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PROTEIN: THE FABRIC OF MEMORY

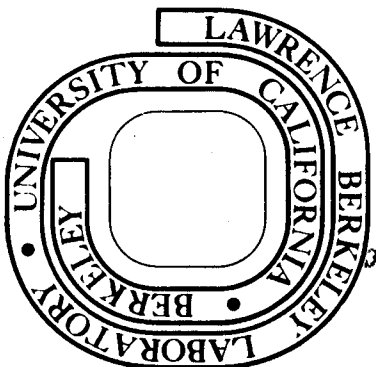
James Felix Flood, II
(Ph. D. Thesis)

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PROTEIN: THE FABRIC OF MEMORY

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ABSTRACT

Inhibitors of cerebral protein synthesis were employed to investigate the role of protein synthesis in memory formation. Cycloheximide, acetoxycycloheximide, and anisomycin, inhibitors of protein synthesis, were employed in studies of inhibition of brain protein synthesis and in studies of learning and memory in mice.

Cycloheximide- and anisomycin-treated mice and saline controls were given one-trial training in a black and white, step-through, passive avoidance task. The following training parameters were found to affect retention in drug and saline-control mice in six genetically distinct strains: (a) shock intensity, (b) shock duration, (c) original latency to enter the shock compartment, and (d) retention interval. When these parameters are measured and controlled, highly consistent amnesic effects can be obtained.

Anisomycin and cycloheximide were compared as inhibitors of cerebral protein synthesis and as amnesic agents in several strains of mice. Both inhibitors were effective at stopping 80 to 98% of the cerebral protein synthesis in several strains of mice. Anisomycin has never proved toxic. However, even minimal

doses of cycloheximide were found to be quite toxic and, under certain conditions of training, lethal. Anisomycin could be given repeatedly at 2-hour intervals, thus permitting an extension of 80% or greater inhibition for several hours (e.g., 2-12 hrs). Mice receiving two successive injections of cycloheximide die within 24-48 hours. Evidence was obtained that cycloheximide caused some mild impairment of acquisition for passive avoidance; yet anisomycin appeared not to disrupt acquisition of this task.

In several strains of mice, it was shown that the longer the inhibition of protein synthesis, the greater the percentage of amnesic subjects. This was true across 6 strains in which widely differing conditions of training were required in order for 90-100% of the mice in each strain to learn one-trial passive avoidance. Small increases in shock strength reduced the amnesic effect, but an additional 2 hours of inhibition reestablished amnesia.

It was found that anisomycin could be used to control the time and duration of protein synthesis after mice were trained in a one-trial passive avoidance task. If small amounts of protein synthesis were permitted to occur after training by permitting some recovery of protein synthesis, then memory could be established hours after training. Without this protein synthesis, a high percentage of the subjects were found to be amnesic. The longer the duration of this post-training protein synthesis and the closer it occurred to training, the greater the percentage of subjects remembering the training.

Some of the amnestic trends in passive avoidance had direct counterparts in a left-right active avoidance task: (a) longer durations of inhibition caused a higher percentage of subjects to be amnestic, (b) stronger training reduced the percentage of amnestic subjects but (c) further increases in the duration of inhibition could reestablish the amnesia. The most important training parameters for this active avoidance task were the rate of avoidance learning (i.e., number of trials to make the first avoidance response) and, rate being constant, the amount of practice (i.e., the number of avoidance responses). Fast rates of learning and/or more practice reduced the amnestic effect of a given duration of inhibition. Under marginal conditions of learning, retention for the escape component of the avoidance task could be disrupted, but no effect was seen even with longer periods of inhibition if more training was used.

This work is seen as strong support for the hypothesis that protein synthesis has a necessary role in memory formation.

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TABLE OF CONTENTS

	<u>Page</u>
Title Page	i
Abstract	ii
Acknowledgments	v
Table of Contents	vi
List of Figures	x
List of Tables	xii
Chapter I. Protein: The Fabric of Memory	
Introduction	1
Scope of the Biochemical Research Approaches	1
Effects of Inhibition of Protein Synthesis on Memory	6
Purposes of the Thesis Research	16
General Description of Methods	
Biochemical	18
Behavioral	
Apparatus	19
Training	20
Retention Test	21
Injection Procedure	22
Statistics	23
Chapter II. Influence of Training Strength on Amnesia Induced by Pretraining Injections of Cycloheximide	
Introduction	28
Inhibition of Protein Synthesis	
Procedures	30
Results	30

	<u>Page</u>
Behavioral Effects	
Experiment 1	35
Experiment 2	40
Discussion	
Controlling Inconsistency	51
Overtraining	51
Control Problems	52
Related Studies	56
Speculation	57
Conclusions	58
Chapter III. The Influence of Duration of Protein Synthesis	
Inhibition on Memory	
Introduction	61
Inhibition of Protein Synthesis	
Procedures	62
Results	63
Behavioral Effects	
Experiment 3	68
Experiment 4	69
Experiment 5	73
Experiment 6	74
Experiment 7	76
Experiment 8	80
Experiment 9	80

	<u>Page</u>
Discussion	
Amnestic Effects of Multiple Injections	81
Comparison of Ani and Cyclo	82
Side Effects of Ani and Cyclo	85
Overtraining	87
Comment	87
Conclusions	89
Chapter IV. Comparison of the Effects of Anisomycin on Memory Across Six Lines of Mice	
Introduction	91
Inhibition of Protein Synthesis	92
Behavioral Effects	
Experiment 10	97
Discussion	101
Experiment 11	106
Experiment 12	108
Discussion	110
Chapter V. The Relation of Memory Formation to Controlled Amounts of Brain Protein Synthesis	
Introduction	113
Inhibition of Protein Synthesis	113
Behavioral Effects	
Experiment 13	114
Experiment 14	118
Experiment 15	120

	<u>Page</u>
Discussion	122
Chapter VI. Effects of Protein Synthesis Inhibition on Memory for Active Avoidance Training	
Introduction	129
Inhibition of Protein Synthesis	130
Behavioral Effects	132
Acquisition of the Avoidance Task	135
Experiment 16	135
Experiment 17	144
Experiment 18	155
Discussion	158
Chapter VII. Discussion	
The Research Problems	165
Problem of Interpretation	167
Training	167
Permanent Incapacity of Impairment of Memory Formation	172
Amnesia by Three Inhibitors	173
References	174

LIST OF FIGURES

Figure No.	Subject	Page
1	Effect of acetoxycycloheximide on memory	12
2	Effect of cycloheximide on memory	14
3a,b	Inhibition of protein synthesis by cycloheximide	32,33
4	Distribution of escape latencies for cyclo- and saline-injected mice	37
5	Amnesia as a function of escape latency	44
6	Amnesia as a function of retention period	45
7	Amnesia as a function of training latency	47
8	Retention as a function of drug, retention period and escape latency	48
9	Optimal training strength	53
10	Retention as a function of drug condition and training latency	54
11	Inhibition of protein synthesis in brain and liver by anisomycin at 3 doses	64
12	Effect of successive injections of anisomycin on protein synthesis	66
13	Comparison of anisomycin and cycloheximide inhibition	67
14	Retention as a function of drug condition and shock intensity	70
15	Retention as a function of drug condition and training condition	71
16	Effect of two successive anisomycin injections on memory and control conditions	75

		Page
17	Retention as a function of drug condition and training latency	77
18	Retention as a function of duration of inhibition of protein synthesis and training condition	79
19	Comparison of the distribution of training latency in anisomycin-, cycloheximide- and saline-injected mice	86
20	Time course of inhibition caused by one or two injections of anisomycin	94
21	Acquisition curves for seven mouse strains trained on passive avoidance	100
22	Effects of controlled protein synthesis on retention	117
23	Effect of time and duration of protein synthesis on retention	124
24	Acquisition curves of NaCl- and Ani-injected subjects for active avoidance	137
25	Effect of duration of Ani-produced inhibition on retention for active avoidance training	140
26	Distribution of active avoidance retention scores for NaCl- and Ani-injected mice	142
27	Distribution of retention scores for NaCl, Ani and long duration of inhibition groups	148
28	Simple and complex escape pathways	163
29,30	Frequency of simple versus complex escape responses for experiments 16 and 17	165,166

LIST OF TABLES

Table No.		Page
1,2	Effects of puromycin on memory	7,8
3,4	Amnesia as a function of shock intensity	38,39
5	Distribution of test latencies for cyclo- and saline-injected mice	43
6	Retention as a function of drug, latency to enter and escape, and retention period	49,50
7	Time course of inhibition of brain protein synthesis by anisomycin in 7 strains	93
8	Description of mouse strains	96
9	Distribution of short escape latency across six mouse strains	103
10	Distribution of short versus long training latencies across six mouse strains	104
11	Retention as a function of strain and drug condition	107
12	Retention as a function of strain, duration of inhibition and drug condition	109
13	Effect of duration and time of protein synthesis on the percent amnesia	119
14	Percent and duration of inhibition of protein synthesis by Ani and AXM	131
15	Effect of Ani on retention for escape	143
16	Time and types of injections for experiment 17	145
17	Retention as a function of rate of acquisition	149

	Page
18 Retention as a function of the number of training trials	149
19 Retention as a function of rate of acquisition and number of errors	152
20 Retention as a function of the duration of inhibition	153
21 Retention as a function of the number of training trials and duration of inhibition	154

I. PROTEIN: THE FABRIC OF MEMORY

Introduction

SCOPE OF THE BIOCHEMICAL RESEARCH APPROACHES

In an effort to discover the possible role of biochemical processes in memory formation a number of techniques have been employed. The following main types of research will be described briefly in this section -- measurement of RNA or protein synthesis, RNA-DNA hybridization, interanimal transfer, the use of pharmacological agents that affect the release of synaptic transmitter, and the use of inhibitors of protein synthesis.

To demonstrate that RNA or protein synthesis is involved in learning and memory storage, investigators have labelled the RNA or protein being synthesized with radioactive material during or shortly after training (Glassman, 1969; Hydén and Lange, 1968; Uphouse, Macinnes, and Schlesinger, 1972a,b,c). In all cases, it must be assumed that the labelling period is one during which part of the RNA or protein synthesis is activated by training and/or by memory processing.

Aside from isolating the RNA or protein and determining its radioactivity relative to some form of a control, autoradiography has been employed to detect the possible role and localization of increased RNA or protein synthesis which is important for learning and memory (Zemp, Wilson and Glassman, 1967; Hydén and Lange, 1972).

Some investigators feel that memory-related macromolecules are formed as the result of training and that these unique molecules play a role in the storage and retrieval of memory. One technique used to study this type of hypothesis is competitive hybridization of RNA with DNA (Gaito and Bonnet, 1971). In the hybridization experiments, RNA binding to DNA is compared in control and trained subjects in the hope of finding evidence for the existence of a unique species of RNA being synthesized as the result of learning. The assumption is being made that the time at which labelling occurs and at which the RNA is isolated, represents a period of time during which synthesis relevant to training and memory processes is occurring.

The control groups that are usually employed in the two approaches described above take three forms -- cage, yoked, or well-trained control. A cage control receives no experimental treatment but is housed and fed in the same manner as the trained subjects. Ideally, the yoked control experiences everything the trained subject does, except that the behavior of the yoked control is not systematically affected by the CS (light, buzzer, the training apparatus itself) or the US (usually shock). In most studies the yoked control is more or less a stress control as some aspect of the experience is missing (e.g., no shelf as in the Glassman training box, or the subject is just isolated in a shock box). The well-trained control is a subject trained to a high criterion of responding and then retrained at the time at which the experimental subject is being trained. The rationale is that a well-trained subject has little more to learn; therefore, learning

and memory processes should not be very active. The assumption that yoked controls do not activate learning and memory processes because they are presumed not to learn and that well-trained control subjects will not activate such processes because they have already learned seems to me suspect; and these assumptions are too important to go untested. It is sufficient to say that poor control groups seriously hamper one's ability to draw concrete conclusions about the processes of learning and memory from these research efforts.

Another memory molecule approach has been that of interanimal transfer of memory (Byrne, 1970; Adam, 1971). The interanimal transfer of memory studies, with one exception (Ungar, 1970, 1972), are not really biochemical in approach -- rather they involve a perverse kind of pharmacology. The hope is that if one trains an animal, isolates the RNA or protein produced as the result of the training and then injects it into another untrained subject (usually of a different species), that the naive subject will show savings in learning the task. The assumptions and the rationale behind this approach are questionable on many grounds (e.g., When the trained-RNA is administered intraperitoneally, does the RNA enter the CNS, neurons, etc.? Given that the molecules could enter the CNS, are they biologically active after the isolation procedures? Does the CNS use the molecules as whole units or subunits?).

In a modification to this approach, Ungar started with interanimal transfer, isolated the active protein agent responsible for the effect and subsequently reported the structure of the polypeptide (Ungar, 1970, 1972). This protein has been reported to cause

fear of darkness in mice (i.e., naive mice showing a light aversion will show a dark aversion after receiving the polypeptide). The protein was originally isolated and characterized from rats trained to avoid stepping into a dark compartment by receiving footshock when they entered the dark compartment from the light compartment. This approach has many of the same difficulties of other interanimal transfer studies including some question regarding the ability of these results to be reproduced in other laboratories.

Another approach has been to employ pharmacological agents that will modify synaptic activity by enhancing or disrupting transmitter release (picrotoxin: Breen and McGaugh, 1961; strychnine: Andry and Luttges, 1971; McGaugh and Krivanek, 1970; bemegride: Luttges and McGaugh, 1971). The general problem with this approach is that few studies report the physiological effects of the drugs on electrophysiological or biosynthetic activity. Therefore, one has to take on faith that the dose of the drugs used in the behavioral studies is altering the synaptic activity and subsequent biochemical activity.

In one pharmacological approach using inhibitors of protein synthesis, care has been taken to determine the degree and time course of the inhibition of brain protein synthesis. These inhibitors have been used to test the hypothesis that protein synthesis is necessary for memory formation. The drugs (i.e., antibiotics) are non-specific inhibitors of protein synthesis. Some of these inhibitors are rather toxic and have side effects that have complicated the interpretation of the results. Usually the drugs are administered prior to training, which constantly raises the

possibility that impairment of memory is actually impairment of acquisition. However, it is generally the case that impairment of acquisition is difficult to demonstrate and where it exists it seems too small in magnitude to account for the large loss of memory. The most important assumptions in this approach are that (a) inhibition lasts as long or longer than the capacity of the CNS to synthesize memory related protein(s), (b) inhibition is uniform across brain areas, cell types and types of protein, and (c) inhibition of protein synthesis and not some unknown side effect of the inhibition is the active agent.

In this first section, I have indicated what I felt were the main assumptions and problems of the principal research efforts directed toward the biochemical processes underlying memory. I have selected inhibition of protein synthesis as the means of studying the role of biochemistry in memory processing because this area has definable control groups and does not suffer from problems of interpretation that plague the other approaches. In the incorporation studies, the control should be a subject that experiences everything that the learning partner does but itself does not learn or remember; no one has yet succeeded in defining such a condition, and it is not clear that this can be done. The incorporation studies have to assume that the period they are examining is one during which learning and/or memory processes are occurring. It is necessary to inject radioactive uridine directly into the brain; to what extent does the injection effect the biochemical processes of learning and memory? Does the labelled material diffuse evenly throughout the brain? Some pilot

work that we have undertaken has shown that the intracerebral injection (a) blocks learning of avoidance tasks (except those in which the CS had little effect on acquisition), (b) prolongs even simple escape from foot shock learning with a light-dark discrimination, and (c) if the subject learns to escape, recall is very poor. The interanimal transfer studies assume also that the period during which RNA or protein is being produced is one during which the processes of memory are activated. In addition, many assumptions must be made as to the fate of the injected material. Because positive results are obtained, it should not be construed to mean that the assumptions are justified. If the rationale behind the procedures and design are not valid, then the results are uninterpretable.

To me, the work using the inhibitors seems to be the least plagued by complex assumptions and unavailable control groups. Thus the possibility of making a concrete and interpretable contribution to our understanding of memory seems best served at this time by the use of inhibitors of protein synthesis.

EFFECTS OF INHIBITION OF PROTEIN SYNTHESIS ON MEMORY

In the following section, rather than present a historical overview on the effects of protein synthesis inhibition on memory, I will present selected studies which demonstrate the type of results that have been reported.

Flexner, Flexner and Roberts (1967) were the first to report amnesic effects with brain protein synthesis inhibition. Puro-mycin was injected bilaterally into either the temporal cortex,

the frontal cortex, or the cerebral ventricles. Combinations of these injection sites were also employed. The injections were given to mice 1 to 43 days after training on a left-right discrimination to avoid shock run in a Y-maze; the subjects were trained to a 9 out of 10 criterion. The results presented in Tables 1 and 2 showed that only the bitemporal injections were critical in obtaining significant amnesia (defined as a savings score of 3% or less) 1 to 3 days after training. Greater delays between the training and the injection of puromycin required giving the drug in all three pairs of the injection sites to obtain significant amnesia.

Table 1

Puromycin Injections		No. of mice in which memory was		
Site	Days	L	I	R
T+V+F	1	7	0	0
T	1	10	0	0
V	1	0	0	5
F	1	0	0	5
V+F	1	0	1	2
T+V+F	11 - 60	17	2	0
T	11 - 35	0	0	7
V	12 - 38	0	0	3
F	16 - 27	0	0	3
V+F	28	0	2	2
V+T	28 - 43	1	1	2
T+F	28	0	0	3

Table 2

<u>Puromycin Injections</u>		<u>No. of mice in which memory was</u>		
<u>Site</u>	<u>Days</u>	<u>L</u>	<u>I</u>	<u>R</u>
T	2	3	0	0
T	3	4	0	1
T	4	0	1	1
T	5	0	1	2
T	6	0	0	3

Tables 1 and 2. Effects of Puromycin on Memory.

All injections were given bilaterally. T, temporal cortex; F, frontal cortex; V, ventricles. Days = days after training that the injections were given. Memory: L, lost - less than 3% savings; I, impaired - 20 to 40% savings; R, retained - 85% savings or greater. Two saline injected mice (intracerebral injection) showed slightly better than 90% savings. The time of the injection was not stated. The authors do not indicate how the subjects were distributed across injection intervals such as 16 - 27 days, 3 subjects.

(Tables taken from Flexner et al., 1967).

As can be seen, the behavioral effects reported depend on data from a relatively few subjects in each group. None of the other inhibitors of brain protein synthesis that will be discussed have had such a disturbing effect upon memory when administered after training.

Barondes and Cohen (1967) and Cohen and Barondes (1968a) introduced the use of acetoxycycloheximide (AXM), another inhibitor of brain protein synthesis. The drug was administered intracerebrally 5 hrs prior to training mice in a left-right discrimination to escape shock run in a T-maze; groups were trained to either a 3-out-of-4 or 9 out-of-10 correct response criterion. The mice trained on this task were given a retention test (retraining to the previous criterion) either 3 hrs, 6 hrs, 7 days or 6 weeks after the original training. The percent savings to reach the training criterion on the retention test was 75 - 80% for the saline-injected subjects. The AXM-injected subjects were not affected at the 3 hr retention test, but for the remaining test times (6 hrs to 6 weeks) the drug-injected subjects showed only a 35% savings when they had been trained to a criterion of 3-out-of-4 correct responses. Those subjects originally trained to a criterion of 9-out-of-10 correct responses and tested 7 days later were not affected significantly by AXM. Thus, training subjects to too high a criterion of acquisition blocked the drug's amnesic effect.

Similar "overtraining" effects were reported for a light-dark discrimination to escape shock in a T-maze with a pretraining injection of AXM, except that the criteria were 5-out-of-6, 9-out-of-10, or 15-out-of-16. Those subjects trained to a 5/6 or 9/10

criteria showed about 35% savings on retraining a week later. However, those subjects trained to a 15/16 criterion were not affected by the drug as they did not have significantly different savings scores from the saline-injected control subjects. Comparing the results of 9/10 groups for spatial and light-dark discrimination, it can be seen that it is not the number of trials per se that is responsible for blocking the amnesic effect of the drug but the degree of learning (i.e., number or percent CR's). Since the light-dark discrimination task was considerably more difficult than the left-right discrimination task, more trials could be given in the light-dark discrimination without training the subjects to too high a degree.

In Barondes and Cohen's studies, it is of some concern that subjects had to be given the drug 5 hrs prior to training because this greatly increased the chances that systemic effects of AXM might impair acquisition. AXM had to be given 5 hrs prior to training so that the inhibition of brain protein synthesis would be at least 90% at the time of training. To test whether acquisition was affected, the authors compared on a trial-by-trial basis the mean number of CR's and found that the training curves for saline and AXM-injected subjects could be completely superimposed; there was no indication that the subjects given AXM had any difficulty in learning the discrimination.

Barondes and Cohen (1968) later reported that when mice were given AXM subcutaneously instead of intracerebrally, inhibition reached 90% within 10 minutes of the injection. The inhibition remained above 80% for 8 hrs and then slowly returned to normal

over the next 4 to 8 hrs. The mice were trained on a light-dark discrimination to escape shock in a T-maze to a 5-out-of-6 criterion of correct responses. The subjects were retrained a week later and the graph below (Figure 1) shows the percent savings as a function of the time the drug was administered.

It can be seen that the greatest amnesic effect still allowed 30% mean savings. Thus, AXM given intracerebrally or subcutaneously caused significant impairment of retention but not complete amnesia. It can also be seen that the drug is less effective when it is given either immediately before or after training than when it is given 5, 30 or 300 minutes prior to training. The authors reported that diarrhea was present 3 to 4 hrs after the injection and that 7% of the mice died within 24 hrs (none thereafter).

Neither puromycin or AXM is used today. Puromycin was found to have many side effects (Kerkut, Oliver, Rick and Walker, 1970; Barondes, 1970); it caused abnormal changes in the morphology and electrophysiology of the brain. In addition, post training amnesic effects obtained with puromycin were found to be reversed by intracerebral injections of saline many days after the drug had been administered (Flexner and Flexner, 1968). AXM would probably still be used, but it is generally not available.

The next inhibitor of brain protein synthesis to be widely studied was cycloheximide (Cyclo); it is a compound closely related to AXM. The main difference between the drugs for the purpose of this work is that Cyclo inhibits protein synthesis for only 2 hrs at 80% or greater, while AXM inhibits protein synthesis for 8 hrs at 80% or greater.

