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### Publication Date

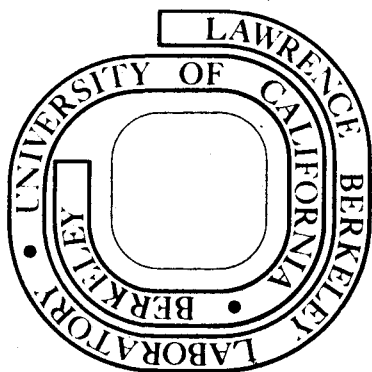
1972-08-01

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INHIBITION ON MEMORY

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August 1972

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



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# The Influence of Duration of Protein Synthesis Inhibition on Memory

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(Received 29 August 1972)

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(3) 555-562, 1973.—Anisomycin and cycloheximide were compared as inhibitors of cerebral protein synthesis and as amnesic agents in mice. Anisomycin is an effective, nontoxic inhibitor of cerebral protein synthesis. Cycloheximide, while also an effective inhibitor of cerebral protein synthesis, is far more toxic. Successive injections of anisomycin permit variation of duration of inhibition from 2-8 hr. With C57B1/Jf mice trained in passive avoidance, the longer the inhibition, the greater the percentage of amnesic subjects. The greater the training strength, the less the amnesia. Evidence indicates that cycloheximide impairs acquisition.

Anisomycin	Cycloheximide	Protein inhibition	Memory	Passive avoidance	Amnesia
Mice	Protein inhibition and amnesia	Training parameters	and memory		

THE PURPOSES of these experiments were to test the hypothesis that the length of protein synthesis inhibition is an important parameter in producing amnesia and to demonstrate the value of a new amnesic agent--anisomycin (Ani).

It has been implied in reports using inhibitors of protein synthesis to block memory formation that the duration of inhibition is an important factor in the effectiveness of the amnesic agent [1, 5, 10]. Previously it had not been feasible to test the hypothesis, since puromycin and acetoxycycloheximide had a long duration of inhibition--7-9 hr at 80% or greater inhibition depending on dose [3, 4, 6]. Furthermore, the dose of cycloheximide (Cyclo) that results in inhibition of 80% or greater for 2 hr is very nearly lethal in mice, preventing increases in dosage or the use of multiple injections.

Anisomycin offers far greater flexibility in the design of experiments on the role of protein synthesis and memory because at doses high enough to produce 80% inhibition of protein synthesis of 2 hr duration, Ani has not been found to be toxic, even when injected four times at 2-hr intervals. Anisomycin, alone or in combination with cycloheximide or streptovitacin A, can achieve various lengths of inhibition from 2-24 hr, where inhibition is maintained continuously above 80%. The subjects survive the prolonged

inhibition of protein synthesis without any obvious signs of illness.

The use of a variety of protein inhibitors in memory research is of interest to help rule out unique side effects which may interfere with the interpretation of the results. Puromycin has been found to cause many effects besides inhibition of protein synthesis such as causing hippocampal seizure, swelling of mitochondria, and disaggregation of ribosomes [9,12]. A single injection of Cyclo frequently causes animals to become ill. Behaviorally, Cyclo causes differences in locomotor activity of mice exposed to an open field [14] and in the distribution of latencies in a step-through passive avoidance apparatus [8]. Anisomycin was not found to produce such latency changes.

## GENERAL DESCRIPTION - BIOCHEMISTRY

### *Procedures*

Anisomycin (2-p-methoxyphenyl-3-acetoxy-4-hydroxy-pyrrolidine) was a gift from Charles Pfizer Co., Groton, Connecticut, through the generosity of Dr. N. Belcher. Solutions were prepared at appropriate concentrations in 0.9% NaCl. In order to dissolve Ani, an approximately equal molar amount of HCl was added, and the pH was finally adjusted to 6-7. Under these conditions, solubility

was at least 40 mg/ml.

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 C57Bl/Jf female mice [8]. Unless otherwise noted, period of precursor incorporation was 30 min.

### Results

Anisomycin is relatively nontoxic in mice. The lethal dose was not established; mice showed no obvious ill effect, except for diarrhea, upon subcutaneous administration of 10 mg, which is 20 times the dose needed to produce effective protein synthesis inhibition, or when a total of 0.4 mg was injected bilaterally into the brain.

The inhibition of protein synthesis in brain and liver was determined as a function of the dose ranging from 0.5–3.0 mg (Fig. 1). Subcutaneous injections yielded greater than 90% inhibition in brain during the first 2 hr. There was little dose dependence during this period. By intracerebral injection, a dose of at least 0.1 mg was required for 90% inhibition. By both routes of injection, the inhibition levels in liver were well below those for whole brain and marked dependence on dose was evident. These results indicate either a selective inhibition of brain protein synthesis or more efficient elimination of the inhibitor and faster recovery of synthesis in the liver.

Since increased doses of Ani did not significantly increase the duration of maximal inhibition of protein synthesis in mice, we studied the effect of repeated subcutaneous injections of Ani at 2-hr intervals. The inhibition curves for 3 successive injections showed that protein synthesis can be inhibited at least 85% for up to 6 hr, and that there is little cumulative effect of the drug (Fig. 2). Each of the injections inhibited at 80% or greater for about 2 hr. Thus, the duration of protein synthesis inhibition can be readily controlled and prolonged by an appropriate schedule of injections, with little or no illness observed.

