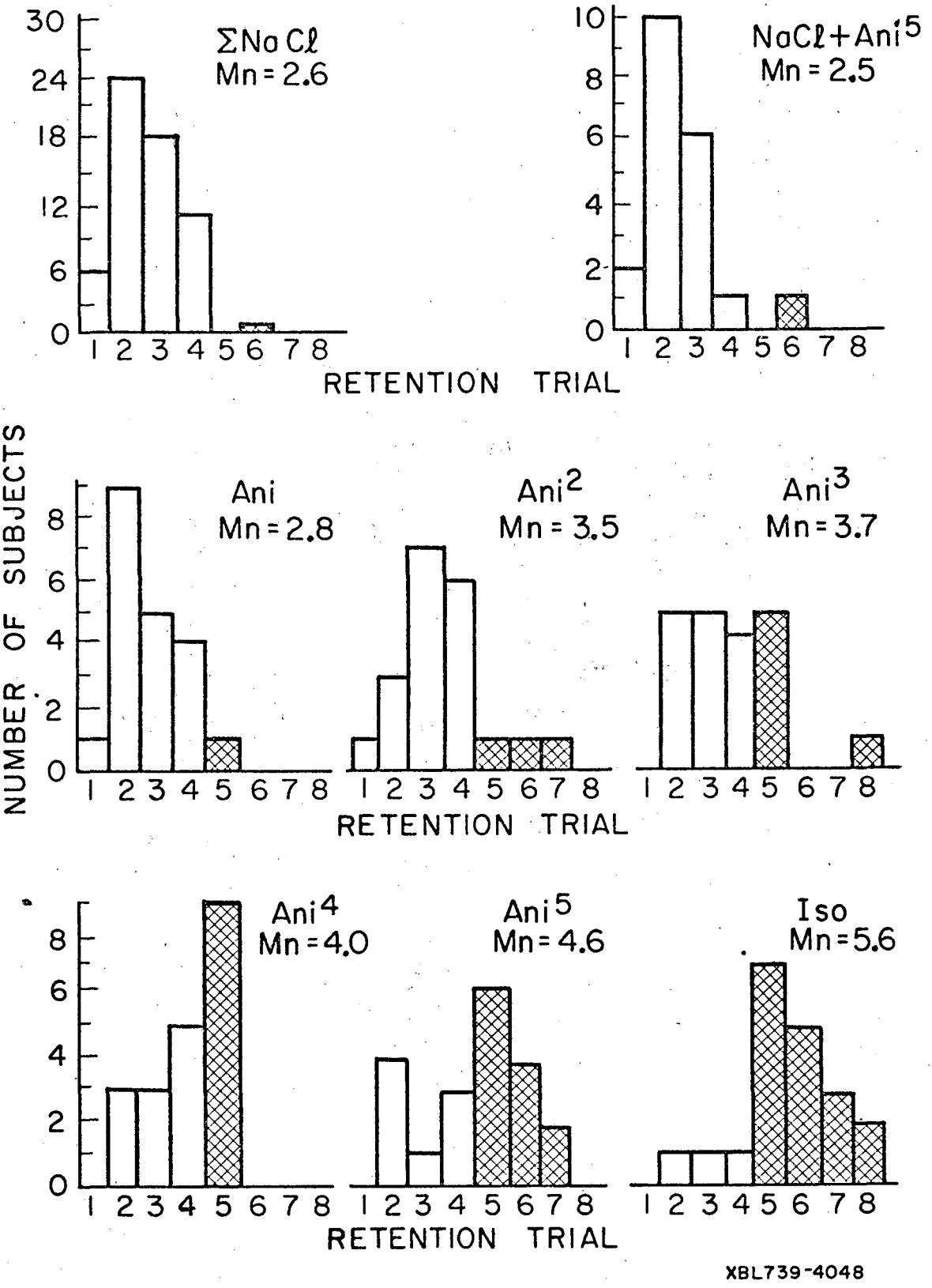

ΣNaCl
Mn = 2.6
NaCl+Ani5
Mn = 2.5
RETENTION TRIAL
NUMBER OF SUBJECTS
Ani
Mn = 2.8
Ani2
Mn = 3.5
Ani3
Mn = 3.7
RETENTION TRIAL
Ani4
Mn = 4.0
Ani5
Mn = 4.6
Iso
Mn = 5.6
RETENTION TRIAL
XBL739-4048