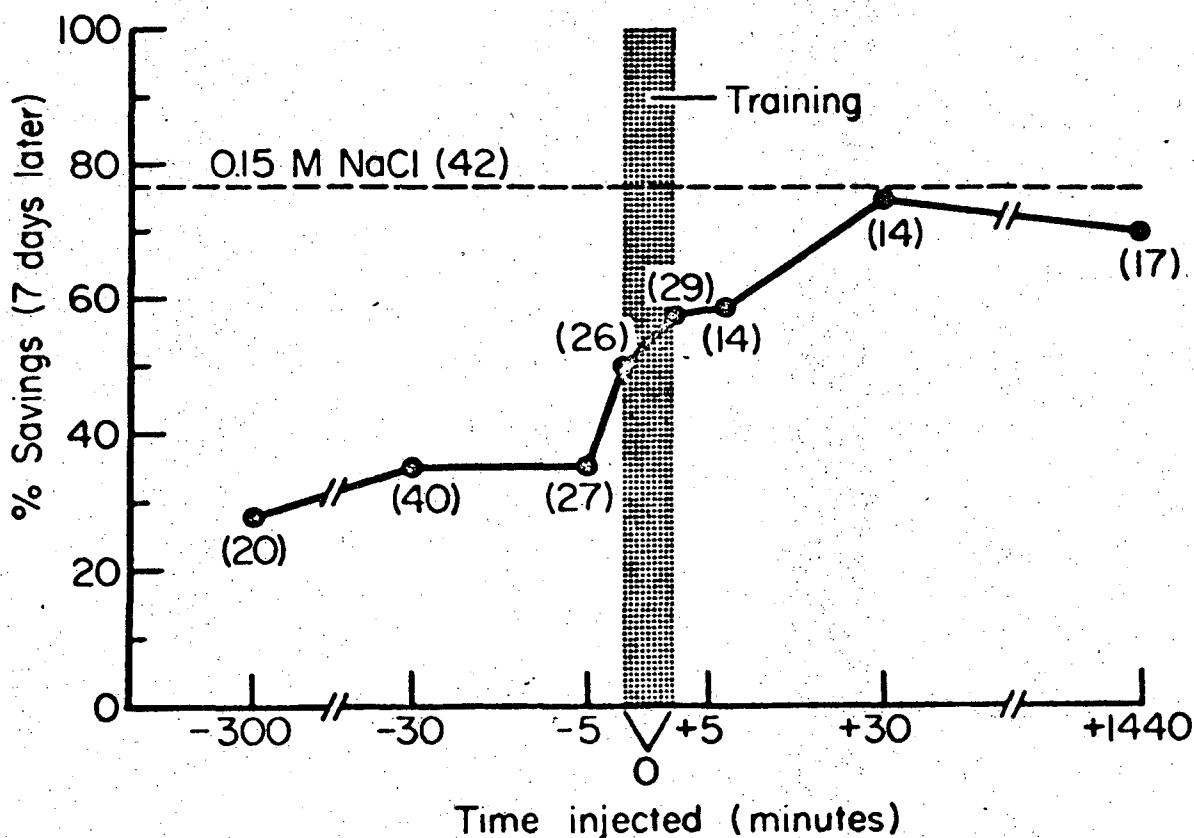


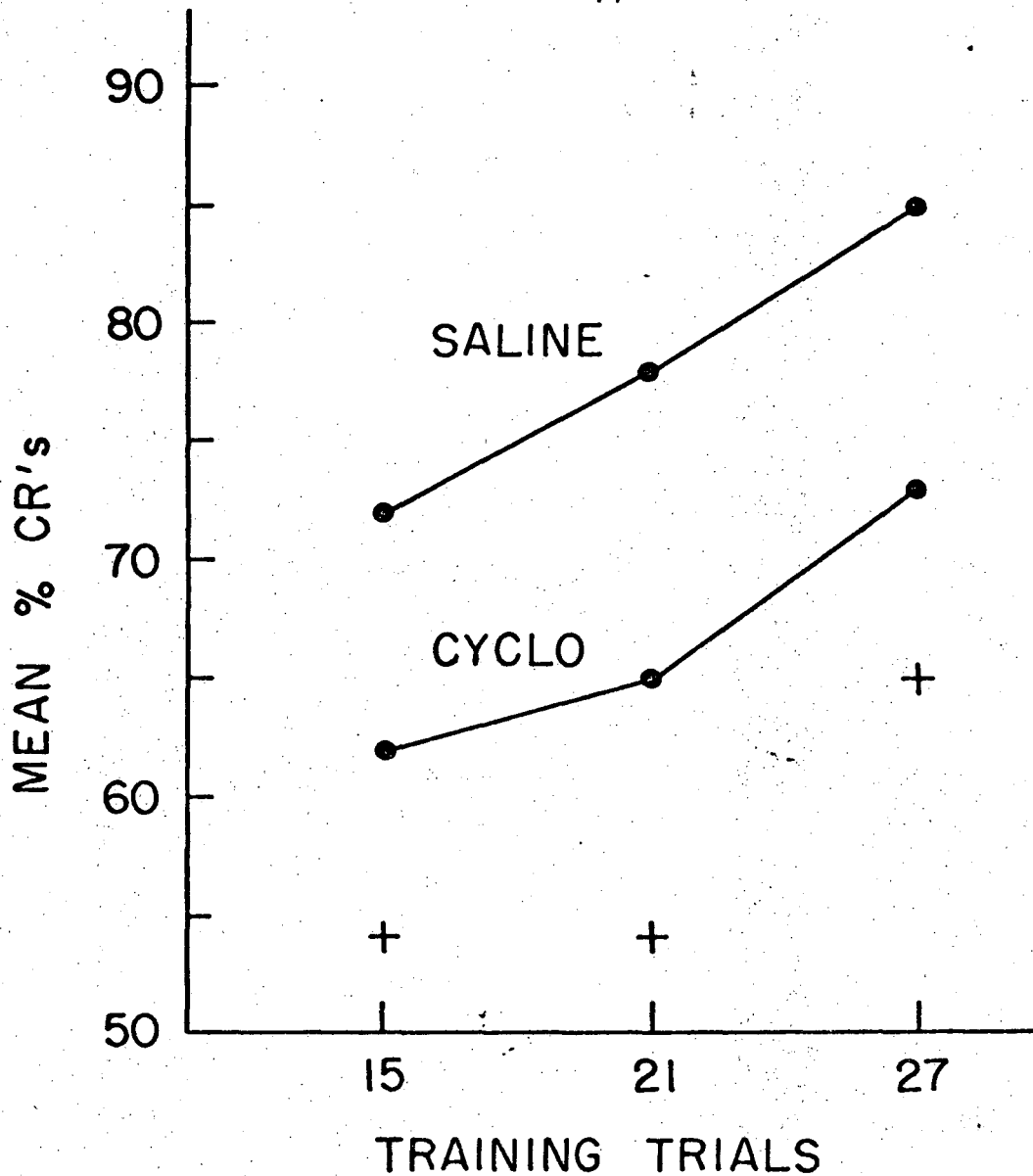
Figure 1

XBL 733-346

Effect of subcutaneous administration of acetoxycycloheximide at various times before or after training on memory. Mice were injected subcutaneously with 240 μ g of acetoxycycloheximide at the indicated time relative to training. They were trained to escape shock by choosing the lighted limb of a T-maze to a criterion of 5-out-of-6 correct responses. Training took an average of eight minutes. Approximately 90% of cerebral protein synthesis was inhibited within 10-15 minutes of subcutaneous injection of acetoxycycloheximide. All mice were tested for retention seven days after training, long after they had recovered from the drug. The mice injected before or within 5 minutes after training all had significantly less savings ($P < 0.05$ or less, Mann-Whitney U test) than those injected 30 or more minutes after training (Barondes and Cohen, 1968).

Squire and Barondes (1972b) have employed this drug in a large rod, small rod discrimination task, run in an automated Deutsch Carousel to escape shock. Subjects were given a subcutaneous injection of Cyclo 30 min prior to training, at which time inhibition was at least at 85%. Subjects were given a fixed number of trials (15, 21, or 27) in the apparatus and retrained a week later. The graph below (Figure 2) shows the mean percent CR's on the retention test as a function of the number of training trials.

It can be seen that the effects, while significant for 15 and 21 trials (saline versus Cyclo, $P < 0.05$), are of a small magnitude and that the Cyclo-injected subjects are almost as far above the naive subjects' performance level as they are below the saline-injected subjects' performance. Saline- and Cyclo-injected subjects did not differ significantly on testing when they were originally trained for 27 trials. There is a slight trend for more training trials to decrease the amnesic effect. The number of CR's achieved during original training did not differ significantly between Cyclo- and saline-injected subjects except over the last few trials in the group given 27 trials where Cyclo-injected subjects made slightly fewer CR's. One problem with this task is that the magnitude of the possible effect can only be a very small one. This is because the number of CR's on the original training for saline-injected subjects was 8 out of 15 trials, while the retention test showed almost 11 CR's. This is only a 3 trial difference and thus one has very little sensitivity available to detect a drug effect. Unfortunately, no measure of variability was reported, so we cannot see to what extent the groups were overlapping.



XBL733-4730

Figure 2. Effect of a pretraining injection of cycloheximide on retention for discriminated shock escape learning. Plotted are the mean percent CR's as a function of the number of original training trials. The saline - cycloheximide differences in mean percent CR's are significant ($P < 0.05$) for 15 and 21 trials, but the difference at 27 was not significant. The +'s indicate the percent CR's on original training for the saline-injected subjects. (Based on tabled data from Squire and Barondes, 1972b.)

Geller, Robustelli, Barondes, Cohen and Jarvik (1969) have employed step-through passive avoidance to test the effects of pretraining injections of Cyclo on retention. Mice were injected subcutaneously 30 minutes prior to training. Training consisted of shocking a mouse when it stepped from a small lighted compartment into a larger black compartment. On the retention test, the subject was again placed into the lighted compartment and the latency to step into the dark box was recorded. It was inferred that if the test latency was longer than the training latency, then the subject remembered having been shocked in the black compartment. The retention test was given 7 days after training. Saline injected subjects had a median test latency of 600 sec (the cut-off latency) with very low variability. The Cyclo-injected subjects had a median test latency of 200, ranging from 150 to 450 sec. The median step-through latency for all subjects at training was 25 sec. Thus it was clear that Cyclo-injected subjects were not "naive", but that their retention was impaired. Two other papers using the same task showed similar impairing effects of Cyclo on memory (Geller, Robustelli and Jarvik, 1970b, 1971).

Quartermain, McEwen and Azmitia (1970) have reported the best amnesic effects in the literature using Cyclo administered 30 minutes prior to training in a step-through passive avoidance task. In this case subjects stepped from a small black box into a larger illuminated box. The type and level of shock differ considerably for Geller et al. (1969, 1970b, 1971) in which subjects escaped from 0.35 ma shock and for Quartermain et al. (1970) in which subjects received 2 seconds of confined shock at 0.16 ma. In the

Quartermain et al. experiment, Cyclo- and saline-injected subjects showed similar median training latencies of 5.3 and 6.6 sec respectively and on the retention test 8.6 and 180 sec (the cut-off latency). No measure of variability was given, but the use of a median measure indicates that it was probably considerable. In this experiment, at least 50% of the subjects would by their test latencies seem to have completely forgotten the training experience.

Purposes of the Thesis Research

The hypothesis that newly synthesized protein is necessary for longterm memory formation demands that if synthesis is prevented, then subjects will show complete amnesia. Some may argue that since inhibition is never complete, some protein synthesis is possible; this we can refer to as the leakage hypothesis. While this remains a possibility, it is an untestable hypothesis; therefore, it is an excuse for failing to obtain the desired or expected degree of amnesia. If we invoke the leakage hypothesis whenever the results of an experiment are not as predicted, then we are attempting to test a hypothesis that cannot be disproven. Thus, the literature cited above can only be considered as limited support of the hypothesis that newly synthesized protein is necessary for longterm memory formation, since the effect of protein synthesis inhibition seems to be only that of impairment of memory rather than amnesia. From the results cited above, it is clear that Cyclo-treated subjects remembered a great deal about the passive avoidance training, even though retention differed significantly

between saline- and Cyclo-injected subjects. From this data, we must conclude that protein synthesis is involved in memory, but is not necessary for memory formation. Thus as the hypothesis exists, only when amnesia is complete can we make the statement that protein synthesis is necessary for memory to be formed.

Also important is the variability of the amnestic effect from subject to subject. While the literature has few reports concerning variability, indications show that it is considerable (i.e., frequent use of median measures).

A third major difficulty for the hypothesis is that training subjects to a high criterion of responding in multiple trial training tasks or the use of high shock intensity in passive avoidance will block the amnestic effect, or at least substantially reduce it.

These three problems -- (a) lack of total amnesia, (b) variable amnestic effects across subjects given the same treatment, and (c) the "overtraining" effect -- have provided the focus of the research that I have undertaken in collaboration with Dr. E. L. Bennett, Ann E. Orme, and Dr. M. R. Rosenzweig. The goal of this research is to determine the sources of these problems and from this knowledge to modify or delimit the hypothesis that protein synthesis is necessary for longterm memory formation.

GENERAL DESCRIPTION OF METHODS

Biochemical

The degree and duration of inhibition of brain protein synthesis by cycloheximide (Cyclo) and anisomycin (Ani) was determined in several strains of mice (see specific experiments for strains used). To determine the inhibition of protein synthesis, we followed in general the methods described by Barondes and Cohen (1967). The inhibitor was injected subcutaneously on the back. Thirty minutes prior to sacrifice (in most cases) valine-U-¹⁴C (200 mC/mM, New England Nuclear Corp.) was injected subcutaneously (5 μC/mouse, 0.05 ml). At the designated time, the mouse was decapitated; the brain was removed and frozen on dry ice until analyzed. Each sample was homogenized in sufficient 0.1 NaOH to give a concentration of 15 mg tissue/ml for liver and 20 mg/ml for brain. Six ml of 12% trichloroacetic acid (TCA) was added to a 2 ml aliquot to precipitate the protein. Two further washes, resuspensions, and centrifugations were used to remove the free valine. One ml of Biosolv BSS-3 solubilizer (Beckman Instruments, Inc.) was added to the TCA-precipitate, which was mixed and allowed to dissolve for at least 30 min. This mixture was then quantitatively transferred to vials with repeated aliquots of Toluene-Fluor II scintillation fluid and the radioactivity determined. The radioactivity of 1 ml aliquots of the TCA-supernatant was measured using dioxane-Fluor II scintillation fluid. Appropriate corrections for counting efficiencies were made.

The "total drug time" is defined as the duration from injection of the drug to decapitation time. The valine-¹⁴C incorporation time in most cases was 30 minutes. For example, in the case of a total drug time of 60 min, the drug was injected at "0" time, valine was injected at 30 min, and decapitation was at 60 min, giving an average measure of inhibition over the last 30 min period. The degree of inhibition was calculated by determining the ratio of valine-¹⁴C incorporated into the precipitate to the total incorporation in the precipitate plus supernatant relative to the subjects given saline and then valine-¹⁴C.

Behavioral

The mice used in the following experiments and in determining the degree of inhibition of brain protein synthesis were both males and females, 60-74 days of age. Strains or lines not raised in the Department of Psychology, University of California, Berkeley were received at 6 weeks of age and housed by us until the proper age. The specific strains used will be described in each experiment. Subjects were housed individually in small plastic or metal cages three days prior to training. Immediately after training, a subject was returned to its cage in a quiet room. Subjects were not handled or disturbed after training until the retention test.

Apparatus

The training task was step-through passive avoidance. The training apparatus consisted of a 17-3/4" long alley divided into a small black start box (3-1/2" long and 4" wide) and a long white shock box (14" long and 3-1/4" wide). The walls of the box were

5" high and 4" wide. The black start box had a masonite floor; the floor of the white shock box consisted of 3/32" diameter brass rod at 3/8" centers. The start box and shock box were separated by a black plexiglass wall with a 1-1/2" diameter "mouse hole" situated 3/8" off the grid and a white translucent plexiglass guillotine door. The white shock box was illuminated by an automobile signal lamp in series with a 7 ohm resistance; the lamp was situated behind a white translucent plexiglass panel at the end of the start box. The top of the apparatus was covered with clear plexiglass.

Shock was delivered by a high voltage, constant current, 18 pole shock-scrambler. The apparatus was wiped dry after each subject was run; after 6 or fewer subjects had been trained or tested, the apparatus was washed with a dilute alcohol solution and then with hot water. The apparatus was allowed to dry before training or testing the next group.

Training

Fifteen min after receiving its injection of a drug or saline, a mouse 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 seconds. The white guillotine door separating the two compartments was then removed when the mouse was facing away from the "mouse hole". This was done because it was noticed that if the mouse was facing the hole when the door was raised there were frequently long latencies to enter the white box because the subject either (a) backed away from the hole or (b) spent several seconds sniffing the area around the hole. With the guillotine door

removed, the mouse had access to the white box when it turned around. A stop watch was started when the subject 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 subject had all four paws on the grid of the white box. At this point, the experimenter flipped a switch that delivered continuous shock 5 seconds 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 seconds after returning to the start box, the subject was removed to its home cage.

Some variations of this training procedure were used and will be described in the pertinent experiments to follow.

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 subject oriented toward the mouse hole. Subjects not crossing into the white box within 300 or 180 sec (see specific experiment for the cut-off latency used) were removed to their home cages.

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. Throughout, 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 latency-to-enter in most strains for un-injected naive mice. The escape latency is the time from shock onset until the mouse returns to the black start box. Training strength refers to the ability of particular training

parameters used to elicit passive avoidance responses on the retention test. The term is used in the relative sense; therefore, no specific values of shock intensity, shock duration, or training latency are necessarily associated with high or low training strength.

Injection Procedure

All injections were given subcutaneously in a 0.25 ml volume. Cyclo was administered at 2.5 mg/male mouse, 2.3 mg/female mouse, Ani at 0.5 mg/mouse/injection. In all cases, single injections were given 15 minutes prior to training. Thus Cyclo- and Ani-injected subjects were under high levels of protein synthesis inhibition at the time of training. Where post-training injections were given, they were administered at 1-3/4 or 3-3/4 or 5-3/4 hrs after training for 2nd, 3rd and 4th injections respectively. In some cases the injection schedule was altered and this change will be discussed at the appropriate time.

Injections, pre- or post-training, 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.

Injections could not be given immediately after training on passive avoidance because it was found in two strains that the injection itself could interact with the shock punishment to first

strengthen passive avoidance (1-5 min after training) and then progressively weaken the passive avoidance (5-30 min after training). While a complex interaction between training and injecting existed for 1 to 30 min, it was found that no effect was obtained with injection given 1 hr or longer after training. For this reason, immediate post-training injections were not used. It was felt that the interaction between training and the injections was too difficult to control or to interpret in terms of the biochemistry of memory.

Statistics

The experimental designs frequently take the form used in the analysis of variance. However, the statistic itself was not used because it was known beforehand that the distribution of latencies to enter on the test day was bimodal. In addition, the complication arose that the variance was frequently zero.

Statistics were used only to verify questionable differences. Rank order statistics such as the Mann-Whitney test could not be employed because the number of ties were too great. The most frequently used statistic was the Chi-Square Test. However, this test was not used when a theoretical frequency of any one of the cells was below 6. Instead, Fischer's Exact Probability Test was used.

Passive Avoidance as a Measure of Retention

Assumptions

In passive avoidance, it is assumed that on the test days subjects that step quickly into the white shock box (20 sec or less)

have forgotten that they were shocked in the white box. Also subjects that do not step into the white box are assumed to do so because they remember having been shocked. Thus, the latency-to-enter on the training day is used as a measure of retention.

Critique and Test of the Assumptions

One criticism of the above assumptions about the latency measure is that subjects for one of a number of possible reasons may not want to enter the white box. In the C57Bl line, we have trained several thousand subjects. I can only remember 2 or 3 subjects that did not enter within the 20 sec period defined as the upper limit of amnesia on the training day. The high consistency with which naive subjects enter the white box suggests that mice of this strain are not as capricious as the above criticism would imply. In one strain (DBA/2J) we found that 30% of the naive subjects would not enter the white box within 180 sec. Therefore, it seems advisable to establish that a strain show a consistently short latency to enter on the training day.

Another criticism might be that it is questionable that retention and the test latency are directly related. That is, subjects that are classified as amnesic consistently forget the shock and those not stepping through consistently remember. To see if the classification of subjects into amnesic and not amnesic reliably predicts retention, I did the following experiments in which the generality of avoiding a light compartment was tested.

Three groups of subjects were obtained from some of the experiments that follow. The groups were these: (a) saline-injected subjects that were classified as retaining passive avoidance

training (virtually no saline subject forgot), (b) Ani-injected subjects that retained, and (c) Ani-injected subjects that were classified as having forgotten. The fourth group was naive subjects housed in isolation but not trained or previously injected. The four groups were tested on a light-dark preference in a Y-maze. The Y-maze was totally constructed from plexiglass and its alleys were half as wide and twice as high as those of the passive avoidance apparatus. The alleys were about 12 inches long; the start alley was gray, one alley black and the other white and illuminated from overhead. The subjects were placed in the gray alley and allowed to move into one of the two other alleys (black or white). This took no longer than 1 min. When the subject entered one of the alleys, the alley was removed and the animal allowed to climb out of the alley back into its home cage. The animal was not picked up! On subsequent trials, 10 to 15 minutes apart, the black and white alleys were randomly placed on the left or right. The results showed that naive and Ani-injected subjects that were classified as amnesic did not have a preference for either alley. By contrast, the saline and Ani-injected subjects that were classified as remembering in passive avoidance had a dark-side preference in the Y-maze (90% of the saline and 85% of the Ani-injected subjects). A preference was defined as making 8 out of the 10 responses to the same alley. No subject had a light-side preference.

In another test, four similar groups were formed with new subjects. In this experiment, the mice were trained in a shock escape left-right T-maze. One of the alleys was light-cued and the other dark-cued; the light-cued alley was correct. The naive

and Ani-injected subjects classified as amnesic gave 55% and 60% of their responses to the light alley on the first trial. No subject in either group failed to respond to the light side on the second trial. One hundred percent of the saline- and Ani-injected subjects, classified as having remembered, responded to the dark alley on the first trial. Some of the subjects required as many as 6 trials before they made their first response to the light side. It is important to note that the subjects were shocked continuously until they reached the light alley on each trial. Considering that naive subjects learned to respond to the light alley after making only one error, this dark-side preference can be seen to be very strong.

From these two experiments, we conclude that (a) the subjects learn a discriminated light-dark response, and (b) the relationship between the latency measure on the passive avoidance retention test and the existence of a dark side preference was very reliable; therefore, it is concluded that the latency measure that classifies subjects into amnesic or not amnesic is a reliable measure of retention.

Chorover and Schiller (1966) reported that they felt that rats trained in passive avoidance with confined shock (we are using escape from shock procedure) learned a conditioned emotional response of which the characteristic response was freezing. By freezing, one generally means that the subject remains motionless as long as the aversive stimulus is present. Clearly from my observations of our mice, this is not the case. Typically mice are very active

when they are not stepping into the white compartment on the retention test. They will repeatedly approach the mouse hole, back away and approach again. Often times they will extend themselves as far into the white box as possible without removing their back feet from the black box. We conclude that in mice with an escape from shock procedure "freezing" is not the general response. Rather, the subjects are very active in their "passive" avoidance.

II. INFLUENCE OF TRAINING STRENGTH ON AMNESIA
INDUCED BY PRETRAINING INJECTIONS OF
CYCLOHEXIMIDE

Introduction

In most experiments which employ inhibitors of brain protein synthesis as the disruptive agent, the effects are not consistent across all treated subjects. Also, when subjects treated with cycloheximide and acetoxycycloheximide are given too much training, the amnesic effect of these drugs is blocked (Barondes, 1970; Barondes and Cohen, 1967; Cohen and Barondes, 1968b; Flexner, Flexner and Roberts, 1966). These observations have led to skepticism as to whether protein synthesis is critical to the changes in the nervous system that are involved in the formation of memory. The purpose of the two experiments being reported was to determine the source of the inconsistency and to see if cycloheximide given to "overtrained" subjects has adverse effects on memory. As Barondes (1970) has used the term, subjects are "overtrained" if amnesia cannot be induced by the drug. We will show that appropriate testing conditions will often yield amnesia even with "overtrained" animals, and we will propose a more rigorous definition of "overtraining" that is based on the learning performance of control subjects.

In the first experiment, pretraining injections of cycloheximide (Cyclo) produced significant degrees of memory impairment for

a step-through passive avoidance habit. The effects of shock strength on the effectiveness of Cyclo as an amnestic agent are reported.

In the second experiment, overtrained subjects were found to suffer a memory loss only when the training-to-testing interval was extended beyond that commonly employed. Gradients relating degree of training to degree of memory impairment are shown.

In this section, I will not attempt to deal with the question of why overtraining blocks the amnestic effect of cycloheximide. I am concerned only with showing that memory processes of overtrained subjects have been impaired, but that this impairment is best detected with long retention intervals. Secondly, I want to show that there is at least an indication that the inconsistent amnestic effects obtained with Cyclo are not a failure on the part of the drug but the result of uncontrolled sources of variation in training which overtrain the subjects.

Although several investigators have published data on inhibition of protein synthesis by Cyclo in the brains of mice, there were several reasons for undertaking a more thorough study of this. First, it has not been clear whether there are real strain differences since, with the exception of the Randt, Barret, McEwen and Quartermain (1971) report on two strains, all reports have been based on a single strain. Secondly, because experiments have shown inconsistencies in the behavioral effects, it was important to determine whether there are significant individual differences in inhibition within a strain.

INHIBITION OF PROTEIN SYNTHESIS

Procedures

Dose and time dependence of protein inhibition by cycloheximide has been determined in young adult Swiss (Simonsen, Gilroy, Calif.), C3H/HeJ, DBA/2J (Jackson Laboratory, Bar Harbor, Maine), and DBA/DeCgr1 male mice (Cancer Research Laboratory, University of California, Berkeley, Calif.), and in C57Bl control subjects from the behavioral experiments (colony maintained in the Psychology Department, University of California, Berkeley, Calif.). All mice were maintained in the laboratory for at least two weeks subsequent to receipt. A minimum of 6 mice per point was used.