Our best determination of the relative effectiveness of Ani and Cyclo is given in Fig. 3. The inhibition at short times after administration of the drugs was based on 10-min incorporation periods and is presented in the inset of Fig. 3. A slight difference is noted between Ani and Cyclo inhibition during the first 20 min. Since a data point represents an average for the preceding interval, it is estimated that Ani takes about 4 min to reach 80% inhibition while Cyclo takes about 2 min. During the next 90 min Ani appears to be at least as effective as Cyclo. After 3 hr, the inhibition by Ani drops off more rapidly than that caused by Cyclo.

### GENERAL DESCRIPTION - BEHAVIOR

#### Animals

The mice were females from our colony in its 14th generation of inbreeding (except in Experiment 2). They are designated C57Bl/Jf to distinguish them from the parent stock of C57Bl maintained at Cancer Research Genetics Laboratory, University of California, Berkeley. Our colony was started from a single inbred pair of C57Bl/Crgl. The animals were between 60 and 70 days of age and weighed between 18 and 21 g at training. Animal assignment was random; where several groups were employed, each condition was represented at each training and testing session.

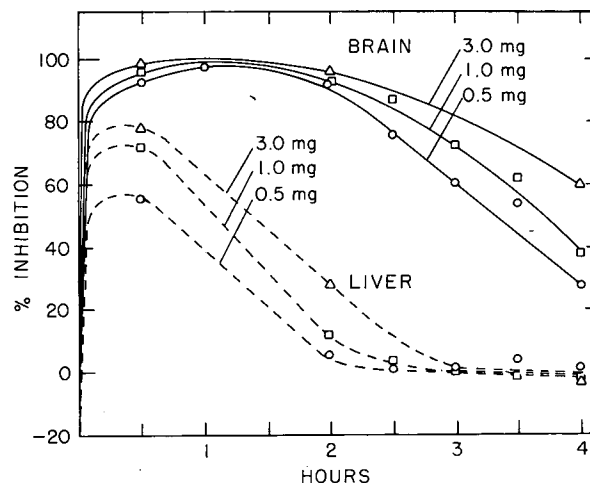


FIG. 1. Inhibition of protein synthesis by subcutaneous injection of anisomycin in C57Bl male mice for liver and whole brain.

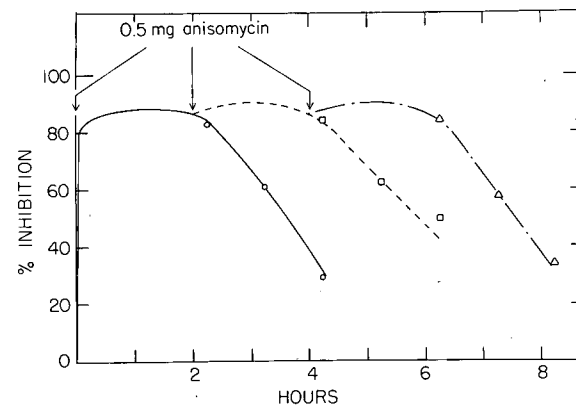


FIG. 2. A summary of the effect on cerebral protein synthesis of one (○—○), two (□—□) or three (△—△) subcutaneous injections of 0.5 mg of anisomycin to C57Bl/Jf female mice. Ani was administered at time 0, 2 and 4 hr. The symbols indicate times at which the mice were sacrificed.

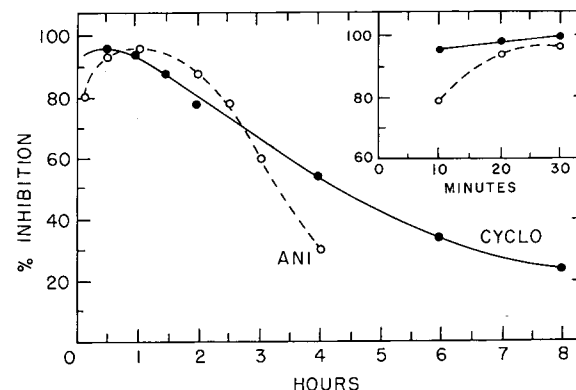


FIG. 3. Comparison of the inhibition of protein synthesis in brain by anisomycin (0.5 mg) or cycloheximide (2.5 mg) determined in C57Bl/Jf female mice.

Animals had food and water available at all times.

*Housing*

Animals were placed in individual metal cages (4x8 in) three days prior to training. Immediately after training, an animal was returned to its cage in a quiet room. Animals were not handled or disturbed until the retention test.

*Apparatus*

The training task was step-through passive avoidance. The training apparatus has been described previously [8]. In brief, it consists of an alley made of Plexiglas divided into a 3-1/2 in. long black start box and a 14 in. long white box illuminated only by a lamp situated behind a translucent panel at the end of the white box. The black and white compartments were separated by a black panel with a 1-1/2 in. dia. mouse hole. A white translucent Plexiglas guillotine door blocked the hole and prevented entry into the white box until training was begun.