Fig. 3

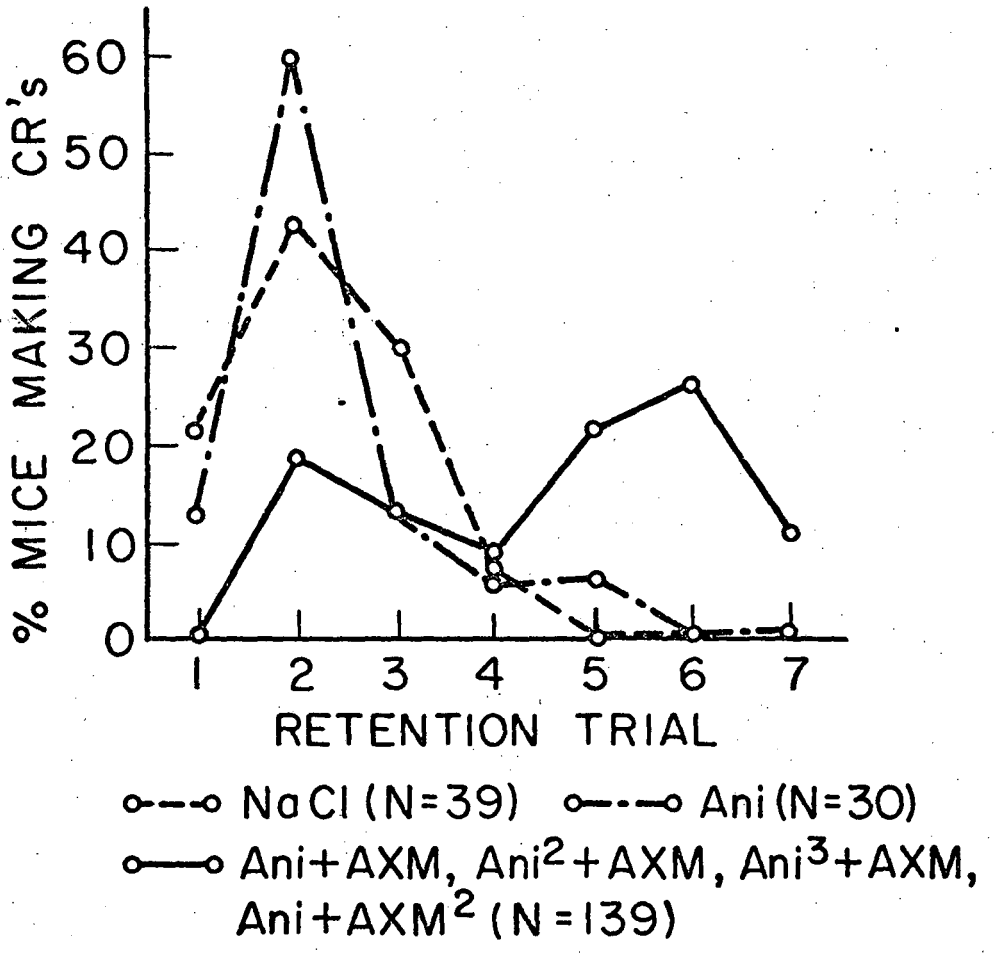

% MICE MAKING CR's
60
50
40
30
20
10
0
1
2
3
4
5
6
7
RETENTION TRIAL
NaCl (N=39)
Ani (N=30)
Ani+AXM, Ani²+AXM, Ani³+AXM,
Ani+AXM² (N=139)
XBL739-4068