Results

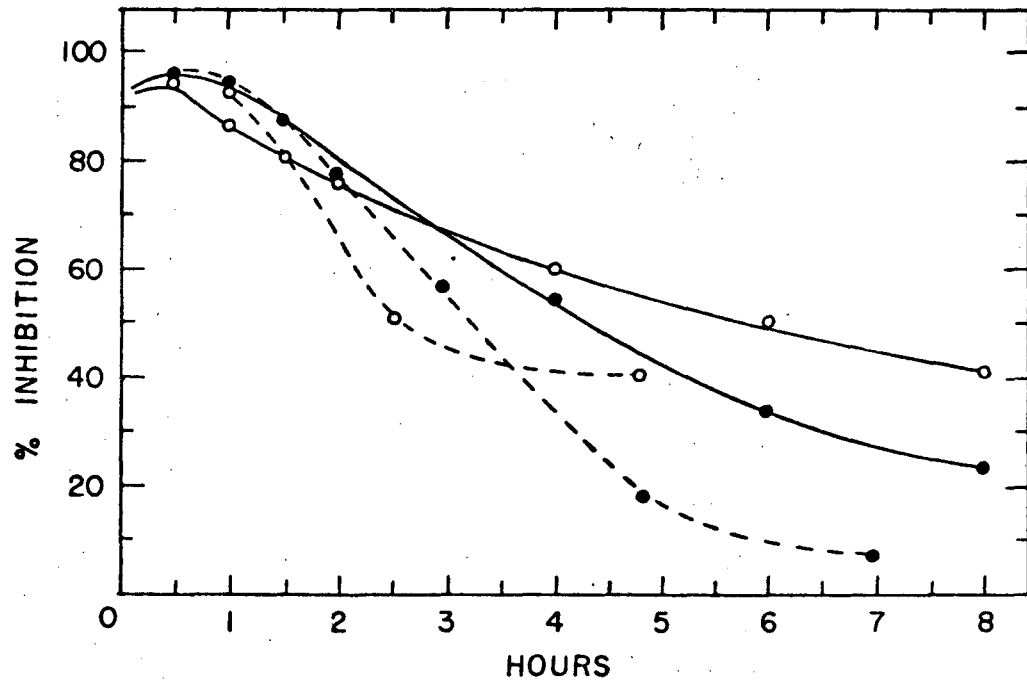
Based upon a number of experiments using Swiss albino and C57Bl mice, our best evaluation of the time course of protein inhibition resulting from a subcutaneous injection of Cyclo is shown in Figure 3a. The degree of inhibition of protein synthesis for the first 90 min after drug administration is greater than 87% for C57Bl and 80% for Swiss albino mice, after which time inhibition falls. Both degree and length of inhibition are nearly dose independent in the range of 1.6 to 8.0 mg/mouse for C57Bl mice. The higher dose is not lethal within 24 hr in the untrained male mouse; there is a direct correlation between training and toxicity, resulting in death at dosages as low as 2.5 mg under certain conditions of training. Inhibition is essentially equivalent in cortex and subcortex, while the inhibition in liver (90%) is slightly

less during the first 30 min than that found in brain (96%) and begins to decline after 1 hr.

In one series of experiments, an incorporation period of amino acid into protein of 10 min was used, and the degree of inhibition was determined at short intervals after drug injection. The results indicated that inhibition was probably achieved within less than 1 min after Cyclo was injected and was very reproducible. Individual and sex differences were found to be negligible; inhibition was between 95.5 and 97.7% for all 20 female C57B1 mice sacrificed at 5 min intervals from 10 to 30 min after injection of 2.3 mg of Cyclo, and inhibition ranged from 96.7 to 98.8% for the 20 male C57B1 mice injected with 3.0 mg of Cyclo.

Inhibition data after subcutaneous injection of Cyclo have also been obtained for DBA/2J, DBA/DeCgr1, and C3H/HeJ male mice (Figure 3b). With C3H/HeJ mice, greater than 80% inhibition is found 2-1/2 hr after Cyclo injection, whereas with DBA/2J and DBA/DeCgr1 mice the inhibition was shorter in duration and remained above 80% for only 1-1/2 hr.

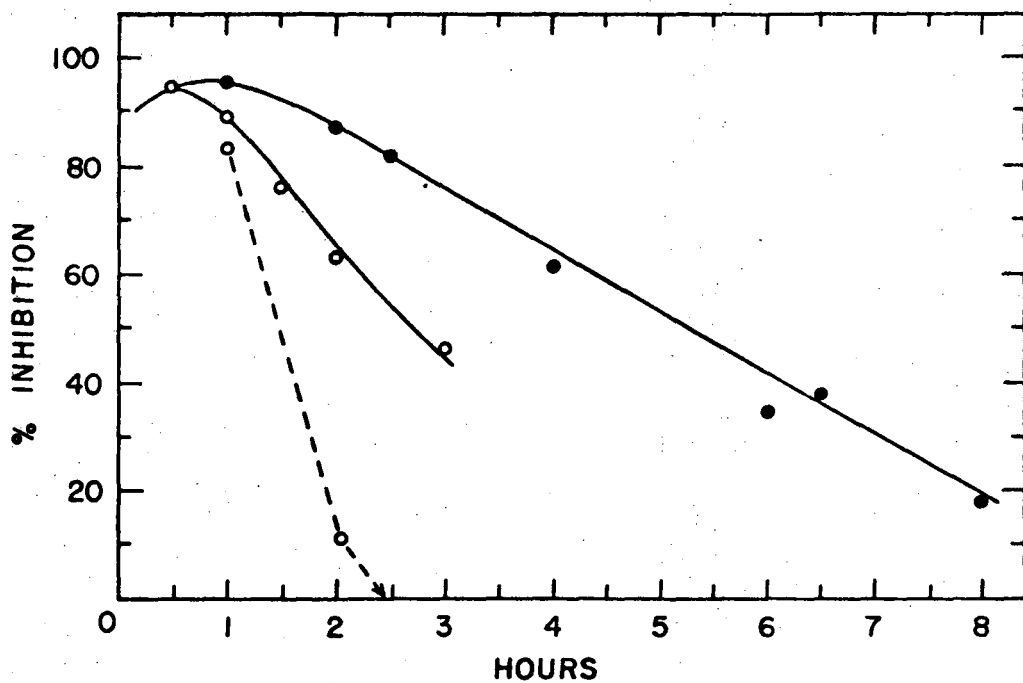
It is generally considered that the duration of inhibition at 80% or above is critical for obtaining amnestic results. Cohen and Barondes (1968a) have reported that greater than 80% inhibition for Swiss albino mice is produced by Cyclo for approximately 1-1/2 hr after injection; we found this degree of inhibition after 1-3/4 hr. During the several hours after this point, the Cohen and Barondes data and our data diverge slightly (Figure 3a). The same trend is observed between the data of Randt et al. (1971)



XBL 721-4517

Figure 3a. Inhibition of protein synthesis by cycloheximide.

- C57B1 male mice, 2.0 to 2.8 mg/mouse, our data.
- - - ● C57B1 male mice, 3.0 mg, Randt et al., 1971.
- Swiss albino mice, 2.5 mg/mouse, our data.
- - - ○ Swiss albino mice, 5.0 mg/mouse, Cohen and Barondes, 1968.



XBL721-4518

Figure 3b. Inhibition of protein synthesis by cycloheximide.

- C3H/HeJ male mice, 2.5 mg/mouse, n=4/point.
- DBA/2J and DBA/DeCrg1, 3.0 mg, our data.
- - -○ DBA/2J, 2.0 mg, Randt *et al.*, 1971.

and our data for C57Bl mice (Figure 3a). However, the data obtained by Randt et al. and our data differ markedly for DBA/2J mice (Figure 3b). At 2-1/2 hr Randt et al. found no inhibition, whereas we observed 55% inhibition. As these authors discuss, differences in methodology and methods of expressing the results may account for some of the discrepancies. To the extent that protein is lost during the period of inhibition, a bias towards a lower value of inhibition may result from their method of expressing the results. In addition, Banker and Cotman (1971) have shown the tritium in leucine-4,5-H³ to be labile; tritium-labelled leucine was used by both Cohen and Barondes (1968a) and Randt et al. (1971). The lability of tritium makes less certain the precursor-product relationships which are assumed in these studies. On the other hand, the carbon-labelled valine which we used does not as readily become incorporated into non-nucleic acid products of metabolism.

Traketellis (1965) has reported high and long lasting inhibition (25 hr) in C3H/HeJ mouse liver after a single intraperitoneal injection of 2.7 mg Cyclo. We were not able to replicate this effect in any strain tested. In Swiss, C57Bl and C3H/HeJ male mice, we have found 18-20% inhibition of protein synthesis in brain 16 hr after administration of Cyclo, and 10-15% inhibition in liver. At 24 hr a slight (5-15%) stimulation of protein synthesis is frequently observed.

In view of the discrepancies among reported values of inhibition, especially over longer time periods, we would recommend that investigators undertaking research in this area determine levels of inhibition in their subjects under their conditions of experimentation.

BEHAVIORAL EFFECTS

Experiment 1

In this experiment, I determined the optimal shock intensity at which Cyclo-injected subjects will have the highest possible percentage amnesia yet the saline-injected control subject the lowest. The experiment employed 4 levels of shock (0.27, 0.30, 0.33, 0.36 ma) and the drug or no drug conditions (Cyclo or saline) for a two-factor design composed of 8 groups of subjects. The subjects used in this and the next experiment were males of the C57B1 strain purchased from Cancer Genetics Research Laboratory, University of California, Berkeley.

Fifteen min prior to training, the mice received an ear punch for identification and an injection of either 0.3 ml saline or 0.3 ml of 10 mg/ml cycloheximide solution prepared in saline. The training and testing were as described in General Procedures. The cutoff latency on the retention test, given 24 hrs after training, was 300 sec. Only healthy animals were tested; 9 out of 174 were discarded.

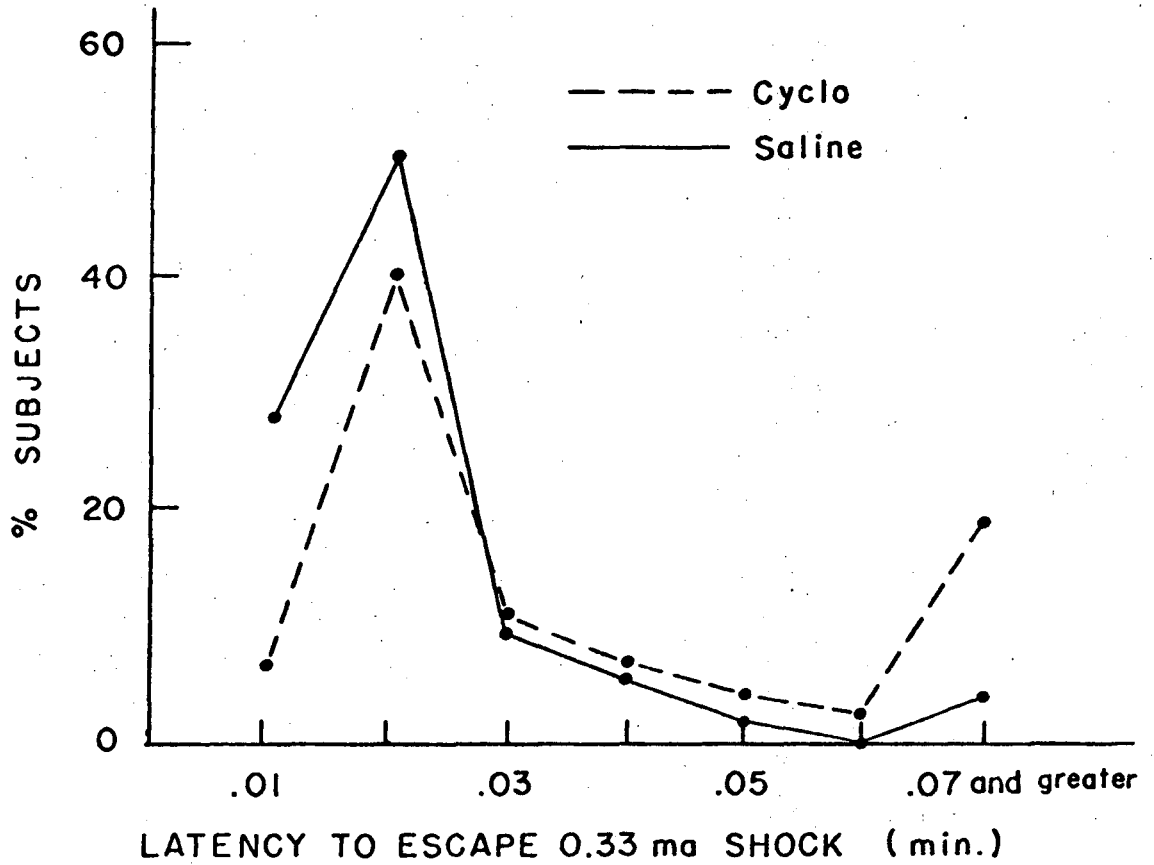
Results

The experimental results demonstrate (a) that shock intensity affects the magnitude and absolute levels of amnesia obtained and (b) that in a procedure commonly used in memory research work considerable variability exists in the values of the parameter of shock escape latency which is important in determining the degree of training.

Figure 4 presents the percentage distribution of escape latencies on the training trial. Only the results for 0.33 ma shock are shown to demonstrate the extent to which the population varies on this important training parameter. The curves for the other shock levels are quite similar; there is some shift toward longer escape latencies with increasing shock intensity. The N for 0.36 ma is smaller for several reasons: (a) subjects had a difficult time escaping from the shock box (some had to be removed), (b) the number of subjects that died or were ill was higher here than in any other group (0.36 ma, 8 replacements; 0.33 ma, 1 replacement; others, none), and (c) it was clear that overtraining had been reached because only 2 out of the 10 useable subjects had test latencies less than 300 sec. The modal escape latency in all groups was 0.02 min.

Table 3 presents the main effects of drug x shock intensity for subjects having the modal escape latency of 0.02 min. Significant amnesia with Cyclo is present at all shock levels except 0.36 ma. The largest differences between Cyclo and saline was at 0.33 ma. Subjects with escape latency of 0.02 min were selected because (a) this was the only value for which there were approximately equal numbers of subjects in experimental and control conditions, and (b) this was the mode for the distribution of escape latencies in each shock condition.

It can be seen that the magnitude of the amnestic effect as measured by the percent difference between experimental and control subjects decreases when the data from all escape latencies are compared (Table 4). This decrease is due to the inclusion of



XBL 7110-5396

Figure 4. The distribution of escape latencies shows that cyclo subjects take longer to escape shock than the saline controls (P <.001 by Fisher Exact Probability Test).

Table 3

Amnesia as a Function of Shock Intensity

Shock Intensity (ma)	0.27	0.30	0.33	0.36
Cyclo				
Amnestic S/Total S	14/18	18/24	25/31	2/10
Percent Amnesia	78%	75%	81%	20%
Saline				
Amnestic S/Total S	5/21	3/24	2/36	0/20
Percent Amnesia	24%	13%	6%	0%
Delta (Cyclo-Saline)	54%	62%	75%	20%
Chi-Square	11.3	19.0	39.0	---
P Value	.001	.001	.001	---

The mode for latencies to escape shock during training in each of the shock intensity groups was 0.02 min, and this table is restricted to subjects who had this latency. Comparing the percent of control and experimental subjects showing amnesia (defined as escape latency of 20 sec or less) significant differences were obtained at 0.27, 0.30, and 0.33 ma. Delta is the difference in percent amnesia for Cyclo subjects minus saline control subjects. The difference at 0.36 ma is probably not significant; the chi-square value cannot be calculated because one of the theoretical frequencies is less than 5.

Table 4

Amnesia as a Function of Shock Intensity

Shock Intensity (ma)	0.27	0.30	0.33	0.36
Cyclo				
Amnestic S/Total S	25/42	18/36	42/69	4/18
Percent Amnesia	60%	50%	61%	22%
Saline				
Amnestic S/Total S	6/47	5/36	5/72	0/20
Percent Amnesia	13%	14%	7%	0%
Delta (Cyclo-Saline)	47%	36%	54%	22%
Chi-Square	21.4	10.8	46.1	---
P Value	.001	.001	.001	---

Disregarding the distributional differences of escape latencies during training shown in Figure 1, and taking all subjects within a shock intensity group, amnesia is still significant at 0.27, 0.30, and 0.33 ma. However, the magnitude of the effect as measured by delta shows a decrease. Here, as in Table 3, the largest delta is found at the shock intensity of 0.33 ma.

Cyclo subjects with escape latencies longer than 0.02 min which causes a decrease in percent amnesia across all shock intensities. The saline subjects show a slightly higher level of forgetting due to the addition of the scores of subjects that escape shock in 0.01 min, some of which did not passively avoid.

Experiment 2

The purpose of this experiment was to see if an amnesic effect could be detected in Cyclo-injected mice that had been overtrained. In addition, various possible parameters of training strength and a parameter of retention were investigated. The experiment employed four factors: drug condition (Cyclo or saline), shock duration, training-to-testing interval, and training latency.

The subjects were C57B1 male mice as in Experiment 1. Other procedures were as described under the heading General Procedures.

Shock Duration

The level of shock employed throughout was 0.33 ma. Pilot studies were done to determine what range of escape latencies would block amnesia in Cyclo-injected subjects when retention was tested 24 hrs after training. Based on these studies, I selected latencies to escape shock from 0.05 min to 0.13 min for study. Since most subjects would not escape shock with latencies less than 0.05 min (see Figure 4), the present experiment used the guillotine door to confine subjects in the shock box for part of the shock period; then the subject was allowed to escape. The confinement and escape procedure set the minimum latency, but the

subjects determined the upper range. For this reason, there are some differences in the number of subjects per latency, but at least 8 subjects were obtained for each escape latency. Subjects escaping with latencies in excess of 0.13 min were disposed of, and replacement was attempted at the next scheduled training session for that group.

Training-to-testing Interval

Tests of avoidance behavior were given at 24 hr, or at 1, 2 or 3 weeks after training. Different subjects were used in each training-testing group.

Training Latency

The training latency was recorded as in Experiment 1. After completing the experiment, it was found that the training latency varied from 0.08 to 10.8 seconds. The variation in training latency was found to interact with other variables being considered (i.e., duration of shock, train-to-test interval, drug vs. saline). Because this interaction was unexpected, the number of subjects/group was not controlled.

Replaced Subjects

Unlike Experiment 1, in which most subjects escaped shock in 0.05 min or less, Cyclo proved to be more toxic in this experiment which employed longer escape latencies; 33 out of 337 were replaced. Subjects that died or showed any signs of illness were replaced without testing at the next training session of their group.

Results

The data for subjects tested at 24 hr have been excluded from the analysis of the amnesic effect in some figures because this group was purposely trained so that little or no amnesia would be present. Since Cyclo-treated animals show amnesia at 24 hr if given less than 0.05 min shock at 0.33 ma and do not show amnesia if given more than 0.05 min shock, "overtraining" in this experiment is defined as experiencing at least 0.05 min shock.

When the data for test period 1-3 weeks were combined, 47% of the 220 Cyclo subjects showed amnesia, while only 2% of the 219 control mice were amnesic. However, the drug's effect overall is inconsistent. A significant percentage of the Cyclo subjects were found not to be amnesic (30%), having test scores in the 281-300 sec range (Table 5). There are relatively few scores between the extreme latencies.

Figure 5 presents the effects of shock duration on the effectiveness of Cyclo as an amnesic agent. Overtraining (i.e., longer escape latencies) progressively blocks amnesia; whereas 83% of the subjects with escape latencies of 0.05 min developed amnesia, only about 30% of those with latencies of 0.12 or 0.13 min show amnesia. Thus, categorizing subjects by their escape latencies accounts for some of the apparently inconsistent effect of the drug.

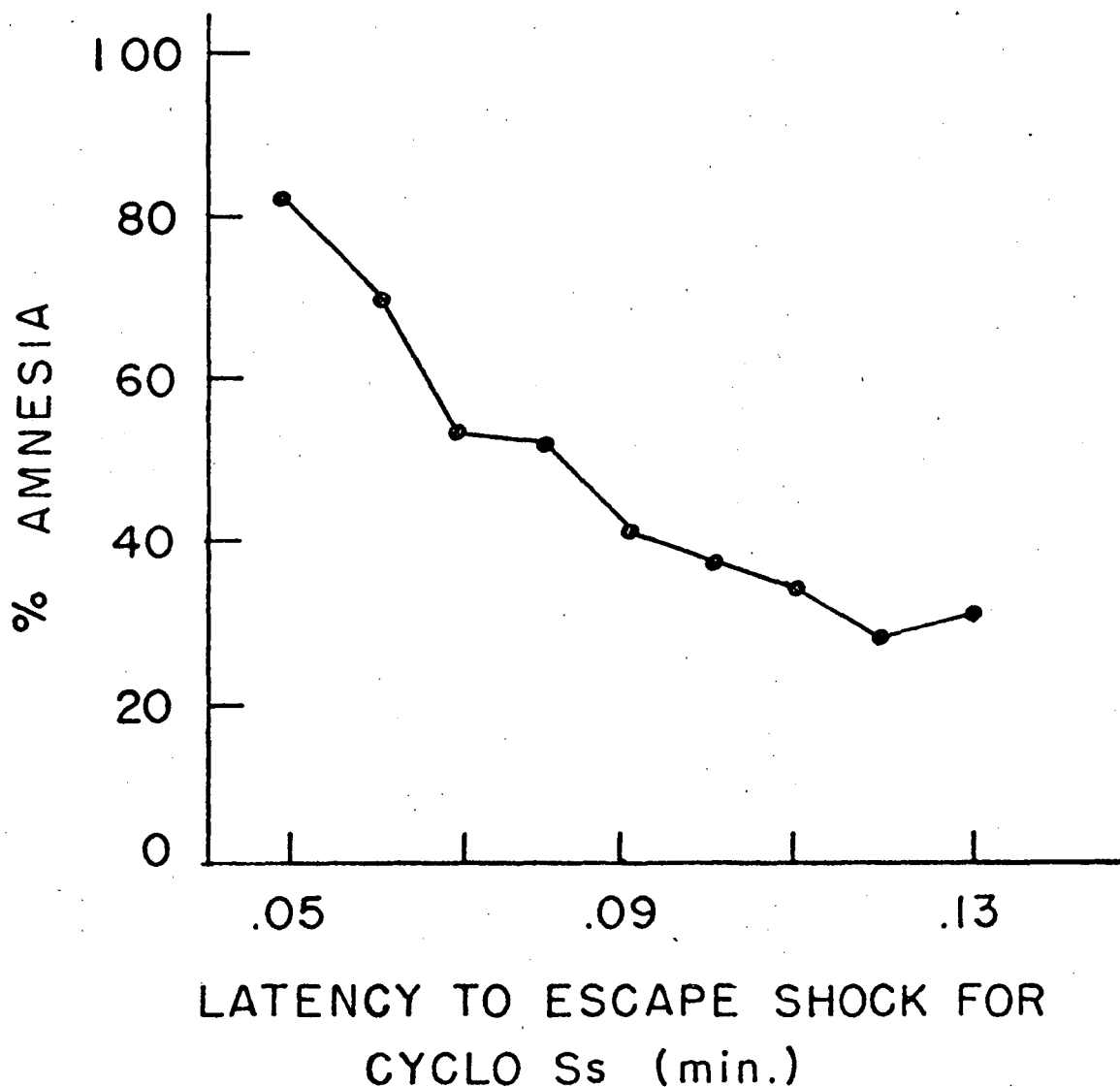
The overall incidence of amnesia in Cyclo subjects, but not in control subjects, increased as the training-to-testing interval was lengthened from 24 hr to 2 wks (Figure 6). At each retention period, less than 3% of the saline subjects showed amnesia.