Shock was delivered by a high voltage, constant current 18 pole shock scrambler. The shock was administered through 3/32 in. brass floor rods in the white box. The intensity of the current was measured to be 0.30, 0.33 or 0.36 ma at its source. The apparatus was wiped dry after every animal was trained and was washed after 6 animals were trained.

*Drug Condition*

All injections, whether administered pre- or posttraining, were given subcutaneously at the following volumes and concentrations (except for Experiments 2 and 7): saline, 0.25 ml; cycloheximide, 0.23 ml of 10 mg/ml solution; and anisomycin, 0.25 ml of 2 mg/ml solution. Drugs were prepared in saline. Injections were given under very light ether anesthesia; the mice recovered motor coordination within a few minutes. The light anesthesia was used in order to reduce variability in behavioral arousal caused by individual differences in reaction to a non-ether injection procedure that was tested. In addition, it was felt that the greater arousal produced by a non-ether injection procedure might interfere with the amnestic treatment given after training.

*Training*

Fifteen min after receiving its injection, an animal was placed into the black start box for 20 sec; next the light illuminating the white shock box and mouse hole was turned on for 20 sec. The white guillotine door separating the two compartments was then removed, giving the mouse access to the white box. A stop watch was started when the animal oriented toward the mouse hole. In most cases, orientation occurred immediately after the guillotine door was removed. The latency-to-enter was recorded when the animal had all four paws on the grid of the white box. As soon as the animal entered the white box, the experimenter flipped a switch that delivered continuous shock 5 sec later. After the mouse escaped from the shock into the black start box, the guillotine door was replaced and the light was turned off. Ten sec after the animal returned to the start box, it was removed to its home cage.

*Retention Test*

The testing procedure was the same as for training

except that no shock was given. Timing of the latency-to-enter started as on the training day when the animal oriented toward the mouse hole. Animals not crossing into the white box within 300 sec were removed to their home cages, except in Experiments 2 and 5 through 7 where 180 sec was the cutoff latency.

We shall refer to the latency-to-enter on the training day as the training latency and to the latency-to-enter on the test day as the test latency. Amnesia will be defined as a test latency of 20 sec or less. Amnesia is defined as a test latency of 20 sec because this represents the maximum range of latencies-to-enter for uninjected naive C57Bl mice. The use of the 180 or 300 sec test period allows a clear distinction between amnesia, impaired memory and retention. Escape latency is the time from shock onset until the mouse returns to the black start box.

COMPARISON OF ANISOMYCIN (ANI) AND CYCLOHEXIMIDE (CYCLO)

*Experiment 1*

*Design.* The purpose of this experiment was to determine the relative effectiveness of the drugs as amnestic agents over a range of shock intensities (0.30, 0.33, or 0.36 ma). The injection was given 15 min prior to training, and the retention test was given 24 hr after training. As in previous experiments [8], the mode of the escape latencies was 0.02 min; only the data from animals escaping shock in 0.02 min will be compared.

*Results.* While Ani and Cyclo caused comparable inhibition of protein synthesis (Fig. 3), Ani proved not to be as effective an amnestic agent as Cyclo (Fig. 4). Ani was only effective as an amnestic agent at 0.30 ma. Cyclo became less effective as the shock intensity increased. At shock intensities 0.30 and 0.33, Cyclo injected groups showed significantly greater percentage of amnesia than Ani injected animals ( $\chi^2 = 9.59, p < 0.005$ ; at 0.36 ma a significant difference was not obtained,  $p = 0.10$ ). Since Ani and Cyclo were found to cause comparable inhibition of protein synthesis, cycloheximide's greater amnestic effect must be due to some factor in addition to inhibition of protein synthesis.

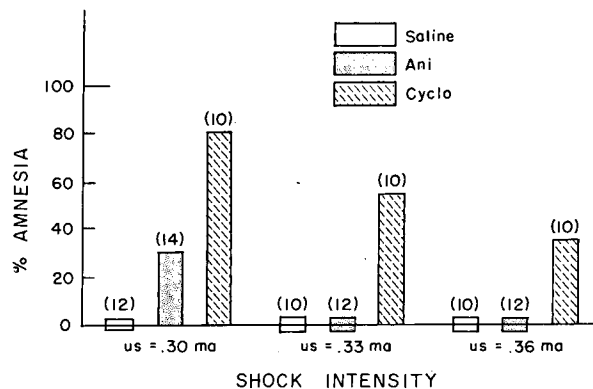


FIG. 4. Effect of shock intensity on anisomycin and cycloheximide induced amnesia. Cyclo vs. Ani at 0.30 ma.  $\chi^2 = 9.6, df = 1, p < 0.005$ ; at 0.33 ma  $p = 0.01^*$ ; at 0.36 ma  $p = 0.10^*$ . Cyclo .30 vs. Cyclo .33 vs. Cyclo .36 ma  $\chi^2 = 9.59, df = 2, p < 0.01$ . \* $p$  calculated by Fisher Exact Probability Test. Retention given 24 hr after training. The number in the parentheses, above the bars, equals the N/group.