Fig. 4

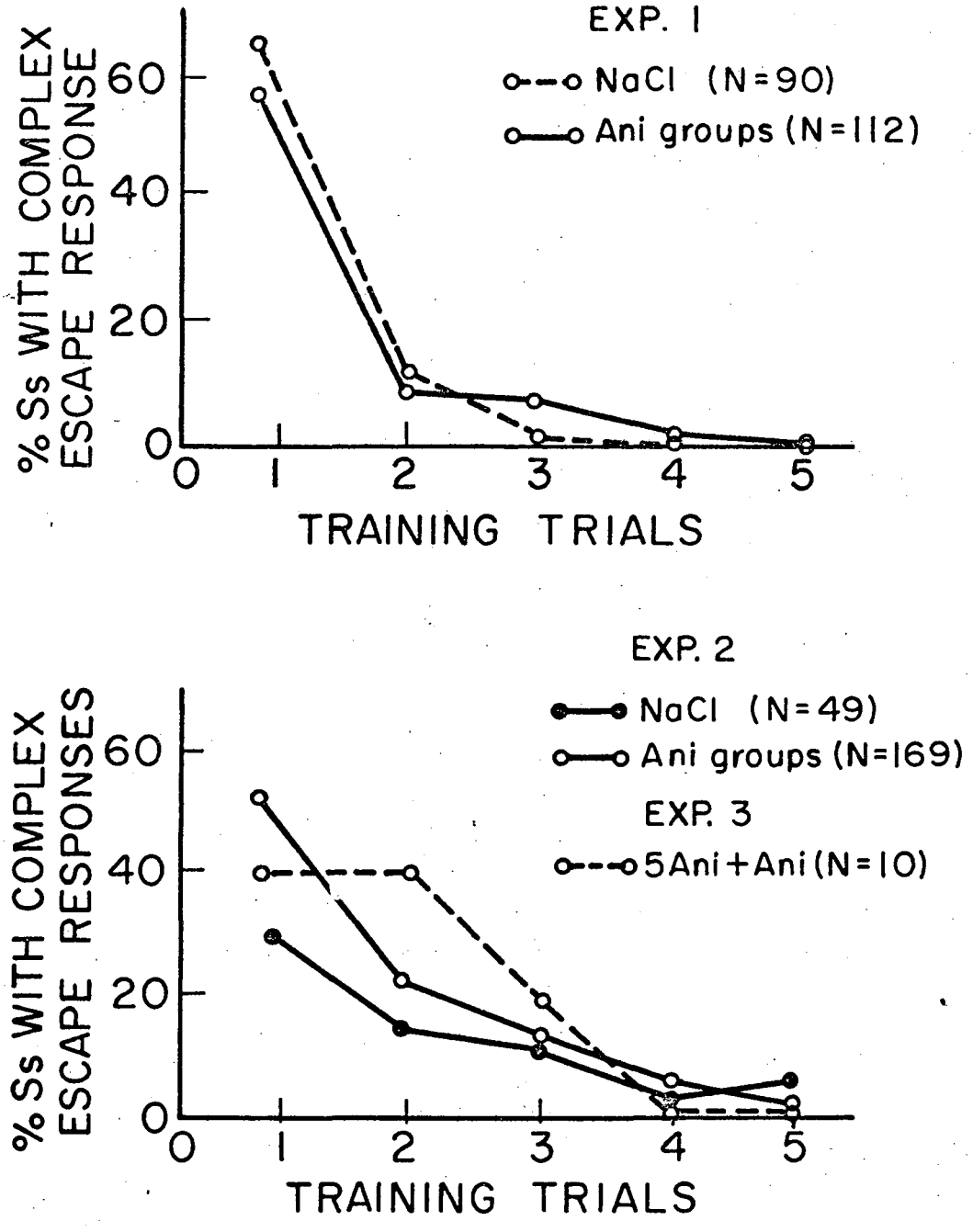

EXP. 1
NaCl (N=90)
Ani groups (N=112)
% Ss WITH COMPLEX ESCAPE RESPONSE
0
20
40
60
0
1
2
3
4
5
TRAINING TRIALS
EXP. 2
NaCl (N=49)
Ani groups (N=169)
EXP. 3
5Ani+Ani (N=10)
% Ss WITH COMPLEX ESCAPE RESPONSES
0
20
40
60
0
1
2
3
4
5
TRAINING TRIALS
XBL739-4073

Fig. 5

TECHNICAL INFORMATION DIVISION
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720