Table 5

Distribution of Test Latencies
Across Test Periods 1 through 3 Weeks

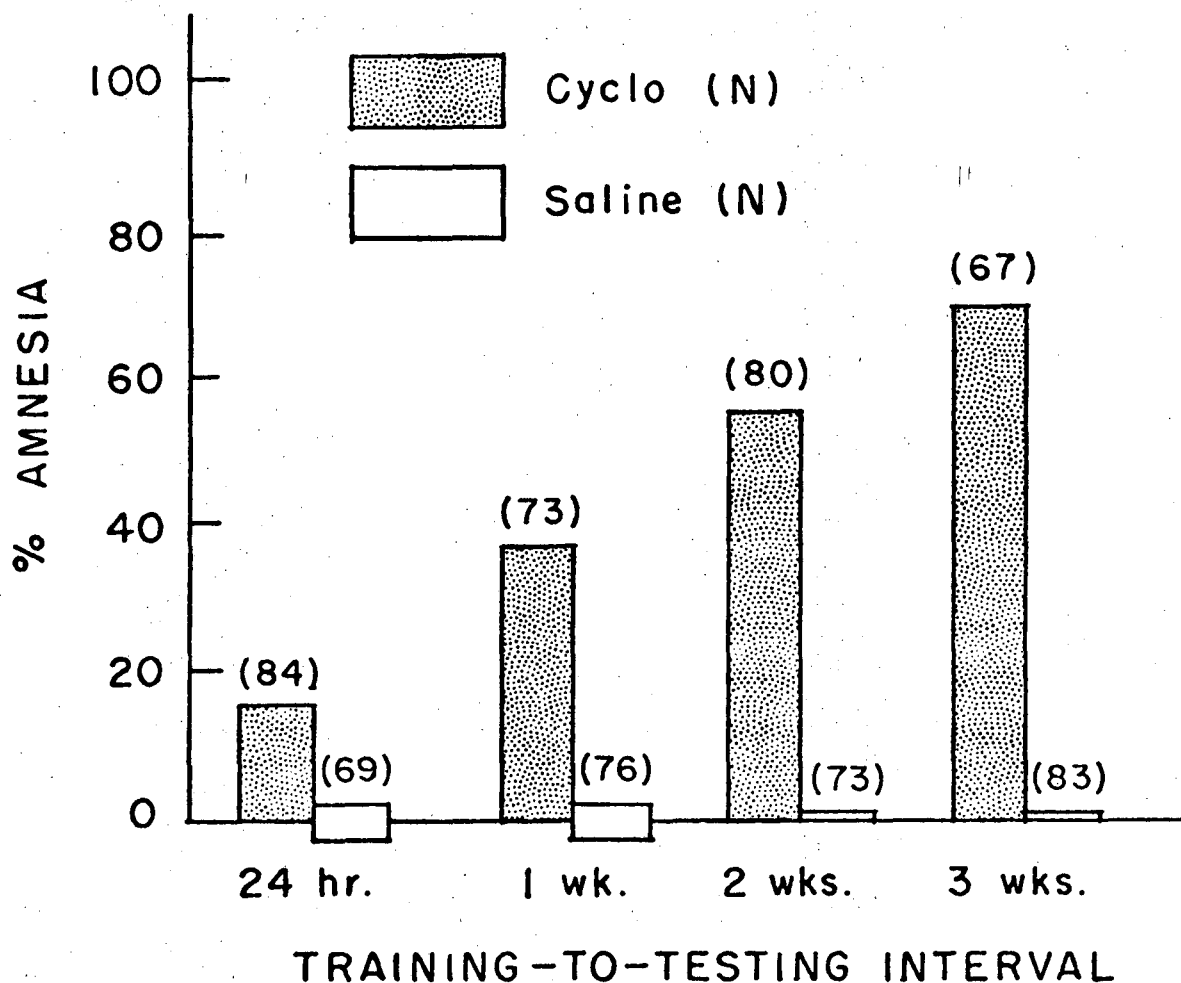
	<u>Cyclo</u>		<u>Saline</u>	
	N	%	N	%
0-20	104	47.3	4	1.8
21-40	10	4.5	3	1.4
41-60	6	2.7	1	0.5
61-80	7	3.2	0	
81-100	5	2.3	2	0.9
101-120	5	2.3	3	1.4
121-140	3	1.4	2	0.9
141-160	5	2.3	2	0.9
161-180	7	4.2	2	0.9
181-200	1	0.5	5	2.3
201-220	0		1	0.5
221-240	1	0.5	2	0.9
241-260	2	0.9	0	
261-280	0		3	1.4
281-300+	<u>64</u>	29.1	<u>189</u>	85.9
	220		219	

Test latency of 0-20 sec is the range of scores representing amnesia. Cyclo was found to have significantly more cases of amnesia ($\chi^2 = 122.2$, $df=1$, $P < .001$). If one considers test latencies of 21-200 seconds as showing impaired memory, 22% of the Cyclo subjects compared to 9% of the control subjects showed impaired memory ($\chi^2 = 14.3$, $df=1$, $P < .001$).



XBL 7110-5395

Figure 5. Main effect of variation in escape latency within the experimental condition. Points represent data summed over test periods 1 through 3 weeks. As the escape latency increases, the probability of amnesia decreases significantly ($\chi^2 = 8.89, P < .005$ for 0.06 min vs. 0.13 min). However, even at 0.13 min experimental subjects differ significantly from controls ($P = .01$).



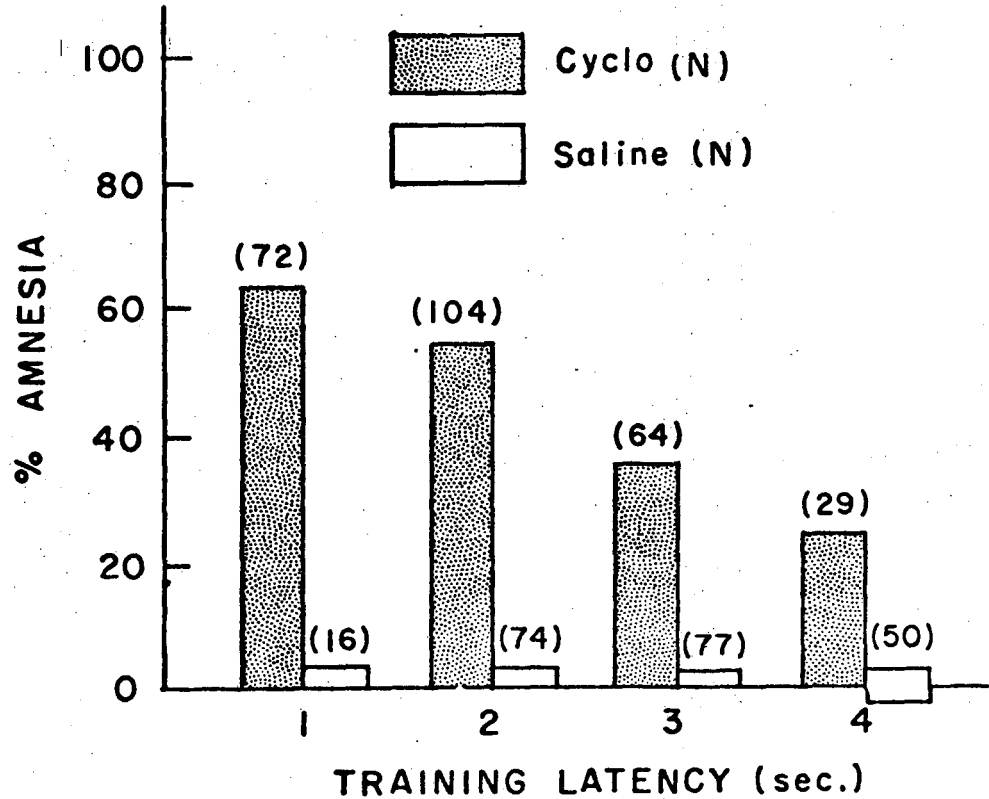
XBL 7110-5392

Figure 6. Percent amnesia as a function of training-to-testing interval. The bars represent all training and escape latencies combined; only the test period is being considered. Cyclo subjects differed from saline controls at 24 hr, 1, 2, and 3 weeks ($\chi^2 = 33.5$, $df=3$, $P < .001$). Within the cyclo condition 24 hr differs from 1 week ($\chi^2 = 6.47$, $P < .01$, 1 week differs from 2 weeks ($\chi^2 = 7.45$, $P < .01$). Bars for saline groups centered on the baseline indicate 0% in this and other graphs.

The latency with which a subject entered the shock box on the training day had a significant effect on the probability that a subject would develop amnesia. The longer the training latency, the less the amnestic effect of Cyclo (Figure 7). Saline subjects had less than 3% amnesia for all these points. Gold, Farrell and King (1971) reported a similar finding using step-through passive avoidance training and transcortical electroconvulsive shock as the amnestic treatment. Cyclo subjects tend to enter the shock box faster than control subjects as reflected by the Ns. A similar observation was also reported by Randt et al. (1971).

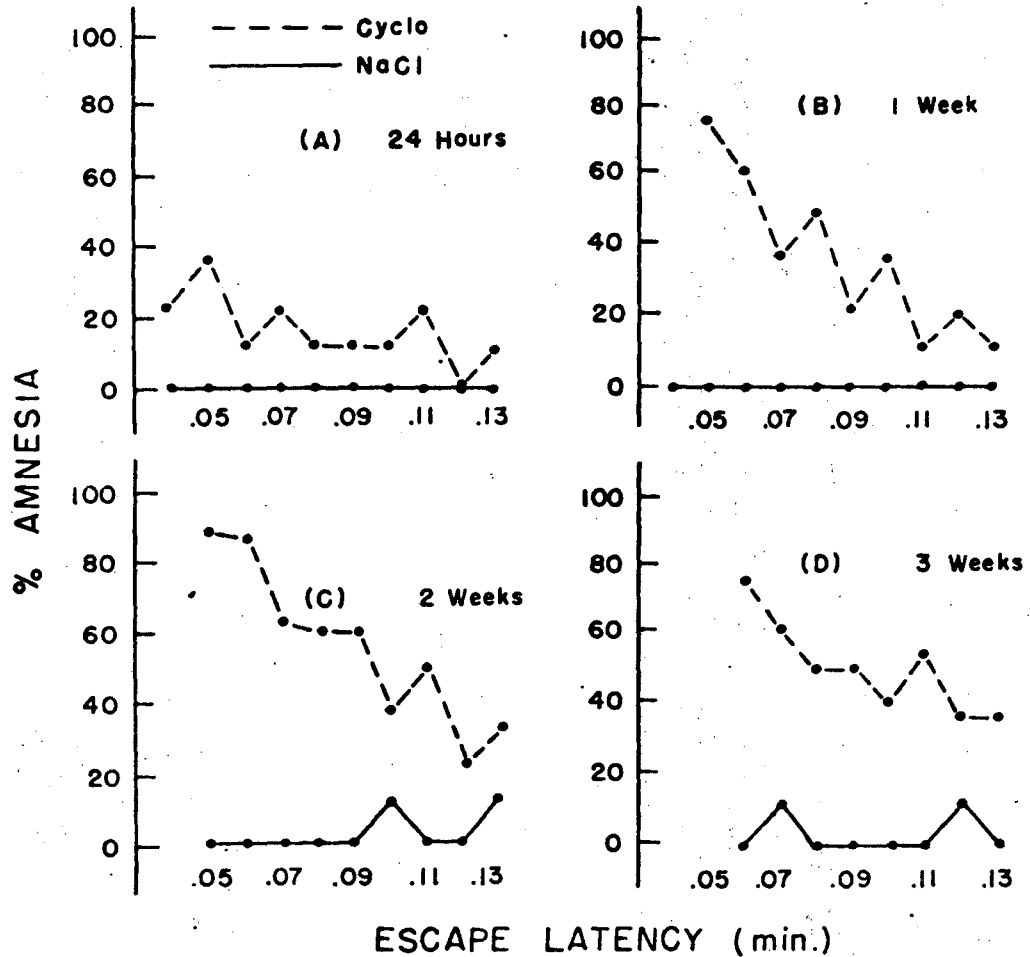
Figure 8 (A-D) represents the complex interaction of drug condition x shock duration x test period. Amnesia is a function of both the time at which testing is done and the shock duration experienced during training. Even at the longest shock latency studied, 0.13 min, amnesia increased from 12.5% after 24 hr to 37.5% after 3 weeks. Control subjects exhibited only a slight tendency to forget at 2 and 3 weeks after original training; only 4 saline subjects in all forgot the training experience.

Table 6 presents the raw data. There is an indication of a 4-way interaction of drug condition x shock duration x test period x training latency. Because the effect of training latency (see Figure 7) was not anticipated, the cells have different numbers of subjects/group, some too small or not represented at all. But the tables give certain indications that are important. For the cycloheximide-injected mice, the values within a cell are generally more consistent than those across rows, columns or tables. Consistent amnesia tends to occur in the upper left hand portion of each



XBL7110-5393

Figure 7. Interaction of training latency x drug condition. Variation in training latency has no detectable effect on control subjects. The data show that for Cyclo subjects, as the latency to enter the shock box increases, the probability of amnesia decreases. For Cyclo vs. saline subjects, $\chi^2 = 17.1$, $P < .001$ at 1 sec; $\chi^2 = 35.3$, $P < .001$ at 2 sec; $\chi^2 = 17.0$, $P < .001$ for 3 sec. At 4 sec χ^2 cannot be calculated; $P = .005$ by Exact Probability Test.



XBL7110-5399

Figure 8. Three way interaction of drug condition, escape latency, and test period. The greatest change occurs from 24 hr to 1 week. The percent amnesia increases slowly at escape latencies 0.09 through 0.13 min over the 2 weeks. $\chi^2 = 13.2$, $P < .001$ for 0.09 - 0.13 min at 24 hr vs. 2 and 3 weeks. $\chi^2 = 8.87$, $P < .005$ for 1 week vs. 2 and 3 weeks. (Each data point represents 8-10 subjects.)

TABLE 6
EFFECTS OF TRAINING LATENCY, ESCAPE LATENCY AND RETENTION INTERVAL ON TEST LATENCIES

A. Saline Control + 24 hours Escape Latency (min)												A'. Cycloheximide + 24 hours Escape Latency (min)											
Training Latency (sec)	0.04	0.05	0.06	0.07	0.08	0.09	0.10	0.11	0.12	0.13	Training Latency (sec)	0.04	0.05	0.06	0.07	0.08	0.09	0.10	0.11	0.12	0.13		
1	300		300 300		300				300		1		7	5	9 26 300	300	2	300	6 300 300		1 300 300		
2	300 300	300	300 300	300 300	300 300	300 300	300 300	300 300	300 300	300	2	3 158	15 138	300 300 300	8	4 149 300 300	300 300		2 300 300 300	277 300 300	226 300		
3	166 300	300	64 300 300	218	300		300	300 300	300 300	300	3	236 276 300	300 300	300	300 300 300	242 300	21 300 300	8		300 300	300		
4	300	300 300	300	300	300	300 300 300		300	300	120 300 300 300	4	300		133	300			300 300	300 300	300 300	300		
over 4 sec	111 300	300 300 300	251	300 300 300	300 300 300	300 300	300	300	300	300 300 300	over 4 sec	8 300	7 300	300		300	300	300		300	300 300		

B. Saline Control + 1 week Escape Latency (min)										B'. Cycloheximide + 1 week Escape Latency (min)											
Training Latency (sec)	0.04	0.05	0.06	0.07	0.08	0.09	0.10	0.11	0.12	0.13	Training Latency (sec)	0.04	0.05	0.06	0.07	0.08	0.09	0.10	0.11	0.12	0.13
1			300			300		29	300		1		10 12	8 10 12 16	13 118	7	300	12 15 300	22 300	2 2	5 96 300 300
2		300 300	300 300	300		231		300 300	300 300	300	2		2 3 12	5 156 172 300	8 9	10 11 300 300	10 23 86 90 170	300	106 153 239 300	300 300	284 300
3		200 300 300	300 300	300	300	300	300 300 300	300 300 300	300 300	300	3		300 300		300 300 300	300 300	300 300	27	300		300
4		300 300	300 300	300	300 300	300 300	300 300	300 300	300	300	4					5		6 172 300 300		300	
over 4 sec		300 300	300	300 300 300	300 300 300	300 300	300	300	300	300	over 4 sec		7		300				300	300 300 300	300

Table 6 (cont.)

Training Latency (sec)	C. Saline Control + 2 weeks Escape Latency (min)										Training Latency (sec)	C'. Cycloheximide + 2 weeks Escape Latency (min)									
	0.04	0.05	0.06	0.07	0.08	0.09	0.10	0.11	0.12	0.13		0.04	0.05	0.06	0.07	0.08	0.09	0.10	0.11	0.12	0.13
1				300		300			124		1		3	4	6	4	4	7	1		2
2			300	300	153	34		225	300	4		4	4	8		159	8	34	70		22
3		171		300	300	300	6	300	300	300	2		4	3	6	11	5	6	12	3	6
4			300	199	300	300	300	300	300			5	115	6	300	300	120			3	72
over 4 sec	300	117	205	84	96	300	300	300	300	300		6	6	10	300	6	6	4	10	300	2
	300	300	300	300	300	300	300	300	300	300	3	3	5	19		6	7	28	128	300	14
	300	300	300	300	300	300	300	300	300	300	4		72		14	36	300				300
	300	300	300	300	300	300	300	300	300	300	over 4 sec			6	43	10	189		6	49	5
																			144	161	242
																			300	300	300
																					300

Training Latency (sec)	D. Saline Control + 3 weeks Escape Latency (min)										Training Latency (sec)	D'. Cycloheximide + 3 weeks Escape Latency (min)									
	0.04	0.05	0.06	0.07	0.08	0.09	0.10	0.11	0.12	0.13		0.04	0.05	0.06	0.07	0.08	0.09	0.10	0.11	0.12	0.13
1							300	300	300		1			2	4	2	69		3	300	2
2			300	12	300	116	300	300	300	300				16	5	44	300		108		300
3		179	300	300	300	197	300	139	3	300	2		2	3	2	2	2	3	4	2	
4			300	190	300					300		2	5	95	4	5	8	4		68	
over 4 sec	265	300	144	300	300	23	182	300	300			9		300	6	20	15	300		300	
	300		300	300	300	300	300	300	300	300	3		96			300	4	4	4	7	55
											4			13	9	180	300	300	159	300	
											over 4 sec				72					5	6
																			300	300	

table for Cyclo subjects. The lower right hand corner tends to contain the longest latencies.

Discussion

The following parameters have been shown to affect the degree of amnesia in Cyclo-treated subjects: (a) shock intensity (Exp. 1), (b) shock duration (Exp. 2), and training latency (Exp. 2). In addition, the sensitivity of the passive avoidance test to forgetting increased markedly for Cyclo subjects as the training-to-testing interval increased (Exp. 2).

Controlling Inconsistency

The inconsistency of cycloheximide's effect has been of concern primarily because the assumption was made that training was the same for all subjects in a given treatment condition. A major source of the previously reported inconsistent results may be the fact that not enough parameters of the training condition were defined and measured. We have observed that variations occur in several parameters of training and that this variation systematically affects the results. When we factored out some of the variability in our training situation (variability likely to be present in other similar experiments), the consistency of the amnesic effect was substantially improved.

Overtraining

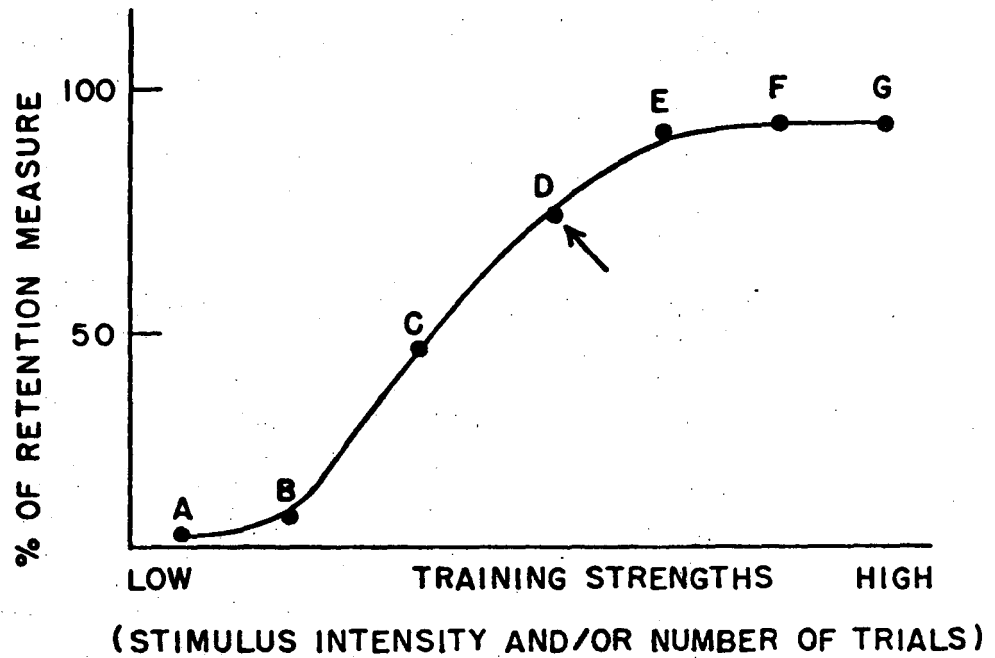
Earlier we defined overtraining loosely as giving Cyclo-injected subjects so much training that the amnesic effect is blocked. This is a circular definition which generates a hypothesis that cannot be easily disproved. The question is, what

level of training should reliably elicit Cyclo's amnestic effect, while still demonstrating memory on the part of the control subjects. The definition or criterion for this level or point of training must be relative to the control subject's performance.

In other studies we have found that if control subject acquisition curves are plotted for successive increases in training strength, the curves tend to look sigmoidal (Figure 9). In passive avoidance, 1-way active avoidance and 2-way shuttlebox tests, we have generally found that Cyclo is most effective as an amnestic agent at the point just prior to the asymptote of the acquisition curve. In the case of Figure 9, D is that point. Looking back to Table 3, the control performance reaches its asymptote at 0.36 ma; at values below this point amnesia is obtained. We have not formally tested this suggested rule, but it has proved reliable in most of our experiments with Cyclo. This rule is not without exceptions, because other variables than the amount of training can affect the occurrence of amnesia (i.e., when the test is given and/or the disruptive agent used).

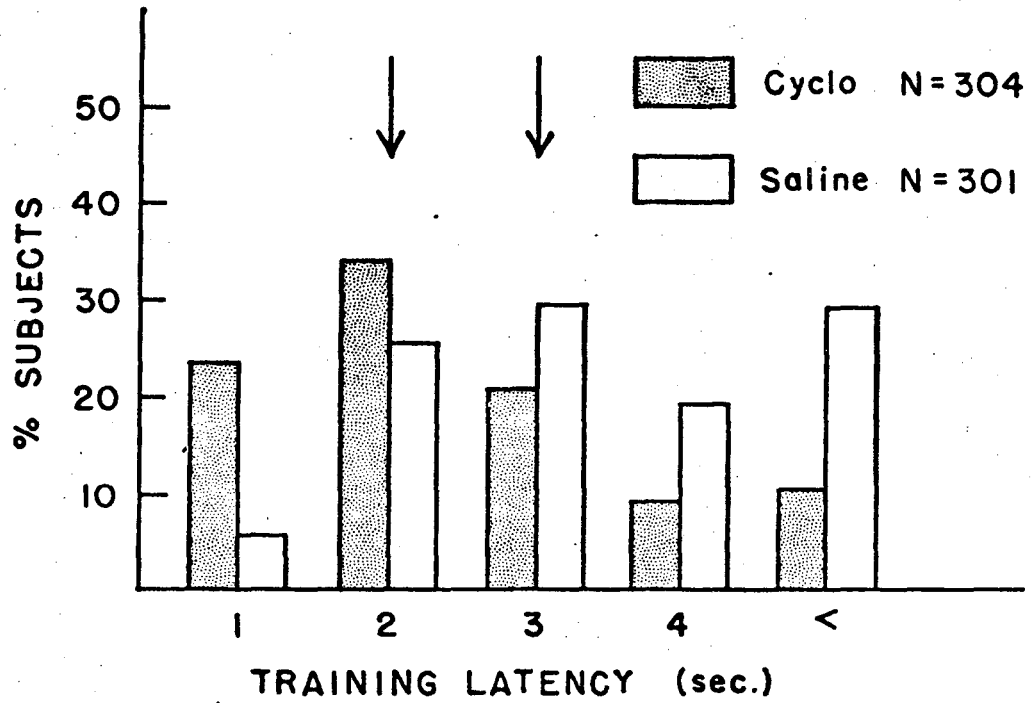
Control Problems

The fact that Cyclo decreases the latency to enter the white compartment during training and that it increases the latency to escape shock leads to problems of control that merit discussion. The distribution of latencies to enter on the training day for Cyclo and saline subjects is shown in Figure 10. Only at latencies to enter of 2 and 3 sec are there both significant numbers and about an equal percent of Cyclo and saline subjects.



XBL7110-5411

Figure 9. Typical acquisition curve. The arrow indicates the point where Cyclo usually has the greatest effect. Training strength may involve stronger stimuli (i.e., brighter light, stronger shock, longer shock) or increases in the number of training trials. ● = hypothetical data point.