### Experiment 2

**Design.** This experiment investigated the effects of training latency and escape latency on the probability of obtaining amnesia with Ani or Cyclo. Animals, C57B1 males, were classified into four groups according to their performance during the training session: (I) short training and short escape latency—this provided minimal training; (II) long training and short escape latency; (III) short training and long escape latency; (IV) long training and long escape latency—this provided the maximal training. The particular latency values for each training condition may be seen in Fig. 5. Ani (0.5 mg), Cyclo (3.0 mg) or saline were injected 15 min prior to training. Males rather than females were used in this experiment because previous studies had shown that the incidence of death or illness in training conditions III and IV would be too high in Cyclo injected females.

In order to generate long training latencies, we placed under the grid of the apparatus paper that had been urinated on by other male mice. The apparatus was washed as usual but placed on dirty paper. Even with this procedure many mice entered with short training latencies. The technique was particularly ineffective with Cyclo injected animals; only 1 in 3 mice could be classified in training conditions II or IV.

Our procedure for generating long escape latencies has been described [8]. It involves replacing the guillotine door after the subject enters the white box and not removing it until a few sec after shock onset. We have compared data from subjects having naturally occurring long latencies with those subjected to this confine-escape procedure and have found that the two procedures do not produce different results. A retention test was given 1 wk after training.

**Results.** In this experiment as in Experiment 1 with single injections, Cyclo proved to be a more effective amnestic agent than Ani. Figure 5 shows that both drugs were highly effective under the lowest conditions of training, both causing 95% amnesia. However, under higher conditions of training Cyclo was significantly more effective than Ani.

The experiment also showed that as training strength increases (i.e., longer duration of training latency or escape latency) the percentage of amnestic animals decreased. This effect is particularly dramatic across the Ani groups: Condition I showed 95% amnesia while Condition IV showed only 10%.

Apparently, the drug has a far greater amnestic effect on the strain used in this experiment (C57B1) than on the substrain (C57B1/Jf) used in all other experiments. Since C57B1 require more intense shock than the C57B1/Jf mice to learn this passive avoidance task, the C57B1 were not as thoroughly trained in this case even though training conditions were the same for the two strains.

#### EFFECTS OF DURATION OF INHIBITION OF PROTEIN SYNTHESIS

### Experiment 3

**Design.** The purpose of this experiment was to see whether doubling the duration of inhibition would cause more animals to become amnestic. All animals were injected at time zero and trained 15 min later. The groups employed can be seen in Fig. 6. The Ani and Na groups received only the pretraining injections. Na+Na, Ani+Ani

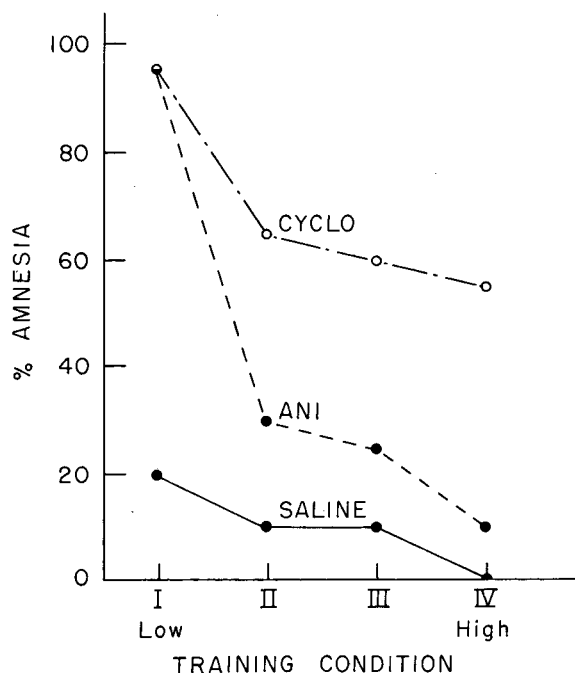


FIG. 5. Comparative amnestic effect of anisomycin or cycloheximide as a function of training strength.  $N = 20/\text{point}$ . Ani vs. Saline at I and II,  $p < 0.001$ ; at III,  $p < 0.002$ ; at IV,  $p = \text{NS}$ . Cyclo vs. Saline at I, II, III, IV,  $p < 0.001$ . Ani vs. Cyclo at I,  $p = \text{NS}$ ; II, III,  $p < 0.025$ ; IV,  $p < 0.001$ . \* $P$  obtained by  $\chi^2$  Test, all others by Fisher Exact Probability Test. Retention given 1 wk after training. The latency values for the four training conditions were:

Training Latency (sec)	Escape Latency (min)
I 1 - 4.9	0.01 - 0.04
II 5 - 8.4	0.01 - 0.04
III 1 - 4.9	0.05 - 0.08
IV 5 - 8.4	0.05 - 0.08

and Na+Ani received one injection before training and another 2 hr after the first injection. Ani+I was injected prior to training and pseudo-injected 2 hr later; nothing was injected. The shock intensity was set at 0.33 ma. The retention test was given 1 wk after training to animals which had an escape latency of 0.02 min with short and long training latency. Other conditions were as described under the heading General Description.