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Figure 10. Percent distribution of training latencies. Cyclo subjects enter the white shock box on the training day significantly faster than controls, $\chi^2 = 73.4$, $df=4$, $P < .001$.

Amnesia is affected by the latency to enter; shorter latencies have a greater probability of producing amnesia (Figure 5). If we included the 1 sec latencies this would tend to overestimate the amnestic effect of cycloheximide. Control subjects have been greatly overtrained--86% had test latencies over 300 sec. Therefore, it is unlikely that the conclusion would be significantly altered had we run enough control subjects at the 1 sec training latency to obtain equal Ns of Cyclo and saline Ss. However, in paradigms in which the control subjects are not highly trained, failure to match groups for latencies to enter could seriously distort the conclusions.

Counting only the scores from 2 and 3 sec training latencies across the 1 to 3 week test, there is 47% amnesia among the 119 Cyclo-injected mice and 3% among the 107 saline-injected mice. This compares well with the overall incidence of amnesia, 47% for Cyclo and 2% for saline. A comparison of the scores of subjects having 2 and 3 sec training latencies did not significantly alter any of the trends reported.

Another control problem was found in Experiment 1; Cyclo subjects take longer to escape from shock than control subjects. In Experiment 2, it was found that as shock duration increases, the probability of a subject developing amnesia decreases. Failing to match Cyclo and saline subjects on the variable of shock duration will underestimate the amnestic effect. This can be seen to occur by comparing Tables 3 and 4 of Experiment 1. In Table 3, which includes only the scores for those subjects escaping shock in 0.02 min, amnesia was 81% for the Cyclo group and 6% for saline--

a difference of 75%. Table 4, based on all subjects whatever their latency to escape shock, shows 61% amnesia for Cyclo and 7% for controls--a difference of 54%.

These findings make it advisable to record latency data when employing the passive avoidance step-through test. This would seem particularly important when lesions or drugs result in any hyperactivity, as is the case with cycloheximide.

Related Studies

Delayed onset of amnesia has been reported in several recent studies: in mice following electroconvulsive shock, by Geller and Jarvik (1970a); Hughes, Barrett and Ray (1970); and McGaugh and Landfield (1970); in goldfish following acetoxycycloheximide or cycloheximide, by Davis and Agranoff (1966) and Agranoff (1971); in chicks following cycloheximide, by Watts and Mark (1971); in mice following acetoxycycloheximide or cycloheximide, by Barondes and Cohen (1967, 1968) and Cohen and Barondes (1968a,b). The delayed onset of amnesia has occurred in some studies over a few hours, in other studies over days, and in our present study, over weeks. It is quite possible that different memory processes are involved in these different time courses, as we will discuss shortly.

In contrast to these experiments reporting the progressive development of amnesia, several studies have reported a transient amnesia following treatment [Flexner, Flexner and Roberts (1966); Quartermain, McEwen and Azmitia (1970); Serota (1971); and Barondes and Cohen (1967)]. Barondes and Cohen reported impaired memory 24 hr after training in mice that had been given acetoxycyclohexi-

mide. They attributed the memory impairment to illness, since the subjects were not found to be amnesic 4 days after the training and drug treatment. Since most of these experiments report the "transient amnesia" to occur at 24 hr after the subjects have received the protein inhibitor, it seems likely that the cause of the apparent amnesia was the lingering effects of the inhibitor rather than a true disruption of memory with subsequent recovery. In this work, there is no indication of recovery of memory once amnesia has developed.

Speculation

Noting that amnesia may develop hours after treatment in some experiments and over days in others, Squire and Barondes (1972a) have recently suggested that different studies may reflect variously the decay of short-term memory and interference with the retrievability of weakly established long-term memory: When protein synthesis is largely inhibited, a long-term memory process might be only weakly established and "might be particularly vulnerable to interference and could rapidly become inaccessible (p.76)." Because of our findings of increasing amnesia over a two-week period, we have been concerned with the same problem and have formulated a related but more strictly chemical hypothesis which is explicitly based on decay rather than interference processes. Four assumptions are made to explain why controls, who synthesize more protein than drug subjects, also remember longer: (a) structural changes supported by protein synthesis induced by training must remain above a threshold level for long-term memory to be expressed; (b) the amount of protein synthesized and thus the extent

of structural change increases with the intensity of training (i.e., with stronger stimuli and/or more trials); (c) over time the structural changes are imperfectly maintained and, if they fall below the threshold, forgetting occurs; (d) therefore, the length of time over which a memory is available is a function of the amount of protein synthesis induced by training. The role of protein is not considered to be that of a "memory molecule", but rather of creating or supporting some structural modification in the neuronal network controlling behavior.

Conclusions

1. The inhibition of incorporation of valine-¹⁴C into protein caused by subcutaneous incorporation of cycloheximide was measured in brains of mice of 5 strains.
2. Amnesia for one-trial passive avoidance training in cycloheximide-injected vs. saline-injected mice was found to vary as a function of several parameters:
 - a. The percentage of amnesia decreased with intensity of the footshock.
 - b. Latency to escape shock varies among animals and is a determinant of how effective the amnestic agent will be. The longer the shock duration, the lower is the percentage of amnestic subjects (Figure 5).
 - c. Latency to enter the shock compartment on the training trial is another determinant of amnesia; the longer the training latency, the less the percentage of amnesia (Figure 7).

d. The incidence of amnesia increased with the training-to-testing interval in Cyclo subjects but not in saline controls; over 50% of Cyclo subjects showed amnesia when tested 2 or 3 weeks after training as against only 17% amnesia when tested 24 hr after training. The controls showed almost no amnesia at any of these times (Figure 6). By controlling these factors, either high or low incidence of amnesia can be obtained. Experiment 2 used the shock level of 0.33 ma, selected from the results of Experiment 1 as optimal for our training procedure with this strain. Selecting animals with short training latencies (1 or 2 sec) and short escape latencies (up to 0.06 min), 25 out of 30 (83%) of Cyclo subjects were amnesic in tests at 1, 2 or 3 weeks. On the other hand, if subjects were selected for long training latencies (4 sec or more) and long escape latencies (0.10 min or more), only 4 out of 29 (14%) were amnesic. No control subject with either set of values was amnesic.

3. It may be useful to define overtraining as the amount or strength of training that brings control subjects to their asymptote of performance. For both passive and active avoidance we have found that cycloheximide is most effective as an amnesic agent when given to subjects just prior to the asymptote of the acquisition curve (see Figure 9).

4. While more consistent amnesic effects can be obtained by controlling some sources of variation in training strength, it should nevertheless be of concern to those who support the protein-memory hypothesis that despite over 90% inhibition of protein synthesis, a slight increase in the degree of training still permits a high level of retention.

III. THE INFLUENCE OF DURATION OF PROTEIN SYNTHESIS INHIBITION ON MEMORY

Introduction

The purposes of the experiments in this section were to test the hypothesis that the length of brain protein synthesis inhibition is an important parameter in producing amnesia, and to demonstrate the value of a new amnestic agent--anisomycin (Ani).

It has been implied in reports using inhibitors of protein synthesis to block memory that the duration of inhibition is an important factor in the effectiveness of the amnestic agent (Agranoff, 1971; Flexner, Flexner, de la Haba and Roberts, 1965; Squire and Barondes, 1972a). Previously it had not been feasible to test the hypothesis, since puromycin and acetoxycycloheximide had a long duration of inhibition--7 to 9 hrs at 80% or greater inhibition depending on the dose (Barondes and Cohen, 1964; Flexner, Flexner, Stellar, Roberts and de la Haba, 1964). Furthermore, the dose of Cyclo that results in inhibition of 80% or greater for 2 hrs 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 hrs duration, Ani has not been found to be toxic, even when injected four times at two-hr intervals. Anisomycin, alone

or in combination with Cyclo or streptovitacin A, can achieve various lengths of inhibition from 2 to 24 hrs, where inhibition is maintained continuously above 80%. The subjects survive the prolonged inhibition of brain 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 (Kerkut *et al.*, 1970; Squire and Barondes, 1972a). A single injection of Cyclo frequently causes subjects to become ill. Behaviorally, Cyclo causes differences in locomotor activity of mice exposed to an open field (Squire, Geller and Jarvik, 1970) and in the distribution of latencies in a step-through passive avoidance apparatus (Flood, Bennett, Rosenzweig and Orme, 1972; see Figures 4 and 10 of this dissertation). Anisomycin has not been found to produce such latency changes.

INHIBITION OF PROTEIN SYNTHESIS

Procedures

Anisomycin (1-p-methoxyphenyl-3-acetoxy-4-hydroxypyrrolidine) was a gift from Charles Pfizer Co., Groton, Conn., through the generosity of Dr. N. Belcher. Solutions were prepared at appropriate concentrations of 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.

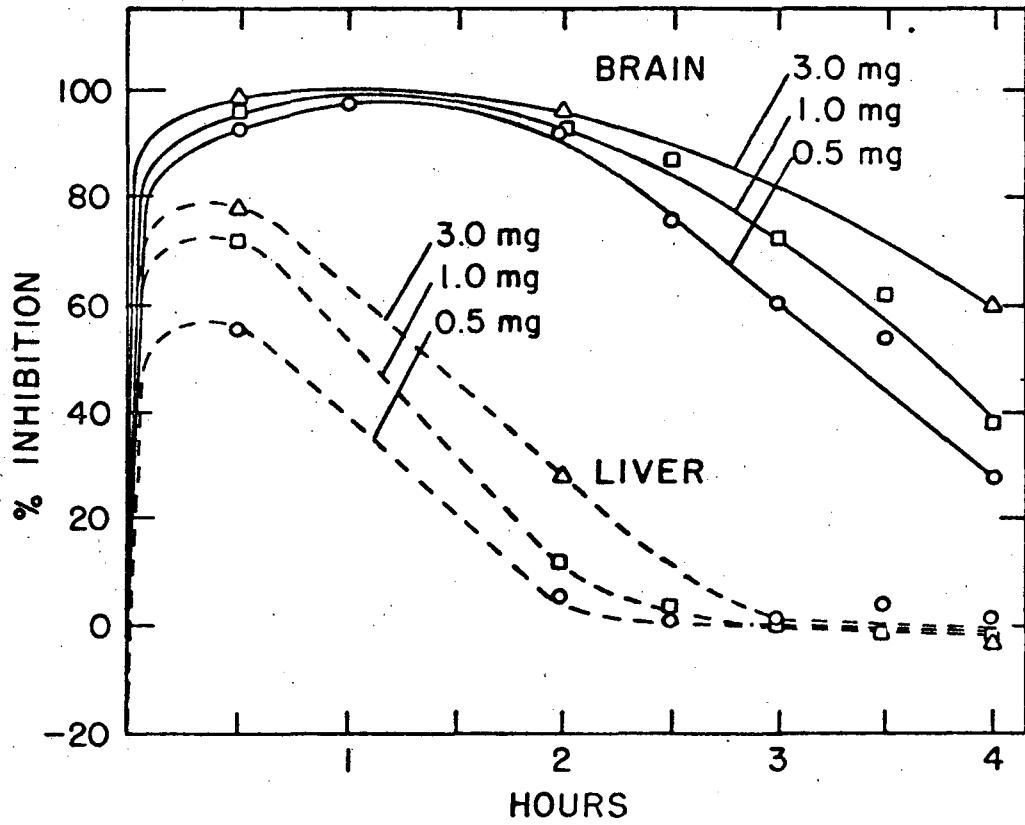
Inhibition of protein synthesis was determined as described under the heading General Methods.

Results

Anisomycin is relatively non-toxic in mice. The lethal dose of the drug 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 in liver was determined as a function of the dose ranging from 0.5 to 3.0 mg (Figure 11). Subcutaneous injections yielded greater than 90% inhibition in brain during the first 2 hrs. 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 brain protein synthesis in mice, we studied the effect of repeated subcutaneous injections of 0.5 mg of Ani at 2-hr intervals. The inhibition curves for 3



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Figure 11. Inhibition of protein synthesis by subcutaneous injection of anisomycin in mice for liver and whole brain.

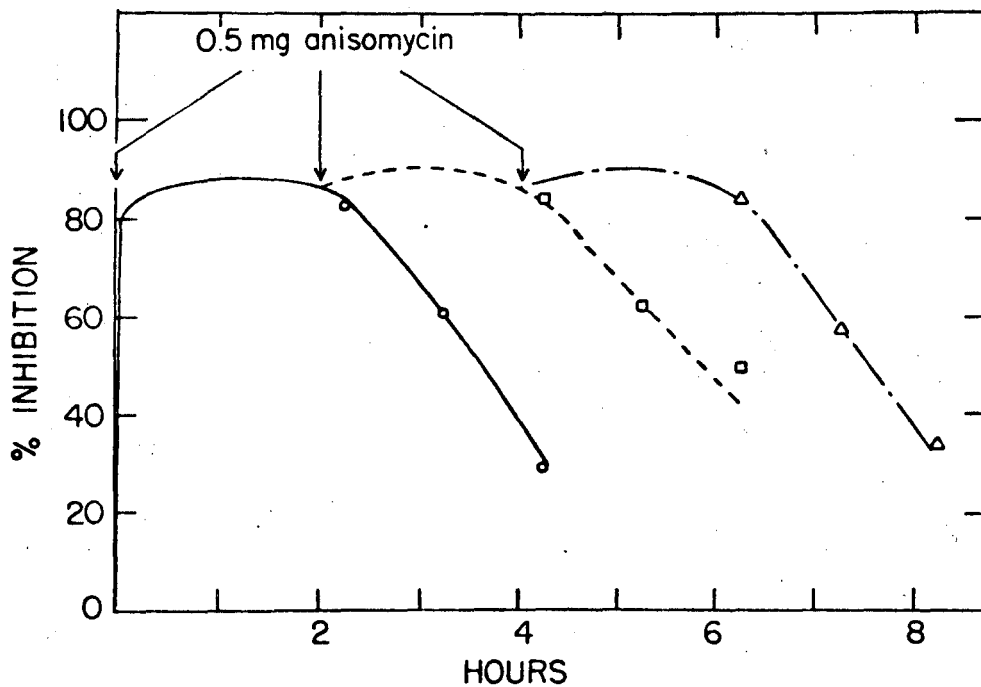
successive injections showed that protein synthesis can be inhibited at least 85% for up to 6 hrs, and that there is little cumulative effect of the drug (Figure 12). Each of the injections inhibited at 80% or greater for about 2 hrs. Thus, the duration of protein synthesis inhibition can be readily 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 Figure 13. The inhibition at short times after administration of the drugs was based on 10 min incorporation periods and is presented in the inset of Figure 13. 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 hrs, the inhibition by Ani drops off more rapidly than that caused by Cyclo.

BEHAVIORAL EFFECTS

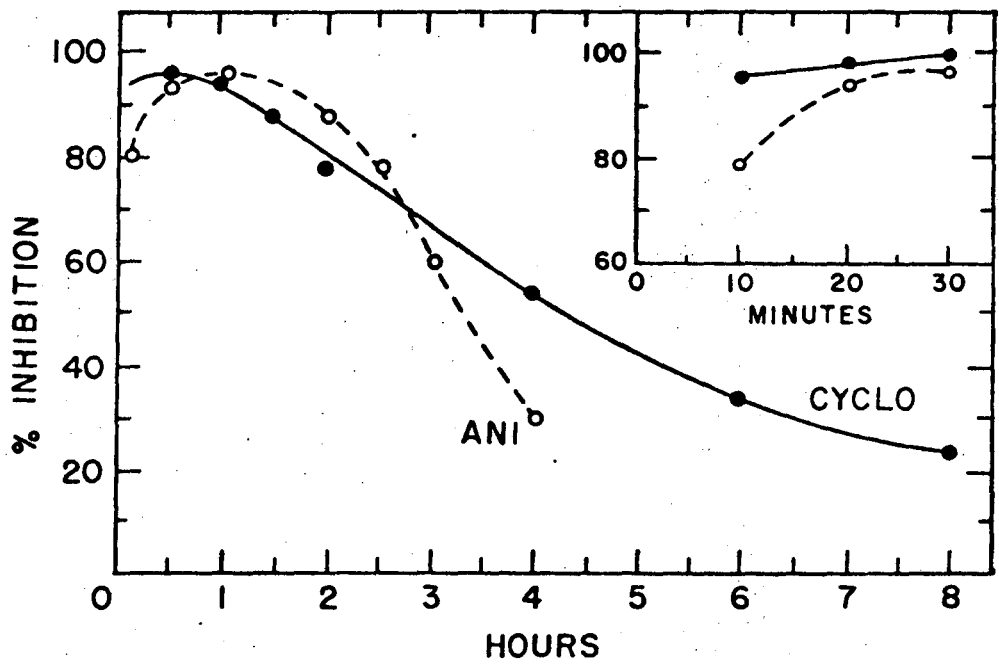
Subjects

For the experiments in this section the subjects were females from our colony in its 14 generations of inbreeding (except in Experiment 4 where males were used). Our colony bears the designation C57B1/Jf to distinguish them from the parent stock of C57B1 maintained at Cancer Genetics Research Laboratory. Our colony was started from a single inbred pair of C57B1/Crg1. The



XBL7111-5435

Figure 12. A summary of the effect on cerebral protein synthesis of one (o—o), two (□-----□), or three (Δ-----Δ) subcutaneous injections of 0.5 mg of anisomycin to C57B1/Jf female mice. Ani was administered at time 0, 2 and 4 hrs. The symbols indicate times at which the mice were sacrificed.



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Figure 13. Comparison of the inhibition of protein synthesis in brain by anisomycin (0.5 mg) or cycloheximide (2.5 mg).

subjects were between 60 and 70 days of age and weighed between 18 and 21 grams at training. Subject assignment was random; where several groups were employed, each condition was represented at each training and testing session. Animals had food and water available at all times.

Drug Conditions

All injections, whether administered pre- or post-training, were given subcutaneously at the following volumes and concentrations (exception noted in Experiments 4 and 9): saline, 0.25 ml; Cyclo, 0.23 ml of 10 mg/ml solution; and Ani, 0.25 ml of 2 mg/ml solution. Drugs were prepared in saline. Injections were given under very light ether anesthesia as described in the Introduction.

Other

The apparatus, training and testing were as previously described unless otherwise noted under specific experiments.

Comparison of Anisomycin (Ani) and Cycloheximide (Cyclo)

Experiment 3

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 hrs after training. As in previous experiments (see Figure 4),

the mode of the escape latencies was 0.02 min; only the data from subjects escaping shock in 0.02 min will be compared.

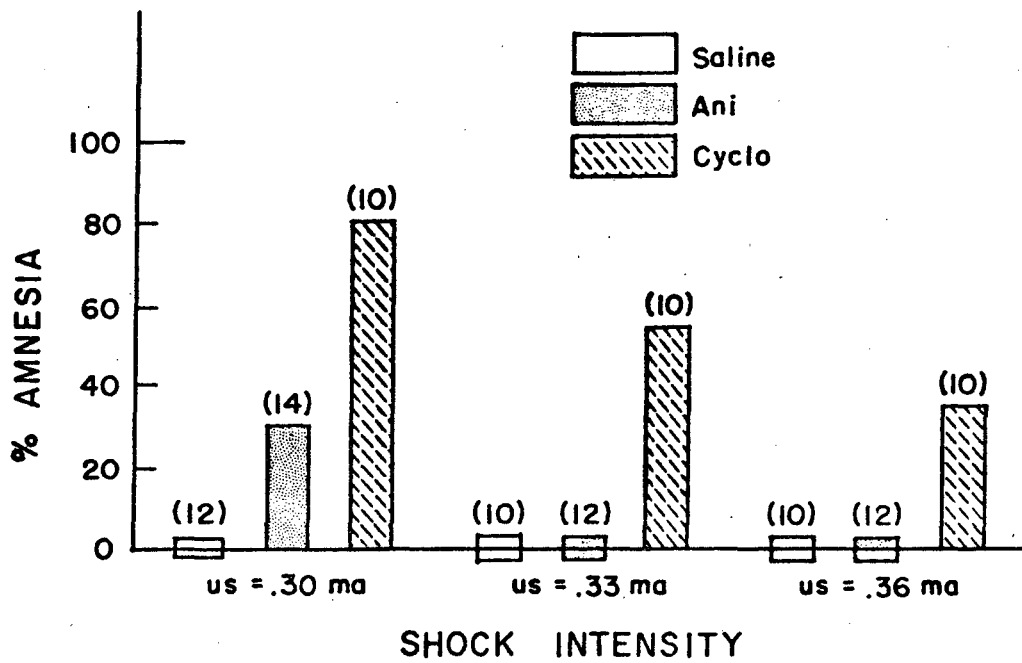
Results

While Ani and Cyclo caused comparable inhibition of protein synthesis (Figure 13), Ani proved not to be as effective an amnesic agent as Cyclo (Figure 14). Ani was only effective as an amnesic 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 subjects ($\chi^2 = 9.59$, $P < .005$; at 0.36 ma a significant difference was not obtained, $P = .10$). Since Ani and Cyclo were found to cause comparable inhibition of protein synthesis, cycloheximide's greater amnesic effect must be due to some factor in addition to inhibition of protein synthesis.

Experiment 4

Design

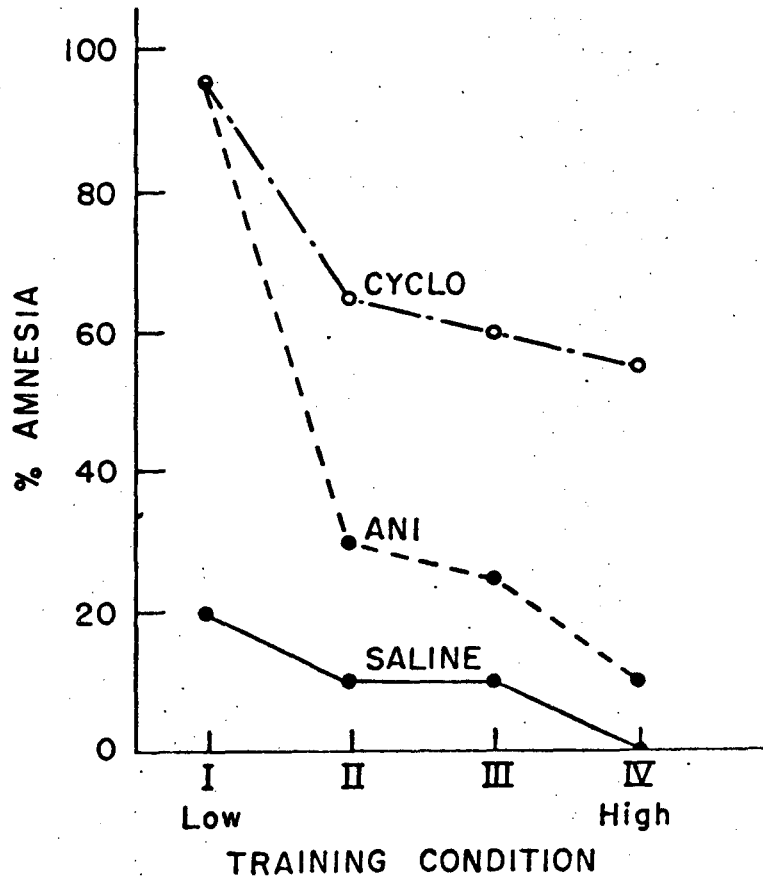
This experiment investigated the effects of training latency and escape latency on the probability of obtaining amnesia with Ani or Cyclo. Subjects, C57B1 males, were classified into four groups according to their performance during the training session: (I) short training and short escape latency--this provided minimal training; (II) long training and short escape latency; (III) short training and long escape latency; and (IV) long training and long escape latency--this provided the maximal training. The particular latency values for each training condition may be seen in Figure 15. Ani (0.5 mg), Cyclo (3.0 mg) or saline were



XBL 721-4516

Figure 14. Effect of shock intensity on anisomycin and cycloheximide induced amnesia. Cyclo vs. Ani at 0.30 ma: $\chi^2 = 9.6$, $df=1$, $P < .005$; at 0.33 ma $P = .10^*$. Cyclo_{.30 ma} vs. Cyclo_{.33 ma} vs. Cyclo_{.36 ma}: $\chi^2 = 9.59$, $df=2$, $P < .01$.

*P calculated by Fisher Exact Probability Test. Retention test given 24 hrs after training.



XBL727-4702

Figure 15. Comparative amnestic effect of anisomycin or cycloheximide as a function of training strength. N=20/point. Ani vs. saline at I and II, $P < .001$; at III, $P < .002$; at IV, $P = NS^*$. Cyclo vs. saline at I, II, III, IV, $P < .001$. Ani vs. Cyclo at I, $P = NS$, II, III, $P < .025$; IV, $P < .001$.

* P obtained by χ^2 Test, all others by Fisher Exact Probability Test. Retention given 1 wk after training. The latency values for the four training conditions were:

	<u>Training Latency (sec)</u>	<u>Escape Latency (min)</u>
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

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 subjects; only 1 in 3 mice could be classified in training conditions II or IV.

Our procedure for generating long escape latencies has been described in Experiment 2. It involves replacing the guillotine door after the subject enters the white box and not removing it until a few seconds 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 3 with single injections, Cyclo proved to be a more effective amnestic agent than Ani. Figure 15 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 amnesic subjects 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 amnesic 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. (For other strain comparisons see Section IV.)

Effects of Duration of Inhibition of Protein Synthesis

Experiment 5

Design

The purpose of this experiment was to see whether doubling the duration of inhibition would cause more subjects to become amnesic. All subjects were injected at time zero and trained 15 min later. The groups employed can be seen in Figure 16. The Ani and Na groups received only the pretraining injections. Na+Na, Ani+Ani, and Na+Ani received one injection before training and another 2 hrs after the first injection. Ani+I was injected prior to training and pseudo-injected 2 hrs later; nothing was injected. The shock intensity was set at 0.33 ma. The retention test was given 1 wk after training to subjects 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 of Methods.

Results

Two successive injections which maintained the inhibition at 80% or greater for 4 hrs caused significantly more amnesia than a single injection of Ani which inhibited protein synthesis for 2 hrs at 80% or greater ($P = .001$, Fisher Test). The injection procedure itself had no significant effect on the percent amnesia as demonstrated by the low percentage of amnestic subjects in the control groups Ani+I, Na+Na and Ani+Na. A second injection of Ani alone did not cause any amnesia (Na+Ani).

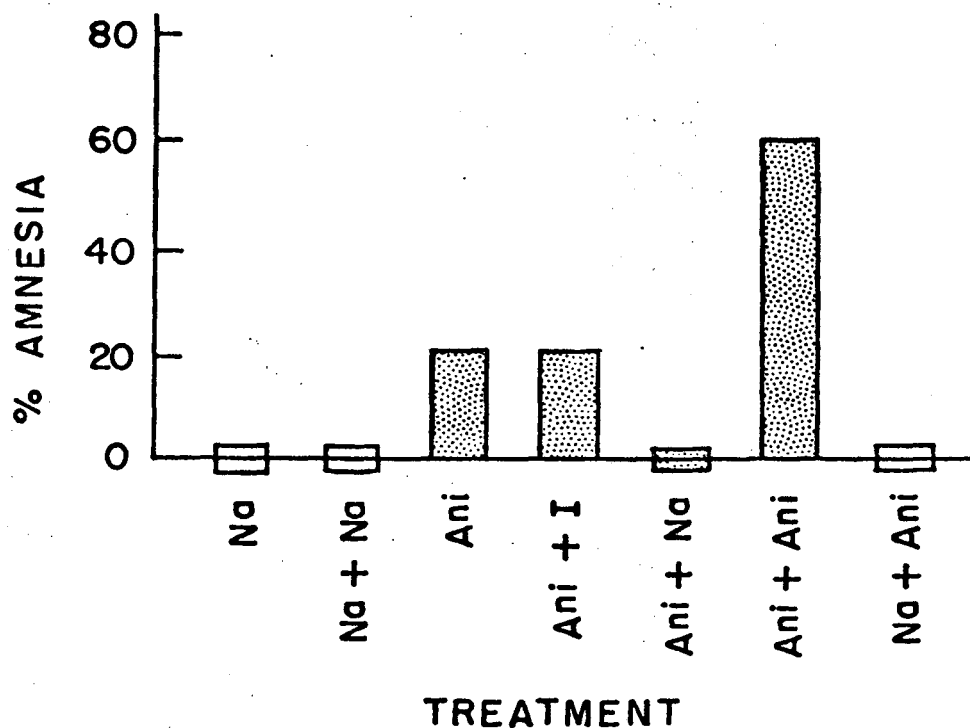
Experiment 6

Design

The purpose of this experiment was to see if the increase in amnestic effects reported in Experiment 5 with a greater duration of inhibition, were 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 5, except that 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 hrs at 80% or greater, the effect of Ani+Cyclo was to block protein synthesis for 5-1/2 hrs at 80% or greater.



XBL 721-4513

Figure 16. 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 <.001. Retention test given 1 week after training.

In keeping with the hypotheses 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 amnestic effect than Ani+Ani for both short and long training latencies, but the difference was not significant (Figure 17). 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, $P < .001$). The saline controls showed no amnesia.

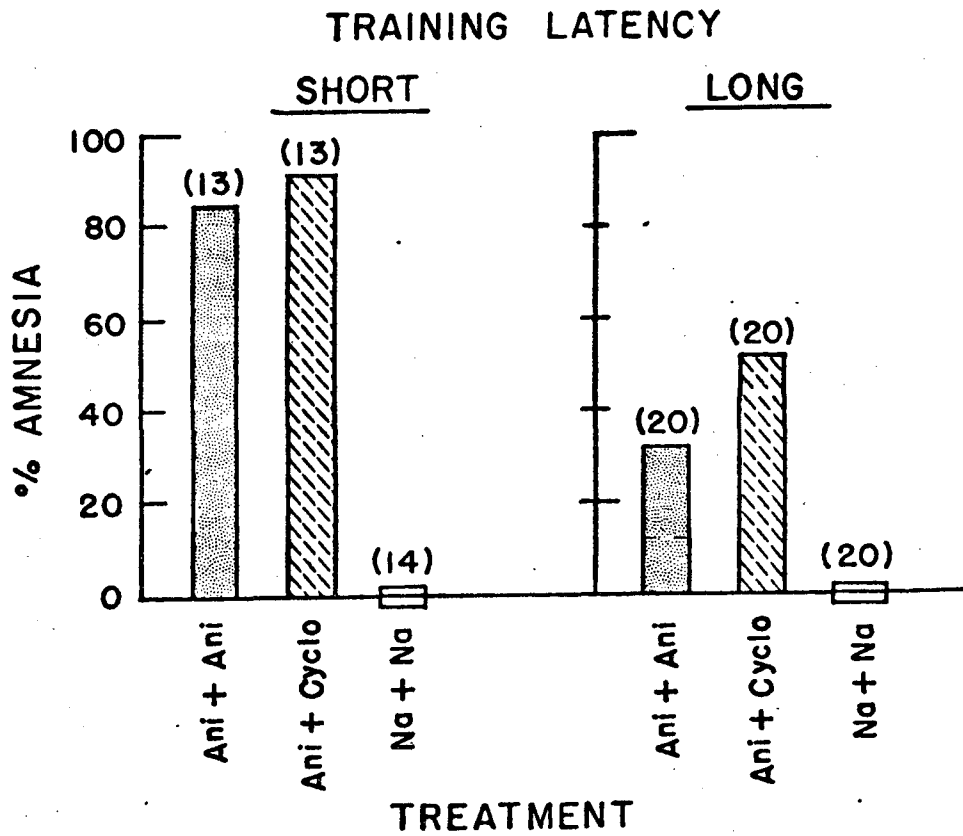
Experiment 7

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 minutes later and the 2nd and 3rd injections at 2 and 4 hrs respectively. To vary the training strength, the 4 training conditions of Experiment 4 were used. The retention test was given 2 weeks after training. The shock intensity was 0.33 ma.

Results

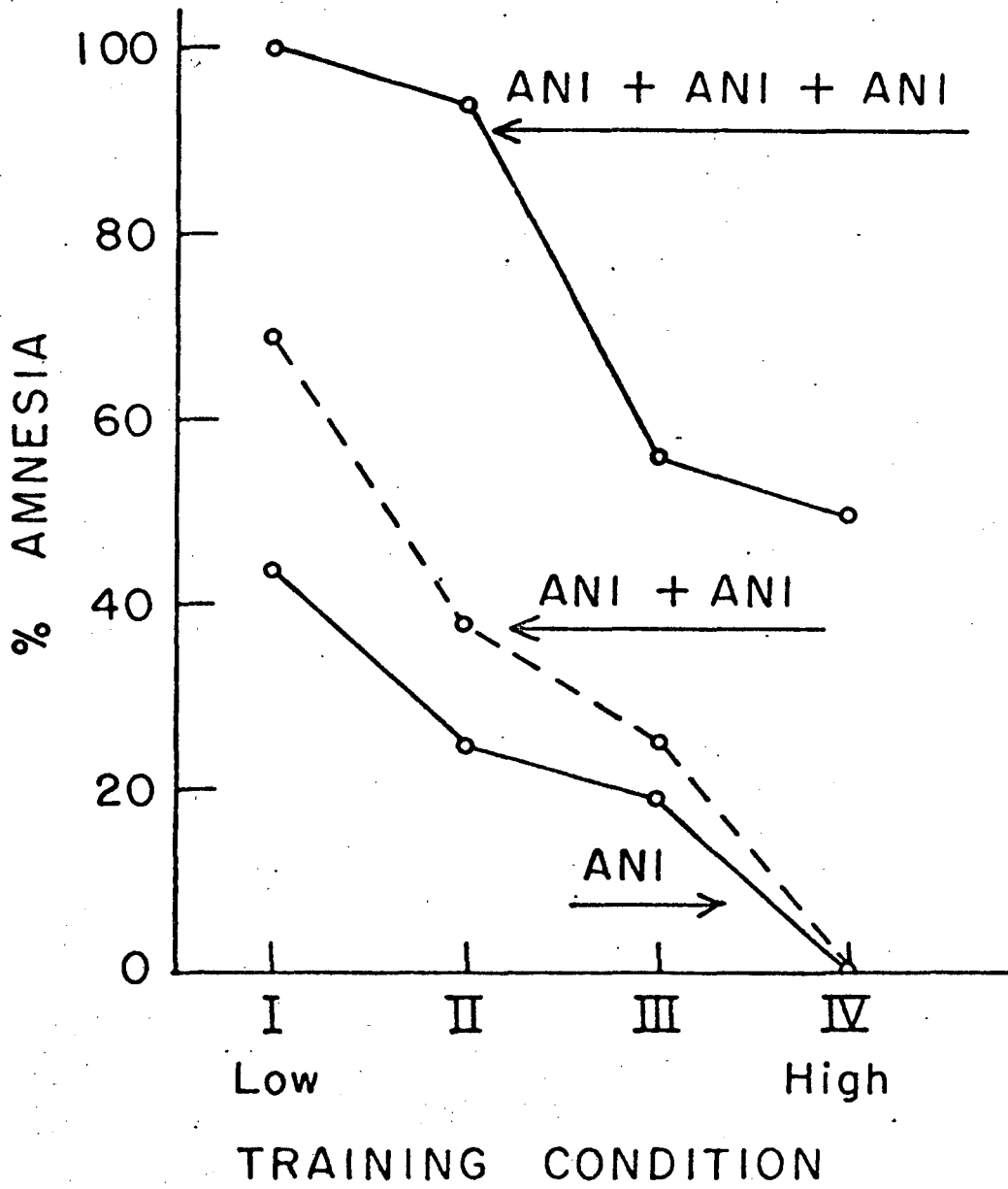
The results of this experiment are presented in Figure 18. The saline controls showed virtually no amnesia; only 2 out of 96 subjects were amnestic. A single pretraining injection of Ani was only effective as an amnestic agent under conditions of minimal training. Two injections of Ani were significantly more



XBL 721-4514

Figure 17. Effects of prolonged inhibition of protein synthesis on memory. Ani+Ani or Ani+Cyclo vs. Na+Na, $P < .001$, for short or long latencies (Fisher Exact Probability Test). Ani+Ani vs. Ani+Cyclo $\chi^2 = 1.67$, $df=1$, $P < .25$. Retention test given 1 week after training.

Figure 18. Percentage of subjects showing amnesia on retest after receiving one of four levels of training and one, two or three successive injections of anisomycin (Ani) at 2 hour intervals. Of 96 saline control mice, only 2 showed amnesia; these control data are not included in the graph. We considered a clear amnestic 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 Figure 15. Retention tests were given 2 weeks after training.



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effective. Three injections of Ani produced significant amnesia in all four training conditions; nearly all subjects were amnesic under conditions I and II.

Experiment 8

Design

The purpose of this experiment was to see if three delayed post-trial injections would cause amnesia. The experiment used the weaker training conditions I and II as described in Experiment 4. Two groups, Ani+Ani+Ani and Na+Na+Na, were given treatments as described in Experiment 7. 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 7. The retention test was given 2 wks 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 amnesic effect of the Ani+Ani+Ani group. The percentage of amnesic subjects was: Ani+Ani+Ani = 100%, I+Ani+Ani+Ani = 10%, and Na+Na+Na = 0%.

Experiment 9

Design

The purpose of this experiment was to see if the greater amnesic 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 4. 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 hrs 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 7. The retention test was given 2 wks after training.

Results

The results demonstrated that the duration of inhibition of protein synthesis (Ani+Ani vs. Ani+3Ani) and not the quantity of drug administered (Ani+3Ani vs. Ani+Ani+Ani+Ani) was responsible for the greater amnestic effect obtained with multiple injections. The percentage of amnestic subjects 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

Post-trial 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 5, 7 and 9).

The experiments show that in principle any increase in training strength that blocks amnesia, can be countered with longer inhibition of protein synthesis to re-establish a high level of amnesia. Observations made in several of the experiments demonstrated that the greater amnesic 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 (Exp. 6). 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 amnesic effect on control or on drugged subjects (Exps. 5-9). The greater quantity of Ani injected in the multiple injection groups was not responsible for the greater percentage of amnesic subjects. The duration of inhibition of protein synthesis, rather than the dose of Ani administered per se, was found to control the percentage of amnesic subjects (Exp. 9). Subjects receiving a large dose of Ani were not incapacitated so as to make memory or recall impossible (Exp. 8).

Comparison of Ani and Cyclo

With the doses used, both Ani and Cyclo produced significant amnesic effects. Both drugs shared the property that they became

less effective amnestic agents as the training strength increased (shock intensity, Exp. 1; duration of training latency, Exps. 2 and 4). From some of the evidence presented above, it can be argued that when Cyclo was injected shortly before training it not only caused amnesia but also produced a mild impairment of acquisition.

With pretraining injections (Exps. 3 and 4), Cyclo was a more effective amnestic agent than was Ani. However, following an initial pretraining injection of Ani, post-training injections of either Cyclo or Ani were found to cause about the same amount of amnesia (Exp. 6). 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 post-training, suggests that Cyclo when administered prior to training caused some impairment of acquisition in addition to blocking memory formation. Squire and Barondes (1972b) have reported that Cyclo impaired acquisition of an active avoidance task in mice. The amnesia caused by a post-training injection of Cyclo (when it could not have interfered with training) demonstrated that Cyclo is a powerful amnestic agent (Exp. 6).

The amnestic effect of Cyclo when given prior to training, while it reflects some impairment of acquisition, seems 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 subjects responded to small changes in training parameters as did Ani-injected

subjects. 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 (Pestka, 1971).

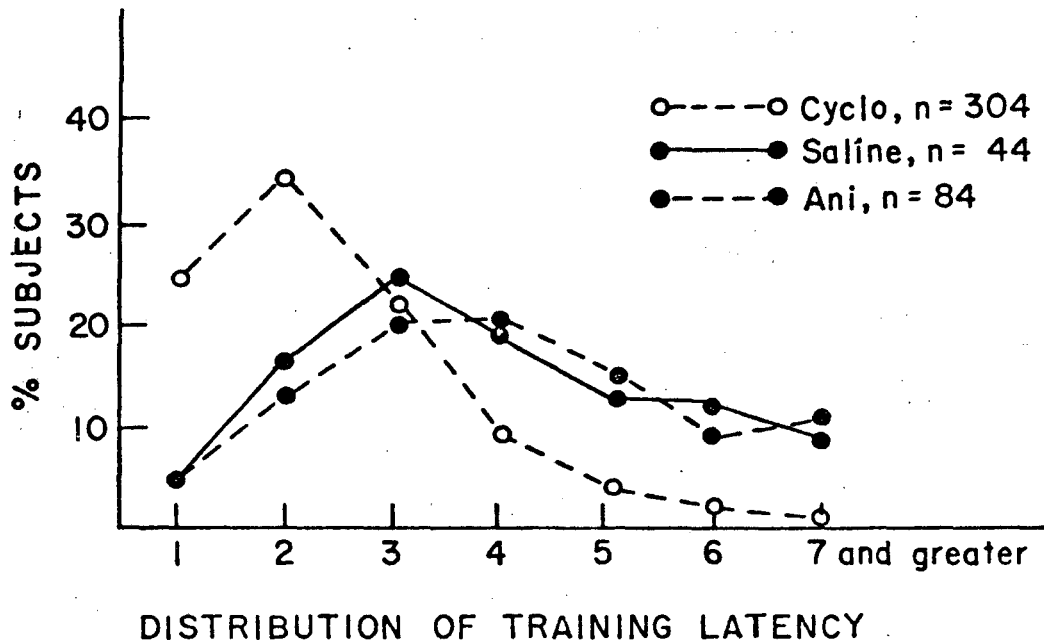
Cyclo, along with the closely related compounds acetoxycycloheximide and streptovitacin A, belongs to the class of antibiotics known as glutarimides. This class of antibiotics inhibits peptide chain initiation as well as chain elongation by interaction with the large 60S ribosomal subunits. They interfere 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 (Squire, Geller and Jarvik, 1970). Hyperactivity among Cyclo-injected subjects is apparent when comparing the distribution of training latencies for Cyclo-, Ani- and saline-injected subjects. Ani- and saline-injected subjects did not differ significantly in their distribution of training latencies, but both differed significantly from Cyclo-injected subjects (Figure 19). As can be seen in Experiments 4 and 7, the training latency is an important parameter of learning, thus failing to match samples of injected subjects for their training latencies could bias the results. In all the experiments reported in this dissertation, samples were matched for latencies when reporting amnestic effects.

With the 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, as in Experiment 2. 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 hrs apart caused death within 24 hrs 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.



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Figure 19. 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 4. The data for Cyclo came from Experiment 2, but the distribution of latencies for the Cyclo-injected subjects of Experiment 4 tended to be the same shape except for certain irregularities, probably due to the much smaller N. Ani vs. saline $\chi^2 = 1.65$, $df=6$, $P = NS$. Ani + saline vs. Cyclo $\chi^2 = 75.14$, $df=6$, $P < .001$.

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 hrs (Barondes, 1970; Flood et al., 1972). These studies have shown that small increases in the strength or amount of training will prevent amnesia from developing where otherwise amnesia would occur. The studies in this section 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" subjects 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" subjects, 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 the preceding experiments testing only healthy subjects, 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 hrs after training, but 7 days later they are not found to be amnesic. While this "transient" amnesia has been reported

by a few investigators (Squire and Barondes, 1972a,b), almost nothing is known about the conditions necessary for reliably obtaining such results. Quartermain et al. (1970) 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 Experiment 2, designed to compare the effects of Cyclo on the memory for passive avoidance over a three-week retention period, it was found that the percent amnesic subjects increased from 16% at 24 hrs, to 37% at 1 week, and 57% at 2 weeks after training (N's = 84, 73, 80 for the three retention periods). In this section, a comparison of the effect of a single pretraining injection of Ani in Experiments 3, 5 and 7 with retention tests given at 24 hrs, 1 and 2 weeks respectively shows that the percentage of amnesic subjects increases from 0% at 24 hrs, to 20% at 1 week, and to 45% at 2 weeks after training. Amnesia reported in both this and in the previous section is not only permanent but also increases progressively in magnitude whether the agent is Cyclo or Ani.

The increase in the percentage of amnesic subjects when a pretraining injection of Ani is followed by post-training injections of Ani demonstrates that protein is required for the formation of longterm memory. While no evidence has been found that Ani impairs acquisition, it is still possible that some impairment occurs. However, the post-training 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 hrs 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 post-training 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 re-establish 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.

IV. COMPARISON OF THE EFFECTS OF ANISOMYCIN ON MEMORY ACROSS SIX LINES OF MICE

Introduction

In the previous section, it was found that Ani caused amnesia for a passive avoidance habit in mice. It was also found that the greater the duration of inhibition the more likely amnesia was to occur. The duration of inhibition, shock intensity, and training and escape latencies were found to interact to determine the degree of amnesia. The generality of these effects is now demonstrated across 6 lines of mice with widely different characteristics of acquisition.

Another purpose of these experiments, beside replicating and extending the results of experiments in the preceding sections to other strains and lines of mice, was to provide a methodology by which other laboratories could obtain amnestic effects using Ani without regard to the specific training parameters or strains of mice used in the experiments being reported here.

It has been reported several times that different lines of mice (inbred and outbred) show differences in acquisition of various tasks. Wahlsten (1972a, 1972b) provides a comprehensive review of the literature. We report widely differing abilities of 6 lines of mice to learning a step-through passive avoidance habit and that these learning differences are apparently not due to differences in shock sensitivity or to faulty mechanisms underlying memory formation.

INHIBITION OF PROTEIN SYNTHESIS

Procedures

Inhibition of protein synthesis was determined by the procedures already described.

Results

Ani was relatively non-toxic in all lines of mice tested. Across lines there are no striking differences in the percent inhibition of brain protein synthesis over the first 2 hr after the injection (Table 7). However, substantial variability exists across strains during the recovery from inhibition (2 to 6 hrs after the injection). It should be noted that in this section a standard dose of 0.5 mg/mouse/injection was used. The differences in inhibition (Table 7) do not seem to be strictly related to the weight differences across lines (Table 8).

In the design of Experiment 13, Ani was administered twice, first at time "0" and then at +2 hr. In Figure 20 (bottom), it can be seen that such an injection schedule in C57B1/Jf female mice doubled the duration of time over which protein synthesis inhibition was at 80% or greater as compared to a single injection (Figure 20, top). Since the strains reported here were similarly affected by a single injection of Ani, it was assumed that two injections would double the inhibition time as it did in the C57B1/Jf females.

Table 7

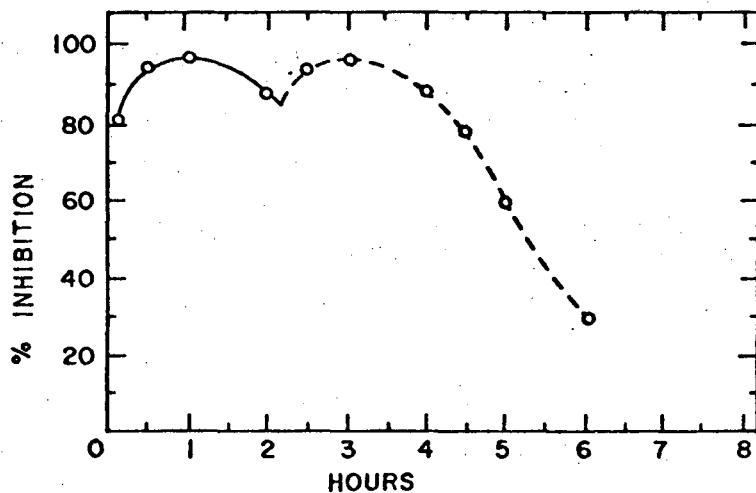
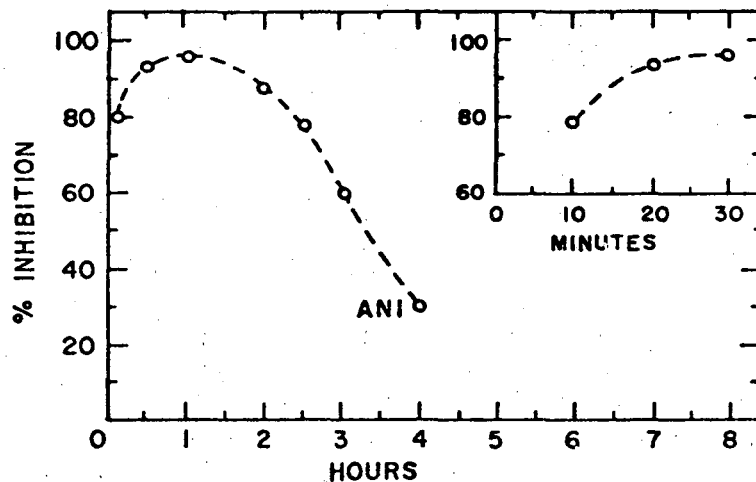
Percent Inhibition of Brain Protein Synthesis by Anisomycin as
a Function of Line and Time Since Injection Time (hr)
after Administration

<u>Strain</u>	<u>1-1/2</u>	<u>2</u>	<u>2-1/2</u>	<u>3</u>
BALB/cJ	92	83	77	55
C57Br/cdJ	91	89	64	46
C57B1/6J	88	77	61	49
C57B1/Jf	85	76	58	46
DBA/2J	84	76	56	32
CB	84	78	59	39
Swiss (CD-1)	83	79	65	56

N = 8/line/sampling time, all males.