**Results.** Two successive injections which maintained the inhibition at 80% or greater for 4 hr caused significantly more amnesia than a single injection of Ani which inhibited protein synthesis for 2 hr at 80% or greater ( $p = 0.001$ , Fisher Test). The injection procedure itself had no significant effect on the percent amnesia as demonstrated by the low percentage of amnestic animals in the control groups Ani+I, Na+Na and Ani+Na. A second injection of Ani alone did not cause any amnesia (Na+Ani).

### Experiment 4

**Design.** The purpose of this experiment was to see if the increase in amnestic effects reported in Experiment 3 with

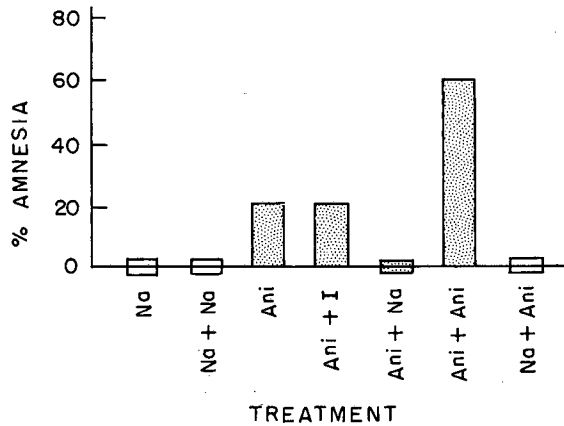


FIG. 6. Effects of two successive injections of Ani on memory and controls for double injecting procedure. N = 10 for each group except Ani+Ani (N = 20). Ani+I + Ani vs. Ani+Ani  $\chi^2 = 6.67$ ,  $df = 1$ ,  $p < 0.01$ . Retention test given 1 wk after training.

a greater duration of inhibition was unique to Ani or whether Cyclo, given as the second injection, would also have greater effects than a single injection of Ani. The drug conditions in this experiment were Ani+Ani, Ani+Cyclo and the control condition Na+Na. The procedures were as described in Experiment 3, except under these training conditions short and long training latency groups were not combined.

**Results.** While the effect of Ani+Ani was to block protein synthesis in the brain for 4 hr at 80% or greater, the effect of Ani+Cyclo was to block protein synthesis for 5-1/2 hr at 80% or greater.

In keeping with the hypothesis that a longer duration of inhibition produces greater amnesia, Cyclo could be substituted for Ani and achieve at least as high a percentage of amnesia. In fact, Ani+Cyclo gave a somewhat greater amnesic effect than Ani+Ani for both short and long training latencies, but the difference was not significant (Fig. 7). Both drug treatments were significantly less effective at long training latencies (between 4.4 and 8.4 sec) than at short training latencies (less than 4.4 sec,  $p < 0.001$ ). The saline controls showed no amnesia.

**Experiment 5**

**Design.** This experiment further tests the effect of the duration of inhibition and training strength on the incidence of amnesia. C57Bl/Jf female mice were assigned to the following groups: Ani, Ani+Ani, Ani+Ani+Ani and their controls Na, Na+Na, Na+Na+Na. The first injection was given at time zero, training 15 min later and the 2nd and 3rd injections at 2 and 4 hr respectively. To vary the training strength, the 4 training conditions of Experiment 2 were used. The retention test was given 2 weeks after training. The shock intensity was 0.33 ma.

**Results.** The results of this experiment are presented in Fig. 8. The saline controls showed virtually no amnesia; only 2 out of 96 animals were amnesic. A single pretraining injection of Ani was only effective as an amnesic agent under conditions of minimal training. Two injections of Ani were significantly more effective. Three injections of Ani produced significant amnesia in all four training conditions; nearly all animals were amnesic under Conditions I and II.

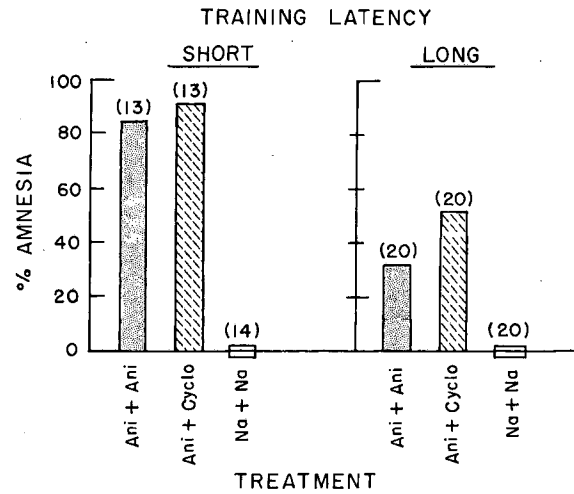


FIG. 7. Effects of prolonged inhibition of protein synthesis on memory. Ani+Ani or Ani+Cyclo vs. Na+Na,  $p < 0.001$ , for short or long latencies (Fisher Exact Probability Test). Ani+Ani vs. Ani+Cyclo  $\chi^2 = 1.67$ ,  $df = 1$ ,  $p < 0.25$ . Retention test given 1 wk after training. The number in parentheses, above the bars, equals the N/group.

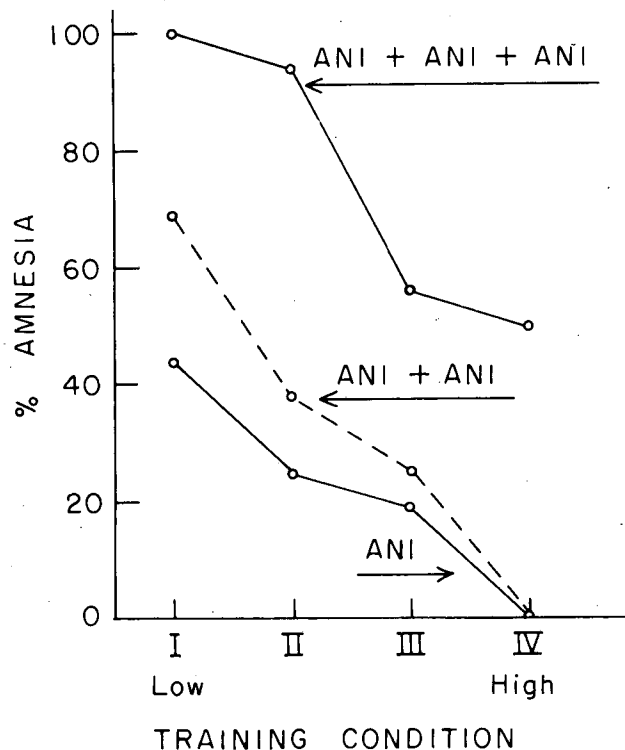


FIG. 8. Percentage of animals showing amnesia on retest after receiving one of four levels of training and one, two or three successive injections of anisomycin (Ani) at 2-hr intervals. Of 96 saline control mice, only 2 showed amnesia; these control data are not included in the graph. We considered a clear amnesic effect to be present when at least 35% of the subjects of a group showed amnesia, although all drug groups showing at least 25% differed at the 0.05 level (Fisher Exact Probability Test) from controls receiving the same training. Parameters of training conditions are shown in the caption of Fig. 5. Retention test given 2 wk after training. Ani, N=16/point; Na, N=8/point.



### Experiment 6

*Design.* The purpose of this experiment was to see if three delayed posttrial injections would cause amnesia. The experiment used the weaker training Conditions I and II as described in Experiment 2. Two groups, Ani+Ani+Ani and Na+Na+Na, were given treatments as described in Experiment 5. The third group, I+Ani+Ani+Ani, received a pseudo injection under light anesthesia 15 min prior to training, the same time the other two groups received their first injection. The series of three Ani injections started 1 hr and 45 min after training, the time for the second injection in the other two groups. Each group had an N = 10. Other training conditions are as described in Experiment 5. The retention test was given 2 wk after training.

*Results.* A series of three injections of Ani started 1 hr and 45 min after training (I+Ani+Ani+Ani) did not have the amnestic effect of the Ani+Ani+Ani group. The percentage of amnestic animals was: Ani+Ani+Ani=100%, I+Ani+Ani+Ani=10%, and Na+Na+Na=0%.

### Experiment 7

*Design.* The purpose of this experiment was to see if the greater amnestic effect of multiple injections was due to the increase in the duration of protein synthesis inhibition or due to the increased quantity of the drug being administered. The experiment used the higher training Conditions III and IV as described in Experiment 2. In each group the first injection was given 15 min prior to training and subsequent injections were given at 2-hr intervals. Two groups received four injections Ani+Ani+Ani+Ani and Na+Na+Na+Na. Another group received three injections Ani+Ani+Ani. Two groups received two injections Ani+Ani and Ani+3Ani; that is, the last group received one standard injection (0.5 mg in 0.25 ml) prior to training and another injection of Ani 3 times as concentrated (1.5 mg in 0.25 ml) 2 hr later. Therefore, Ani+3Ani had as much anisomycin as Ani+Ani+Ani+Ani. The N was equal to 10 for each group except Ani+Ani+Ani which was equal to 20. Other training and testing conditions were as described in Experiment 5. The retention test was given 2 wk after training.

*Results.* The results demonstrated that the duration of inhibition of protein synthesis and not the quantity of drug administered was responsible for the greater amnestic effect obtained with multiple injections. The percentage of amnestic animals was: Ani+Ani = 20%, Ani+3Ani = 30%, Ani+Ani+Ani = 50%, Ani+Ani+Ani+Ani = 80%, and Na+Na+Na+Na = 0%.

## DISCUSSION

### *Amnestic Effects of Multiple Injections*

Posttrial injections were employed to lengthen the duration of inhibition of protein synthesis and thus to test whether duration of inhibition was an important parameter in determining the percentage amnesia obtained. The effect of longer duration of inhibition was to increase the incidence of amnesia when the training conditions remained constant (Experiments 3--5,7).

The experiments show that in principle any increase in training strength that blocks amnesia, can be countered with longer inhibition of protein synthesis to reestablish a high level of amnesia. Observations made in several of the experiments demonstrated that the greater amnestic effects

of longer durations of inhibition of protein synthesis were not restricted to a single inhibitor and could not be attributed to the injection procedure itself, to the total quantity of the drug administered or to illness caused by the drugs. Greater amnesia with increased duration of inhibition could be obtained by giving either Ani or Cyclo after training (Experiment 4). The use of Cyclo in this regard was limited to a single injection because of its toxicity. The injection procedure itself was not found to have any detectable amnestic effect on control or on drugged animals (Experiments 3--7). The greater quantity of Ani injected in the multiple injection groups was not responsible for the greater percentage of amnestic animals. The duration of inhibition of protein synthesis, rather than the dose of Ani administered per se, was found to control the percentage of amnestic animals (Experiment 7). Animals receiving a large dose of Ani were not incapacitated so as to make memory or recall impossible (Experiment 6).