Dose was 0.5 mg Ani/mouse given subcutaneously with an incorporation period of valine-¹⁴C 30 min.

The percentages of inhibition show no major strain differences over the first 2 hr after injection, although some variability exists.



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Figure 20 (top). Effect of a single injection of 0.5 mg of anisomycin on protein synthesis in C57Bl/Jf female mice. The incorporation period was of 30 min duration in the large graph and for 10 min in the inset.

Figure 20 (bottom). Effect of two injections of Ani given at time "0" and 2 hr on protein synthesis in C57Bl/Jf female mice. Two injections of 0.5 mg of Ani given 2 hr apart doubled the duration of inhibition at 80% or greater when compared to the effect of a single Ani injection.

BEHAVIORAL EFFECTS

Subjects

The mice used in the following experiments and in determining the degree of inhibition of brain protein synthesis were males, 60-74 days of age. Lines not raised in the Department of Psychology, University of California, Berkeley, were received at 6 weeks of age. Table 8 presents the relevant data on these lines.

These lines of mice differ in some interesting ways. There are large weight differences across lines. Also represented are inbred, outbred, and randomly bred lines, as well as albino and pigmented lines. Certain other strains, such as the CBA and C3H, were not considered for behavioral experiments because our previous experience and the literature (Sidman and Green, 1965) indicated retinal degeneration occurs as a strain trait at about 50-55 days of age.

Apparatus, Training, and Testing

The apparatus and training and testing procedures were as previously described. Subjects were assigned to a training condition (T.C.) on the basis of their latency-to-enter and -escape from the shock box. T.C. I was defined by 1-4 sec latency-to-enter and 0.01-0.04 min escape latency. T.C. II (in the previous sections this was referred to as T.C. III) was defined by 1-4 sec latency-to-enter and 0.05-0.08 min escape latency. All retention tests were given 1 week after training.

Table 8

Description of Mouse Strains

<u>Strain</u>	<u>Av. Wt. (gm)</u>	<u>Eye</u>	<u>Source</u>	<u>Other Information</u>
CB*	35	dark	UCB-Psych	Hybrid F2-6 of BALB/c and C57B1 obtained from Simon- sen Lab, Gilroy, Calif. Generously supplied by Dr. S. Guth
C57B1/Jf	25	dark	UCB-Psych	Inbred F23-26 from pair obtained from Cancer Genetics Res. Lab, UCB
C57B1/6J	25	dark	JAX	Inbred
Swiss (CD-1)	40	albino	Charles R.	Random bred
C57Br/cdJ	23	dark	JAX	Inbred
BALB/cJ	21	albino	JAX	Inbred
DBA/2J	24	dark	UCB-Psych	Inbred, F2-4 from stock originally obtained from JAX

Key to designations in the table:

UCB-Psych - Univ. of Calif at Berkeley, Dept. of Psychology

JAX - Jackson Memorial Laboratory, Bar Harbor, Maine

Charles R.- Charles River Breeding Laboratory, Inc.,
Wilmington, Massachusetts.

F - generations maintained at UCB-Psychology.

* - an outbred line developed from original hybrid.

Drug Condition

Each injection of Ani was given subcutaneously at a dose and volume of 0.5 mg/mouse in 0.25 ml of saline. This was true whether Ani was administered prior to or after training. In all cases, singly injected subjects received the drug or 0.25 ml of saline 15 min prior to training. Thus Ani-injected mice were under high levels of protein synthesis inhibition at the time of training (Fig. 20, top). When a post-training injection was used, it was given 1-3/4 hr after training. This particular injection time was used so that inhibition of protein synthesis could be maintained at a high level (in most cases 80% or greater) for an additional 2 hr (Fig. 20, bottom). Saline was injected in place of Ani for the control conditions.

COMPARISON OF ACQUISITION

Experiment 10

The usual method of comparing memory across strains is to train the subjects to the same criterion, and then to test retention at the appropriate time after training. In a one-trial training task such as ours, we can translate this into the following: Each strain is trained under those conditions of shock duration and intensity that produce the same degree of retention.

It has been reported that training subjects too highly will block the effects of inhibition of protein synthesis as an amnestic treatment (Barondes and Cohen, 1967; Flood et al., 1972; Flood et al., 1973); in Section II (pg.), it was suggested that inhibitors of brain protein synthesis seemed to have their greatest

effect on memory when subjects are trained to a point just below the asymptote of retention (e.g., the lowest shock intensity that produces 90-100% avoidance in saline-injected subjects for passive avoidance training). Therefore, in this experiment we sought minimal training conditions that produced 90-100% avoidance. Saline-injected subjects were used because these subjects would best reflect the control performance in the subsequent drug experiments.

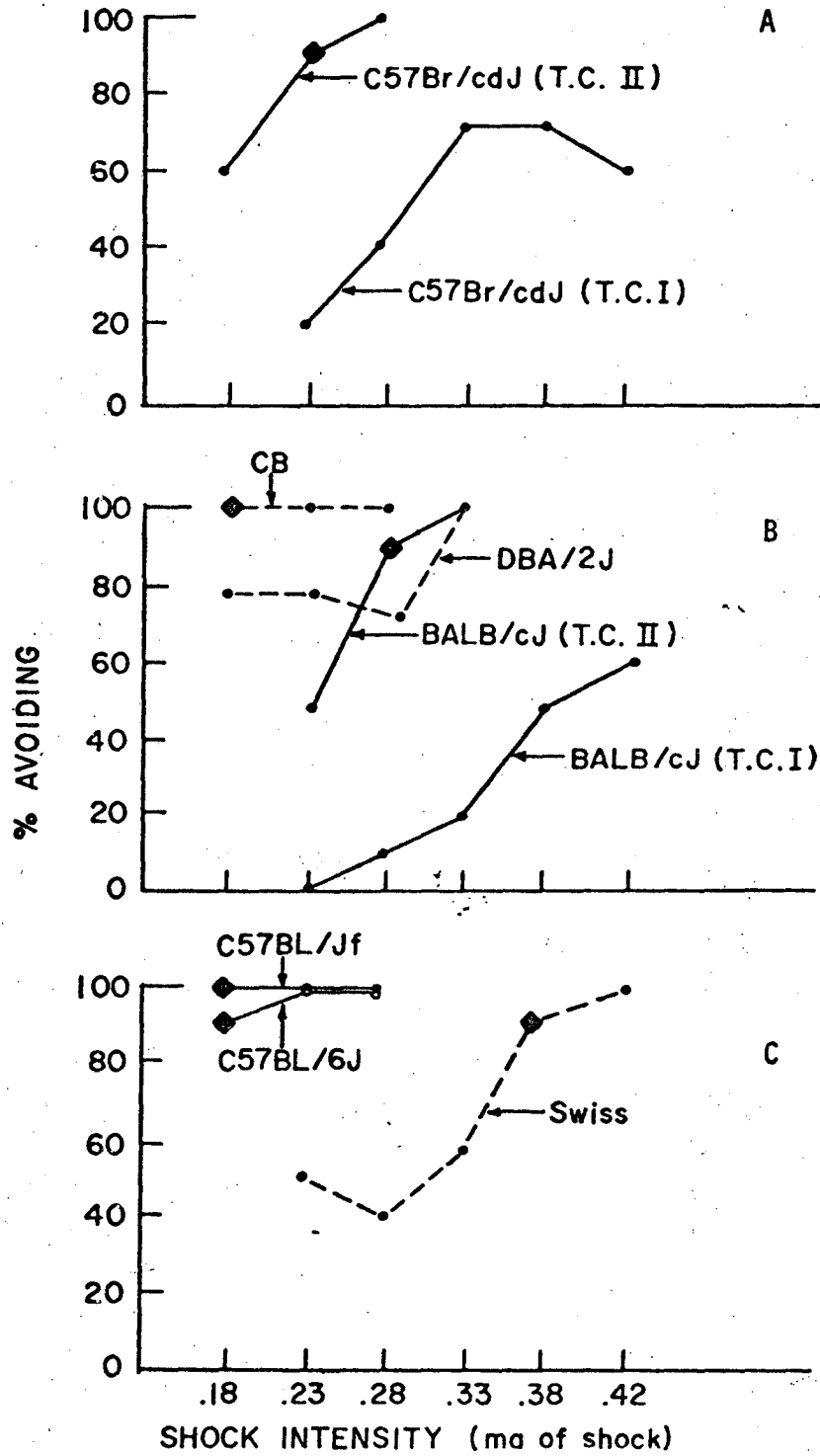
Two parameters of training were manipulated across the 6 lines. Increases in either shock intensity and/or shock duration were employed to increase the percentage of subjects avoiding the white shock box.

Results

The ability of the lines to acquire the passive avoidance habit differed considerably, as is shown in Figure 21 A-C. It can be seen that the desired training condition, shown by the diamond on the curve, was found for each strain. However, greatly different degrees of training were required to find the asymptotes.

C57B1 strains and CB outbred mice learned under the lowest shock intensity (0.18 ma) and the shortest range of escape latencies (0.01-0.04 min, T.C. I). In fact, for the C57B1 and CB mice we cannot be certain that the asymptote has really been found. At lower shock intensities than those reported in Figure 21, most of the mice would not escape from the shock in 0.01-0.04 min. Therefore, we could not train and test subjects at any lower shock intensity to see if retention were significantly below 90-100% avoidance. Swiss mice reached the asymptote at a

Figure 21. Acquisition and retention as a function of line and shock intensity. All lines were trained under T.C. I, in which the latency-to-enter the white box is 1-4 sec and the latency-to-escape shock is 0.01-0.04 min. The C57Br/cdJ and BALB/cJ strains could not be trained to the desired level of retention under T.C. I; therefore, training strength was increased by making the latency-to-escape 0.05-0.08 min (T.C. II). As Figures A and B show under T.C. II, C57Br/cdJ and BALB/cJ strains did reach the criterion of retention. The diamonds are the asymptotic values which were being sought. N = 10-20/strain/shock intensity and/or training condition.



higher shock intensity of 0.38 ma, with a short range of escape latencies. In the case of BABL/cJ and C57Br/cdJ strains, short escape latencies at any shock intensity tested did not produce the desired degree of retention. In order to obtain the asymptotic value, it was necessary to shift both strains to long escape latencies (0.05-0.08 min, T.C. II). In order to do this, the guillotine door was replaced after the subject entered the white shock box; the door was not removed until after shock onset. This delayed escape from the shock box. Shifting subjects from one shock intensity to the next represents a relatively small increase in training strength compared to shifting subjects from short to long shock escape latencies (from our previous results such a shift in latencies is equivalent to increasing the shock intensity by about 0.16 ma). Thus, relative to the other lines, the BALB/cJ and C57Br/cdJ are being trained at a considerably higher training strength.

Discussion

The primary purpose of Experiment 10 was to establish training conditions which would permit the comparison of anisomycin's effect on memory independent of genetic differences affecting acquisition. The training conditions needed for each strain to acquire the passive avoidance habit differed considerably, but in 6 of the lines training conditions were found that produced the same relative degree of retention in saline-injected mice.

It cannot be said what caused the genetic differences in learning or memory for passive avoidance, but we can suggest what is not producing the differences. The differences in learning

are not related to the range of latencies characteristic of each line. If the latency-to-enter and -escape shock are closely matched across lines, the reported retention curves reflect about the same degree of separation. Neither does it appear to be related to shock sensitivity. Taking the percentage of subjects escaping shock with short latencies as a measure of shock sensitivity, Table 9 shows that there are no major differences across strains as the shock intensity decreases. Such differences as exist seem too small to account for the gross differences in training strength needed to find the asymptotes.

Also, there is no consistent pattern which indicates that weight differences are responsible for differences in acquisition. Similarly, whether a line is inbred or outbred, albino or pigmented, there is no consistent relationship to learning ability.

One possible reason for the apparent differences in learning may be genetic differences in the motivation to explore a novel environment. In Table 10, we have taken the percentage of subjects with short versus long training latencies as a measure of motivation to enter a novel environment. To some degree the data suggest that lines that enter quickly require greater shock in order to learn to passively avoid, while lines reluctant to enter require far less intense shock. To minimize strain differences due to this sort of variability, only the data from subjects with training latencies of 1-4 sec were used to make up the acquisition curves. However, learning differences about as large as those reported in Figure 21 exist even if subjects across lines are matched for training latencies on a second-to-second basis.

Table 9

Distribution of Short Escape Latencies across Strains

Shock Intensity (ma)	% Subjects Escaping in 0.01-0.04 min				
	.13	.18	.28	.33	.38
CB	45	86	88	90	--
C57B1/Jf	22	80	88	93	--
C57B1/6J	--	73	90	95	100
Swiss (CD-1)	--	80	80	100	100
C57Br/cdJ	--	85	86	100	100
BALB/cJ	--	80	80	90	100

Taking the percentages as a measure of shock sensitivity, it is clear that none of the differences is large enough to account for the fact that the last three strains on the list require much more shock than the first three to acquire the passive avoidance habit (see Table 10). The presence of a dash (--) means that no training was done at this point; therefore, there are no latencies to calculate the percent escaping in 0.01-0.04 min. The N per cell varied from 20-60.

Table 10

Percentage of Subjects with Short and Long Training Latencies

Latencies to enter (sec)

	1-4	5 and greater	Shock Intensity	Training Condition
CB	56	44	0.18	I
C57B1/Jf	64	36	0.18	I
C57B1/6J	82	18	0.18	I
Swiss (CD-1)	84	16	0.38	I
C57Br/cdJ	98	2	0.23	II
BALB/cF	92	8	0.28	II

The lines are ordered according to the training strength needed to reach the training criterion of 90-100% avoidance. The table shows a fairly strong relationship between the degree of training required and the distribution of latencies to enter, such that for those strains showing little hesitation to enter the white box (training latency 1-4 sec), the training strength must be high. One must keep in mind that for CB and C57B1/Jf the shock intensity used may still be above the minimum required; both strains showed 100% avoidance at this shock level. When going from T.C. I to T.C. II, one is doubling the shock duration. We would estimate that a shift from I to II is the equivalent of a 0.16 ma increase; therefore, BALB/cJ would require at least 0.44 ma in T.C. I.

This is stressed because it has already been demonstrated that longer latencies-to-enter within a line will increase retention, all other conditions remaining constant (Exps. 2, 4, 6, 7).

Thus, in this situation, differences among lines in learning are not due to differences in the distribution of training latencies per se, rather they appear to be due to genetic differences in willingness to explore a novel environment. Of course, one obvious argument against such an interpretation is that we have selected subjects with similar training latencies and therefore similar motivation to explore. However, it may still be the case that a strong enough bias exists even in these selected subjects to influence learning; if not, then we can also rule out motivational differences as being responsible for differences in learning.

The DBA/2J strain was not used in Experiments 11 and 12 because it was found that 30% of the naive subjects would not step-through into the white box within 180 sec. Thus, in this strain the reliability of the latency-to-enter on the test day as a measure of retention is questionable. In addition, from sample to sample DBA/2J mice showed highly variable retention scores (percent subjects passively avoiding); this was probably due to an interaction between the effects of training and the normal reluctance of the mice to enter the white box. Strains having long latencies-to-enter the white box and having highly variable retention scores are undesirable for studying passive avoidance.

In all of the other lines, none of the naive subjects failed to step-through within the 20-sec period defined as amnesia for

the trained subjects. In order to achieve such consistency it is necessary to follow the procedure described above and in particular to wash the apparatus frequently during training and testing. This is important because some lines are highly sensitive to the urine scent of other mice and the presence of this odor will delay entry into the white shock box.

EFFECT OF ANI ON MEMORY

Experiment 11

Having found the desired degree of training for each line, I then proceeded to evaluate the effect of Ani on memory across lines. Subjects were given a 15-min pretraining injection of Ani (0.5 mg/mouse) or saline of 0.25 ml. Each line was then trained under their own training conditions as determined in Experiment 10. Subjects were given a retention test 1 week after training.

Across the 6 lines of mice, Ani-injected subjects showed significantly more amnesia than the saline-injected subjects (Table 11). The C57Bl/Jf mice were somewhat less affected by a single injection of Ani than were the other lines. This may have occurred because (a) we are not actually at a point just below the asymptote as is true for the other lines, or (b) in this strain the mechanisms underlying memory formation are more responsive to training. Because Ani only had a modest amnestic effect on the C57Bl/Jf strain, the duration of inhibition was doubled in one group. To accomplish this, Ani was first given 15 min prior to training and then again 1-3/4 hr after training. Doubling the duration of inhibition caused 85% of the mice to

Table 11

Amnestic Effect of Anisomycin Across Lines of Mice

	<u>T.C.</u>	<u>Shock Intensity (ma)</u>	<u>% Amnesia</u>	
			<u>Na</u>	<u>Ani</u>
CB	I	0.18	10	70
C57B1/Jf	I	0.18	0	45 (Ani+Ani 85)
C57B1/6J	I	0.18	10	90
Swiss (CD-1)	I	0.38	10	80
C57Br/cdJ	II	0.23	10	80
BALB/cJ	II	0.28	15	85

In all strains clear amnestic effects were obtained with a single pretraining injection of anisomycin. Given similar degrees of learning (as demonstrated by the scores of the Na-injected groups), the amnestic effects are about the same for 5 lines in spite of large differences in training strength. The exception is the C57B1/Jf strain in which two injections were required to produce a high level of amnesia (see text). T.C. is the training condition; amnesia is defined as a test latency of 20 sec or less. N=20/line/injection group. The smallest percentage differences are in the C57B1/Jf strain, and even these are highly significant: Ani vs. Na, $P < .001$ (Fisher Probability Test); Ani vs. Ani+Ani, $\chi^2 = 7.04$, $df=1$, $P < 0.01$.

become amnesic. In this replication experiment (N=20), we again found that a single injection still only caused 45% amnesia.

EFFECT OF MULTIPLE ANI-INJECTION

Experiment 12

We have already seen in Experiments 5 and 6 that doubling the duration of inhibition of brain protein synthesis by giving 2 successive injections of Ani (Ani+Ani) caused greater amnesia than a single injection of Ani, when the same training conditions were used for both injection groups. It was also reported that for a given number of Ani injections the percent amnesia decreased as the training strength increased.

In this experiment the effects of increased duration of the inhibition of protein synthesis were investigated across 6 lines of mice. Since in Experiment 11 the percent amnesia in Ani-injected subjects was already quite high, it was necessary to increase the training strength in order to detect what effect doubling the inhibition time (Ani+Ani) would have on memory relative to a single injection (Ani). The training strength was increased by increasing the shock intensity by 0.04-0.05 ma for each line. Ani or saline was injected 15 min prior to training and again 1-3/4 hr after training (Ani+Ani, Na+Na). Also the effect of a single pretraining injection of Ani was assessed at this higher shock intensity.

At the higher shock intensity, Ani+Ani caused significantly more amnesia than Na+Na or Ani across all lines (Table 12). We should like to stress that the difference between the amnesic

Table 12

Effects of Doubling the Duration of Protein Synthesis Inhibition
on Percentage Amnesia

	<u>T.C.</u>	<u>Shock Intensity (ma)</u>	<u>Na+Na</u>	<u>% Amnesia An+Ani</u>	<u>Ani</u>
Cb	I	0.23	0	95	20
C57B1/Jf	I	0.23	0	70	15
C57B1/6J	I	0.23	0	70	30
Swiss (CD-1)	I	0.42	0	80	20
C57Br/cdJ	II	0.28	0	95	30
BALB/cJ	II	0.33	0	95	65

Two injections of anisomycin clearly had a greater amnestic effect than the single pretraining injection alone. By comparing the Ani groups in this table and Table 5, it can be seen that increasing the shock intensity decreased the effectiveness of Ani as an amnestic agent. BALB/cJ strain seems to be particularly sensitive to Ani; this might be interpreted to mean that in this strain the residual effects of training on the CNS are short lived relative to the other strains. N=20/line/injection group. The smallest percentage difference between Ani+Ani and Ani is in the BALB/cJ strain. Even this difference is significant: $\chi^2 = 5.625, df=1, P < 0.05$.

effect of Ani+Ani and Ani is due to differential treatment occurring 1-3/4 hr after training. Thus, the amnestic effect could not be attributed to impairment of acquisition.

DISCUSSION

The results of these experiments demonstrate a considerable degree of generality across 6 mouse lines of findings previously reported for a single strain and sex of mouse (C57B1/Jf, female mice). The principal findings were these: (a) A single pre-training injection of Ani was an effective amnestic treatment when subjects were trained to a point just below the asymptote for acquisition and retention (90-100% avoiding), (b) increasing the shock intensity by 0.04-0.05 ma decreased the percentage of these subjects forgetting the training, and (c) at the higher shock intensity a high percent amnesia could be re-established by giving a second injection of Ani 1-3/4 hr after training.

It was previously suggested that inhibitors of protein synthesis should have their greatest amnestic effect upon memory when the subjects are trained to a point that is just below the asymptote of acquisition and retention (pg. 52). In all 6 of the mouse lines investigated, a single pretraining injection of Ani had a significant amnestic effect in spite of the differences in the training parameters that had to be employed so that each line of mice would have the same relative degree of retention (i.e., 90-100% of the subjects passively avoiding).

In Experiment 10 the differences in acquisition and retention of the passive avoidance training did not seem to be accounted

for by (a) shock sensitivity, (b) differences in latency to enter or to escape from the shock box, (c) weight, pigmentation, or whether the line was inbred, outbred, or randomly bred. Some evidence was presented that suggests differences across the lines of mice in motivation to explore a novel environment affected the amount of training needed to acquire the passive avoidance response. A strain's willingness to explore a novel environment (as measured by the percent subjects entering the white shock box with short latencies) was positively correlated with the intensity of training required to learn the passive avoidance task, such that lines most willing to explore a novel environment required the most intense training (e.g., BALB/cJ).

In the two experiments investigating the amnestic effect of anisomycin, the most notable result was the relatively low variability in amnesia across lines. Only the C57B1/Jf strain showed significantly less amnesia than the other lines. However, this and the CD line were the only ones in which the saline controls were at 100% avoidance. Since no major differences in the degree or time course of inhibition of protein synthesis were found, the C57B1/Jf strain may have been "overtrained" relative to the other lines of mice. Two injections of Ani were required to cause the same level of amnesia in the C57B1/Jf strain as in the other lines. The possibility of overtraining could not be tested, since C57B1/Jf mice would not escape from shock of any lower intensity than already used.

Although the results of Experiment 11 could be interpreted as impairment of acquisition by Ani, I do not believe from the

preceeding work or the results of Experiment 12 that this is likely. In Experiment 12, the pretraining injection of Ani did not have a significant effect except in the BALB/cJ strain. Yet a second injection given 1-3/4 hr after training caused significantly more amnesia in all 6 lines of mice. Thus anisomycin was demonstrated to cause amnesia at a time at which impairment of acquisition was not in question. Also in Experiment 12 it can be seen that most lines show about the same percent amnesia; after a single injection 15-30% of the subjects across lines were amnesic and after two injections of Ani, 70-95% were amnesic. In addition, the previously reported control experiments (Exps. 5, 8, and 9) ruled out illness, the injection procedure itself, or the greater quantity of drug administered as being responsible for the greater amnesic effect of the two injections.

The post-training effect of Ani on memory and the generality of the amnesic effect across lines having different characteristics of acquisition are strong support for the hypothesis that protein synthesis is required for the formation of long-term memory.