### *Comparison of Ani and Cyclo*

With the doses used both Ani and Cyclo produced significant amnestic effects. Both drugs shared the property that they became less effective amnestic agents as the training strength increased (shock intensity, Experiment 1; duration of training latency, Experiments 2,4). From some of the evidence presented above, we would like to argue that when Cyclo was injected shortly before training it not only caused amnesia but also produced a mild impairment of acquisition.

With pretraining injections (Experiments 1 and 2), Cyclo was a more effective amnestic agent than was Ani. However, following an initial pretraining injection of Ani, posttraining injections of either Cyclo or Ani were found to cause about the same amount of amnesia (Experiment 4). Thus Cyclo, relative to Ani, produced greater amnesia when given prior to training than when it was administered after training. The discrepancy in the relative effectiveness of the two drugs, pretraining versus posttraining, suggests that Cyclo when administered prior to training caused some impairment of acquisition in addition to blocking memory formation. Squire and Barondes [13] have reported that Cyclo impaired acquisition of an active avoidance task in mice. The amnesia caused by a posttraining injection of Cyclo (when it could not have interfered with training) demonstrated that Cyclo is a powerful amnestic agent (Experiment 4).

The amnestic effect of Cyclo when given prior to training, while it reflects some impairment of acquisition, seems to us to be due primarily to interference with mechanisms of memory formation. No obvious difficulties were observed in training Cyclo injected mice. Furthermore, groups of Cyclo injected animals responded to small changes in training parameters as did Ani injected animals. In both cases, small increases in training strength could prevent amnesia. Thus, it would appear that mice under the influence of Cyclo or Ani at the time of training are sensitive to small changes in shock intensity, shock duration, and time (training latency); no evidence exists that perception or motor activity are severely disrupted. The impairment of acquisition caused by Cyclo must be mild and could not by itself account for the high levels of amnesia obtained.

Since Ani and Cyclo have effects on biochemistry and behavior that are similar in some ways and that are

different in others, and since this is the first report of the use of Ani in experiments on memory, it is worth noting briefly that Ani and Cyclo inhibit protein synthesis by somewhat different mechanisms [10].

Cyclo, along with the closely related compounds acetoxycycloheximide and streptovitacin A, belongs to the class of antibiotics known as glutarimides. This class of antibiotics inhibits peptide chain initiation as well as chain elongation by interaction with the large 60S ribosomal subunits. They interfere with several steps involved in the translocation of the peptide chain along the ribosomes, including release of transfer RNA and movement of messenger RNA along the ribosome. On the other hand, Ani does not appear to interfere with either peptide chain initiation or translocation, but instead interferes with the process known as transpeptidation both by interfering with the catalytic center and by interaction with the peptidyl transferase.

*Side Effects of Ani and Cyclo*

Cyclo has been reported first to increase and then to decrease locomotor activity when given prior to an open field test [14]. Hyperactivity among Cyclo injected animals is apparent when comparing the distribution of training latencies for Cyclo, Ani and saline injected animals. Ani and saline injected animals did not differ significantly in their distribution of training latencies, but both differed significantly from Cyclo injected animals (Fig. 9). As can be seen in Experiments 2 and 5, the training latency is an important parameter of learning; thus failing to match samples of injected animals for their training latencies could bias the results. In all the experiments reported in this paper, samples were matched for latencies when reporting amnesic effects.

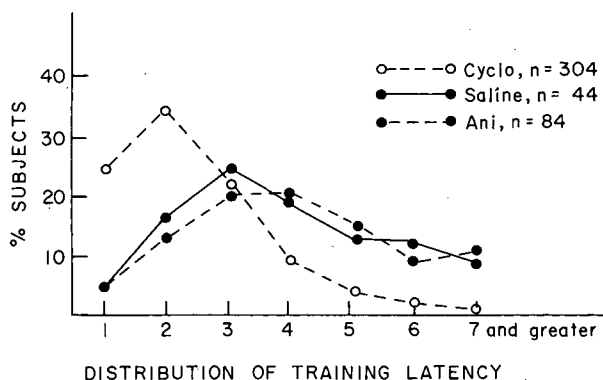


FIG. 9. Effects of anisomycin or cycloheximide on the distribution of latencies to enter the white shock box on the training day. The data points for Saline and Ani came from Experiment 2. The data for Cyclo comes from a previous report [8], but the distribution of latencies for the Cyclo injected animals of Experiment 2 tended to be the same shape except for certain irregularities, probably due to the much smaller N. Ani vs. Saline  $\chi^2 = 1.65, df = 6, p < 0.05$ . Ani+Saline vs. Cyclo  $\chi^2 = 75.14, df = 6, p < 0.001$ .

With a minimum dose of Cyclo that produces 80% or greater inhibition of brain protein synthesis, illness frequently follows injection, and even death may occur with more stressful training [8]. Interestingly, when Cyclo is injected after instead of before training, it usually does not

cause prolonged illness. We have found that two injections of Cyclo given 2 hr apart caused death within 24 hr in 8 out of 8 male mice trained on passive avoidance. In contrast to Cyclo, Ani when injected with a minimum dose that produces 80% or greater inhibition has not been found to produce any gross signs of illness and never has it proved lethal—not even with four successive injections.

The antibiotic nature of Ani and Cyclo causes diarrhea although this is hardly noticeable in Ani injected mice.

*Overtraining*

It has been observed that subjects that are highly trained will not develop amnesia in spite of better than 80% inhibition of cerebral protein synthesis for 2 hr [2,8]. These studies have shown that small increases in the strength or amount of training will prevent amnesia from developing where otherwise amnesia would occur. Our studies have shown that increases in training strength must be coupled with increases in the duration of inhibition if one is to maintain a high level of amnesia. Thus the lack of amnesia in overtrained animals can be seen as being due to a duration of inhibition that is insufficient to counter the effects of the degree of training. In such overtrained animals, protein synthesis related to memory formation would occur after protein synthesis returns to normal.

*Comment*

These results indicate that when protein synthesis is blocked for several hours, synthesis related to memory may still take place upon termination of the inhibition. This does not necessarily imply that consolidation of memory in control subjects takes place over a several hour time span.

In our experiments testing only healthy animals we have always observed the amnesia to be permanent. Some reports from other laboratories have indicated that sometimes Cyclo injected mice and AXM injected mice and rats develop amnesia when tested 24 hr after training, but 7 days later they are not found to be amnesic. While this transient amnesia has been reported by a few investigators [12,13], almost nothing is known about the conditions necessary for reliably obtaining such results. Quartermain *et al.* [11] showed that mice trained at high shock intensity (1.6 ma) developed transient amnesia, while mice trained at low shock intensity (0.16 ma) developed permanent amnesia for step-through passive avoidance training.

In an experiment designed to compare the effects of Cyclo on the memory for passive avoidance over a three-week retention period, we reported [8] that the percent amnesic subjects increased from 16% at 24 hr, to 37% at 1 week, and 57% at 2 weeks after training (N's = 84, 73, 80 for the three retention periods). In the present report, a comparison of the effect of a single pretraining injection of Ani in Experiments 1, 3, and 5 with retention tests given at 24 hr, 1 and 2 weeks respectively shows that the percentage of amnesic animals increases from 0% at 24 hr, to 20% at 1 week, and to 45% at 2 weeks after training. Amnesia reported in both this and the previous paper is not only permanent but also increases progressively in magnitude whether the agent is Cyclo or anisomycin.

The increase in the percentage of amnesic animals when a pretraining injection of Ani is followed by posttraining injections of Ani demonstrates that protein is required for the formation of longterm memory. While we have found no evidence that Ani impairs acquisition, it is still possible

that some impairment occurs. However, the posttraining injections increase the level of amnesia when impairment of acquisition is not possible; these findings are thus strong support for the role of protein synthesis in the formation of those changes in the CNS that serve memory.

### CONCLUSIONS

(1) The duration of inhibition of protein synthesis in mouse brain can be controlled by giving successive injections of anisomycin (Ani) at 2-hr intervals. Each injection was found to produce about 2 hr of inhibition at 80% or greater in the brain at doses far below the lethal toxic dose of the drug. In contrast, cycloheximide (Cyclo) must be used at the near-lethal dose.

(2) Cyclo and Ani were found to cause similar time courses of inhibition of protein synthesis in brain, but not in liver. To the extent that they differed in brain, Cyclo resulted in slower recovery of protein synthesis.

(3) With posttraining injections, Cyclo and Ani were found to cause similar amounts of amnesia for the step-through passive avoidance task, but with a pretraining injection Cyclo was more effective than Ani. It was argued and evidence was presented that Cyclo caused some impairment in acquisition and thus it appeared to be a more effective amnesic agent than Ani.

(4) As training strength increases, single pretraining

injections of Ani or Cyclo were found to become less effective in causing amnesia. This was also true when multiple injections were employed.

(5) With constant conditions of training, increased duration of protein synthesis caused greater amnesia. The greater the duration of inhibition, the greater the amnesia. This greater effectiveness could not be attributed to either the multiple injection procedure or to the greater dose of Ani, per se, that was used in the multiple injection groups.

(6) Within practical limits of increasing training strength and duration of inhibition of brain protein synthesis, it has in principle been demonstrated that for any increase in training strength that blocks amnesia, a duration of inhibition exists that will reestablish the amnesia. Similarly for any duration of inhibition that blocks memory, a greater training strength exists that will block the amnesia.

(7) The effects of increased duration of protein synthesis inhibition in brain on memory supports the hypothesis that protein is required for longterm memory to become established.

### ACKNOWLEDGMENTS

This research received support from the U. S. Atomic Energy Commission and by Grant GB-30368 from the National Science Foundation. We express our appreciation to Mr. Bogdan Roman for assistance in determination of the level of inhibition of protein synthesis.

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