V. THE RELATION OF MEMORY FORMATION TO
CONTROLLED AMOUNTS OF BRAIN PROTEIN SYNTHESIS

Introduction

From the experiments in the previous sections, it was concluded that during inhibition of brain protein synthesis the brain retains the capacity to synthesize specific memory-related protein(s) such that, if inhibition is not sufficiently long, synthesis of memory-related protein(s) will occur after inhibition is terminated. In the experiments that follow, I have used the inhibitor Ani to control the duration and the time at which memory related protein synthesis is able to occur. This was accomplished by permitting a partial recovery from inhibition at various times and for various durations during the inhibition period. This enabled us to test the extent to which the CNS retains the capacity to direct memory-related protein(s) synthesis over an inhibition period that is needed to achieve a high level of amnesia.

INHIBITION OF PROTEIN SYNTHESIS

From the work presented in section III (pp. 62-63) on the inhibition of brain protein synthesis by Ani, it was possible to determine the time course of inhibition of brain protein synthesis used in the experiments that follow. The time courses of inhibition with various schedules of multiple injections are shown in Figure 22.

BEHAVIORAL EFFECTS

Materials and Procedures

The subjects used in the experiments in this section were C57B1/Jf female mice about 60 days of age (18-20 gm). The colony was in its 29-34th generation of inbreeding. The training, testing, and apparatus were as previously described for one-trial passive avoidance training.

Throughout, Ani was administered in 0.25 ml of a 2 mg/ml solution/injection. All injections were given under very light ether anesthesia. The times that injections were given will be described under each experiment. Training and testing were done between the hours of 7 AM and 1 PM which was during the early part of the light cycle.

Experiment 13

Design

In all the experiments previously reported, inhibition was maintained at 80% or greater for several hours by administering Ani at two hour intervals. In this experiment, the injection schedule was altered by delaying the time of the last of three Ani injections. That is, all groups except Ani+Ani received three injections: the first injection at time 0, training at 15 min, the second injection at 2 hrs, and the third injection at 4 hrs or at 4 hrs plus some delay period: 4 hrs + 50 min, 4 hrs + 60 min, 4 hrs + 70 min or at 4 hrs + 90 min. The delay periods (in minutes) permitted a partial recovery of protein synthesis at a time at which protein synthesis had to be blocked

in order to obtain amnesia. Figure 22 graphically illustrates the design of the experiment. Ani+Ani was included (a) to show that under the training conditions employed the third injection of Ani was necessary to obtain amnesia and (b) to determine at what point a delay period was sufficiently long enough so that the third injection of Ani was without effect.

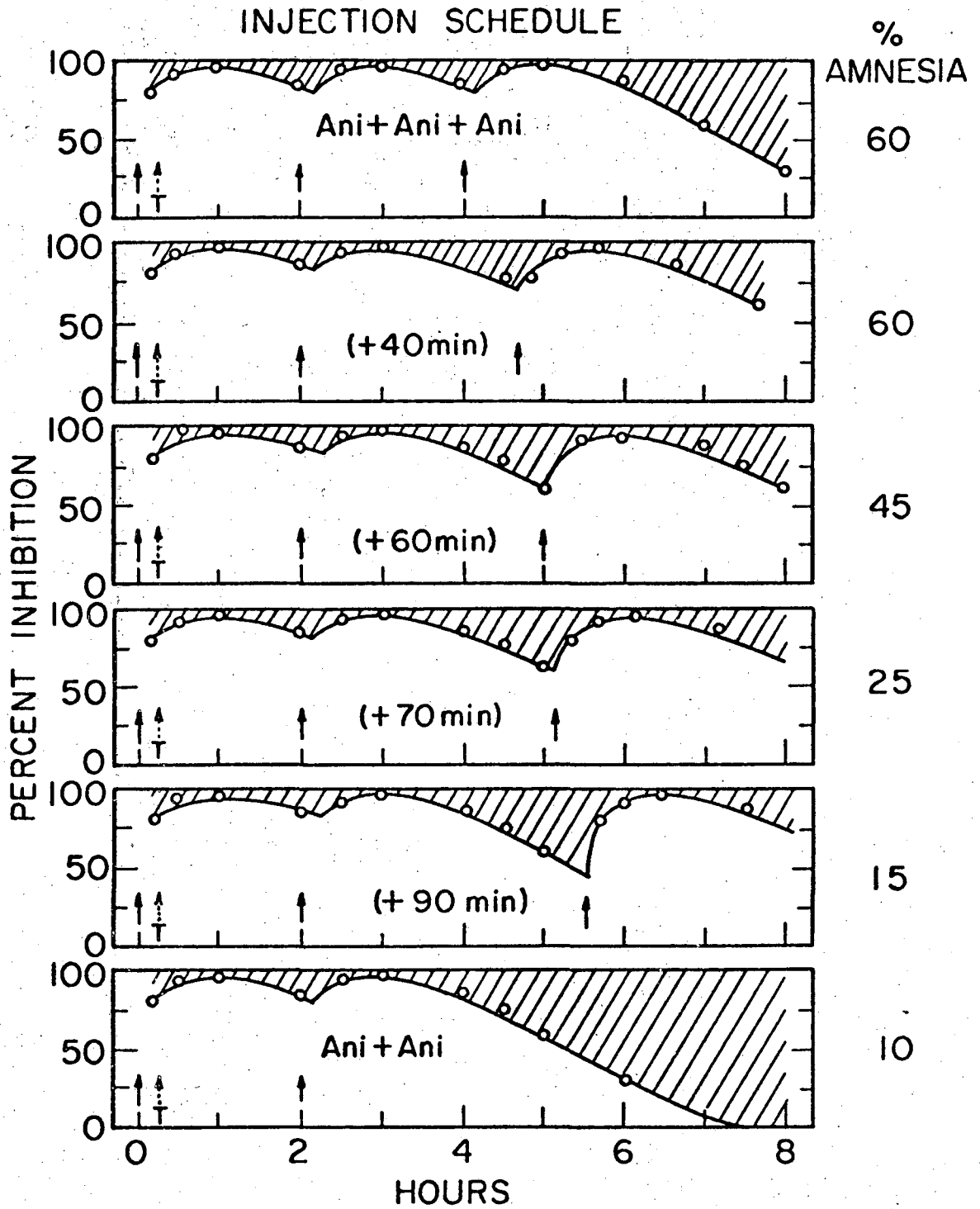
Procedures

Training in all cases was begun 15 min after the subject received its first injection. Subjects were given moderately strong training at a shock intensity of 0.33 ma in training condition III: training latency of 1 - 4.9 sec, escape latency of 0.05 - 0.08 min.

Results

As can be seen in Figure 22 the third injection of Ani was critical in obtaining amnesia, since Ani+Ani showed only 10% amnesia whereas Ani+Ani+Ani showed 60% amnesia. Thus the capacity for synthesizing memory-related protein(s) existed over some portion of the third 2 hr period (i.e., from 3-3/4 to 5-3/4 hrs after training) and in some subject that were not amnesic even longer. When delay periods between the second and third injection were permitted, some protein synthesis occurred. It can be seen that as the duration of this delay period increased, the percentage of amnesic subjects decreased from 60 to 15%. A 90 min delay period completely blocked the effect of the third Ani injection; that is the percent amnesia

Figure 22. The time courses of inhibition of protein synthesis as a function of the injection schedule, and its effects on amnesia. Solid arrows indicate times at which injections were given. T and a dotted arrow indicates the time of training. Where the third injection followed the second by more than 2 hours, the delay interval is shown in parentheses. The shaded areas represent the amount of protein synthesis occurring. The percent of animals showing amnesia upon retest 1 week after training is given in the right-hand column. Where amnesia differs by 30% or more for two conditions, $P < .05$ (Chi-Square, $df = 1$).



did not differ significantly between Ani+Ani and Ani+Ani-90-Ani.

Experiment 14

Design

The purpose of this experiment was to see if there was a decrease in the rate of synthesis of memory-related protein. If this were the case, a short delay period in the inhibition schedule would be more apt to lead to memory formation the closer to training the delay occurred. To test this possibility, delays of 20, 40 or 60 min were used between injections 1 and 2, 2 and 3, and 3 and 4.

Procedures

Subjects were trained at a shock intensity of 0.38 ma in training condition I: training latency of 1 - 4.9 sec, escape latency of 0.01 - 0.04 min. Pilot work had shown that 4 injections of Ani given 2 hours apart were the minimum necessary to obtain significant amnesia under these condition of training (Ani+Ani+Ani = 15% amnesia, Ani+Ani+Ani+Ani = 85% amnesia). The procedures used 9 conditions: 3 delay periods (20, 40, or 60 min) at 3 injection intervals (1-2, 2-3, or 3-4).

Results

Two amnestic trends are present: one occurs across delay times, the other across injection intervals (Table 13). At the somewhat higher training strength, it is clear that the greater the duration of the delay period, the lower the percentage of amnestic subjects. This was true for each of the

Table 13

**Effect of the Duration and Time of Protein Synthesis
on the Percent Amnesia**

Injection Period	Duration of the Delay Period in the Injection Schedule				
	0 min	20 min	40 min	60 min	∞
1-2	85% ^a	55%	35%	15%	-
2-3	85% ^a	65%	40%	15%	-
3-4	85% ^a	75%	65%	35%	15% ^b

^a One group, Ani+Ani+Ani+Ani, had no delay in the schedule, so the results are shown under 0 min for all rows.

^b Ani+Ani+Ani provides, in effect, an indefinitely long delay of the 4th injection. For differences of 20% $P < 0.10$; for differences of 25% or more $P < 0.05$.

times at which the delay period was used (i.e., between injections given at 2, 4, or 6 hrs). The second is a weak but regular trend across the injection intervals. Comparing the effects of protein synthesis on reducing amnesia, we find that none of the comparisons between intervals 1 and 2 and 2 and 3 at 20, 40, or 60 min differ significantly. In the injection period 1-2, even the 20 min delay period reduced amnesia significantly from no delay (Ani+Ani+Ani+Ani = 85% amnesia, Ani-20-Ani+Ani+Ani = 55% amnesia, $P < 0.05$). At the injection interval 2-3, a 20 min delay was not effective at reducing the percentage of amnestic subjects, but a 40 min delay did reduce amnesia significantly ($P < 0.05$). At the injection interval 3-4, only the 60 min delay period significantly reduced amnesia ($P < 0.01$) compared to no delay. The percentage decrease from 20 min to 60 min is about the same across the three injection intervals (40 - 50% decrease in amnesia).

Experiment 15

Design

The purpose of this experiment was to see if delay periods had additive effects in the sense that two short delay periods (45 min) would equal one long period (90 min). If the effects of the delay periods are not additive it might indicate that the quantity of protein synthesized per unit time (rate) is important for memory formation. To answer this question delays were introduced between injections 1 and 2, and between 2 and 3. Over this period the capacity to synthesize the memory-related

protein(s) appears to be nearly constant, since in Experiment 14 the percent amnesia did not differ significantly between injection 1 and 2, and 2 and 3 for the various delay intervals employed (Table 13). The groups used in Experiment 15 were Ani-45-Ani+Ani+Ani, Ani+Ani-45-Ani+Ani, Ani-90-Ani+Ani+Ani, Ani+Ani-90-Ani+Ani, Ani-45-Ani-45-Ani+Ani (the numbers indicate the delay periods in minutes and show between which injections the delays occurred). The training conditions were as for Experiment 14 except that only certain combinations of latencies-to-enter and -to-escape were used so as to maximize the amnesic difference between the 45 min and the 90 min single delay groups. An effect of this selection was to give a higher percentage of amnesia in this experiment than in a similar group (40 min delay) in Experiment 14; thus in Experiment 15 the training condition is in effect slightly lower.

Results

The two groups with single delays of 45 min did not differ significantly from each other (69% vs 75% amnesia). Similarly, the two groups with single delays of 90 min did not differ significantly from each other (30% vs 25%). In agreement with the results of Experiment 14, a gap in the inhibition had a similar effect whether it occurred between injections 1-2 or 2-3. The two 45 min single-delay groups were combined for statistical purposes as were the 90 min delay groups. The

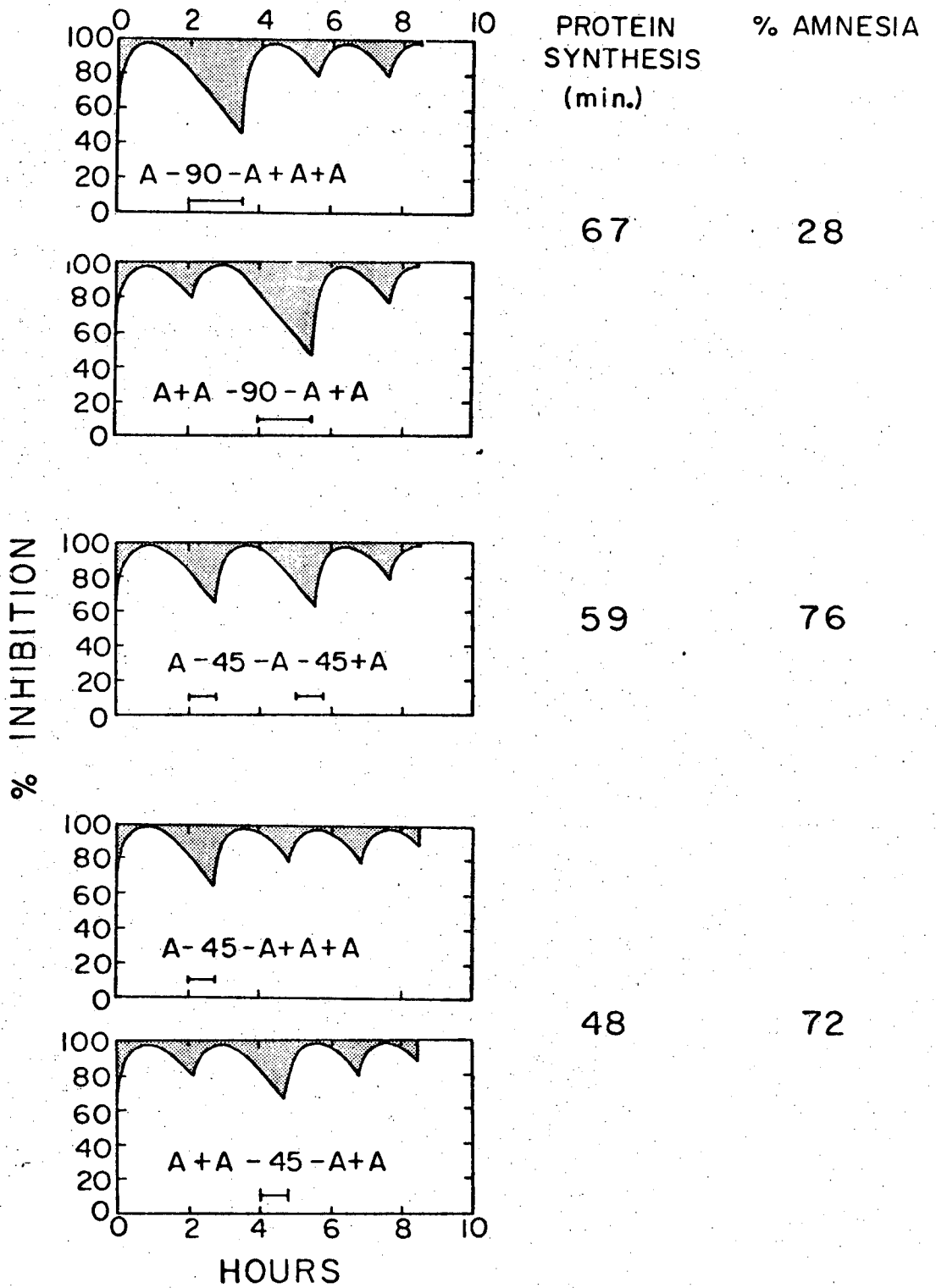
combined 45 and the combined 90 min single-delay groups differed significantly from each other in the percentage of amnesic subjects (72% vs 28%, $P < 0.001$, $N = 24$ /combined group).

The amounts of protein synthesized during the various delay periods in the injection schedule of this experiment are represented by the shaded areas in Figure 23. When a group received two 45 min delay periods (Ani-45-Ani-45-Ani+Ani), the total shaded area representing the protein synthesized did not quite equal that of the 90 min delay period. However, the total shaded area of the two 45 min gaps is clearly closer to that of the 90 min condition than to that of the shaded area of a single 45 min delay. The amnesic effect of the two 45 min delay periods were not additive since the single 45 min delay groups and the Ani-45-Ani-45-Ani+Ani group did not differ significantly (72% vs 76% amnesia respectively). Apparently, the quantity of protein synthesized per unit time is an important factor in memory formation.

Discussion

In Experiment 7 (p.76) in which up to 3 successive injections of Ani were administered, it was concluded as follows: "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

Figure 23. The effect of controlled protein synthesis on retention. The A's in the graphs stand for Ani, the is the time and duration of the delay period before the next injection was given (also given as being either 90 or 45 min duration). The shaded area represents the possible areas of memory related protein synthesis. The total time for protein synthesis is given as an equivalent of 100% protein synthesis. The A-45-A-45-A+A group is almost midway between the single delay groups in total protein synthesis, yet, the percent amnesia indicates that the two short delay periods were not additive in their effects on retention. If the two 45 min delay periods had been additive, we would have expected the percent amnesia for this group to be closer to the 90 min delay groups. The percent amnesia for the single delay 90 and 45 min delay groups is based on the total amnesia for the combined 90 min groups and for the combined 45 min groups. The N's for each group were 12 except for A-45-A-45-A+A which had an N of 24. The results depicted in this figure may indicate that the rate at which memory-related protein(s) are formed is important for memory formation.



that will reestablish the amnesia"(p.90). Experiment 13 of the present study confirms these earlier results and Experiment 14 extends them by showing that with still stronger training, 4 successive injections of Ani were required to produce amnesia (Table 13, Ani+Ani+Ani+Ani = 85% amnesia, Ani+Ani+Ani = 15% amnesia).

The novel aspects of this study were (a) to permit quantifiable amounts of protein synthesis at stipulated times after training and (b) to determine the effect of such controlled amounts of synthesis on memory. Within each of the three experiments, it was seen that as more protein was synthesized, the probability increased that the subjects would remember the training.

The 90 min delay period used in Experiment 13 is equivalent to a rather short period of normal protein synthesis. If we assume that the area of the 90 min delay period is the minimum necessary to establish memory under the training conditions of Experiment 13, and then calculate the time required for such synthesis under normal conditions of protein synthesis, it would take only about 20 min to synthesize enough additional protein to establish memory. In Experiment 14, using more intense shock to provide stronger training, a shorter delay period -- 60 min -- was sufficient to establish memory in most subjects. The protein synthesized during the partial inhibition of the 60 min delay period would correspond to that synthesized during about 8 min of normal protein synthesis.

Apparently only a small amount of protein synthesis, over a short period of time, is required to establish memory.

We have observed repeatedly that the last injection of a series of injections, such as those used in Experiment 13 and 14, is critical to obtaining amnesia in a high percent of the subjects. The results of Experiment 14 suggest that the CNS retains a nearly constant capacity for synthesizing the memory-related protein(s) until this capacity begins to drop off several hours after training. A possible reason for this is that the rate of memory-related protein synthesis remains nearly constant and then drops off. Table 13 showed that it made very little difference in the percent amnesia whether protein synthesis occurred between injections 1 and 2 or between injections 2 and 3 (i.e., 2 or 4 hrs after training). But if protein synthesis was only permitted between injections 3 and 4 (6 hrs after training), then the reduction in the percent amnesia was non-significant except for the 60 min delay period. If we assume that the expression of memory requires a fixed minimal amount of protein, then it would be true that the rate of production of this protein must be slower 6 hours after training than 2 or 4 hours after training since it took more time for subjects in the 6 hour group to synthesize enough protein to show retention (i.e., 60 min) than for the subjects assigned to the 2 or 4 hour groups (i.e., 40 min). It appears that the duration of inhibition must extend over a

period of time long enough for the rate of memory-related protein synthesis to decline significantly, if memory formation is going to be successfully blocked. It will be of considerable interest to know what maintains this capacity in the CNS such that memory formation can occur many hours after training.

Failure to obtain amnesia with inhibitors of protein synthesis has generally been accounted for in two ways: (a) overtraining or (b) leakage of protein synthesis due to incomplete inhibition. In this section and in most of the preceding sections, overtraining has been shown to block amnesia with a given duration of inhibition. However, longer durations of inhibition or protein synthesis have then been shown to cause high levels of amnesia again.

It seems reasonable to assume that anything less than complete inhibition would allow the relevant protein(s) to be synthesized at a low rate but over a considerable time period and that this could eventually establish memory. But the "leakage hypothesis" is not easily tested and, therefore, only remains as an excuse for explaining away negative results. If small amounts of protein could add-up to establish memory as suggested above, then it should have been the case that two 45 min delay periods should have been more like the 90 min delay period in its amnestic effect than like the single 45 min delay periods (Experiment 15). The protein synthesized over two different time periods was not additive and, therefore, this would not seem to support the suggestion

that protein synthesis can leak for some period of time and
thereby establish longterm memory.

VI. EFFECTS OF PROTEIN SYNTHESIS INHIBITION ON MEMORY FOR ACTIVE AVOIDANCE TRAINING

Introduction

In the previous chapters, I have employed only passive avoidance training to evaluate the role of brain protein synthesis on memory formation. In this chapter, I will extend this research to active avoidance. The effects of Ani on retention for passive avoidance and active avoidance conditioning will be compared in the discussion section of this chapter.

In studies of memory formation using protein synthesis inhibition as the amnestic treatment, active avoidance has been used infrequently. Flexner and his co-workers have reported that puromycin will block memory for a left-right shock avoidance habit in a Y-maze (Flexner et al, 1967). However, the amnesia seems to be the result of a disruption of retrieval processes rather than a disruption of longterm memory formation (Flexner and Flexner, 1967,1968). Flexner, Flexner, and Roberts (1966) 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 (Barondes and Cohen, 1968).

INHIBITION OF PROTEIN SYNTHESIS

The procedures for determining the inhibition of protein synthesis are as previously described. Acetoxycycloheximide (AXM) shows a dose dependence such that the greater the amount of AXM administered subcutaneously the greater the duration of inhibition. However, 100 μ g/injection seemed to offer relatively long inhibition at a relatively low dose (Table 14). I feel that the use of the lowest possible effective dose is important because this reduces problems of systemic side effects as the cause of amnesia. Ani was shown to be for the most part dose independent (p. 64). Our dosage of Ani has been set at 500 μ g/injection. AXM is the more potent of the two inhibitors on a gram for gram basis. The 100 μ g injection of AXM inhibits protein synthesis for about 5 hours at 80% or greater, while the 500 μ g injection of Ani inhibits for only about 2 hours at the same level. Ani injections were reported to be additive in the sense that each successive injection prolongs inhibition by an additional 2 hours (p.66). The combination of Ani followed by a 100 μ g dose of AXM (2 hrs later) can be seen to extend inhibition of AXM to 6 hours, 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

Table 14

Percent Inhibition of Brain Protein Synthesis
by AXM and by Ani and AXM

Dose of AXM μ g	AXM		Ani ² +AXM			
	4hr*	6hr	4hr	6hr	8hr	10hr
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 hours. Thus Ani²+AXM at the 100 μ g dose has a total inhibition time of 10 hrs at 80% inhibition or greater (4hrs by Ani² and 6hrs by AXM).

given 6 hours apart. Under these conditions the Ani+AXM² injections were found to inhibit protein synthesis for 13-1/2 to 14 hours at about 80% or greater.

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

BEHAVIORAL EFFECTS

Subjects

The subjects used in these experiments were randomly bred male Swiss (CD-1) mice reared at our colony in Lawrence Berkeley Laboratory. The breeding stock was originally purchased from Charles Rivers Breeding Laboratory Inc, Willmington, Mass. The mice used in these experiments were offspring from the original stock. For other details about this strain see Chapter IV, p.96. Subjects were housed 48 hrs 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.

Apparatus

The training apparatus consisted of a black plexiglass T-maze, (12.5 cm high, 9.8 cm wide alleys, the start alley being 46 cm long, and the goal boxes being 17.5 cm deep). Shock (0.40 ma) was administered through brass floor rods as

previously described (p.20). Each goal box was fitted with a clear plexiglass 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 plexiglass 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 μ g or .5 mg (except in Experiment 18). 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 1 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.

ACQUISITION OF THE AVOIDANCE TASK

It has been my contention throughout this thesis that one cannot best use the inhibitors to test their effects on memory, until 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 24). 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. I will refer to these two groups respectively as mice with fast or slow rates of learning. Also from Figure 24, it is clear that no significant differences in acquisition occurred between the saline- and Ani-injected mice.

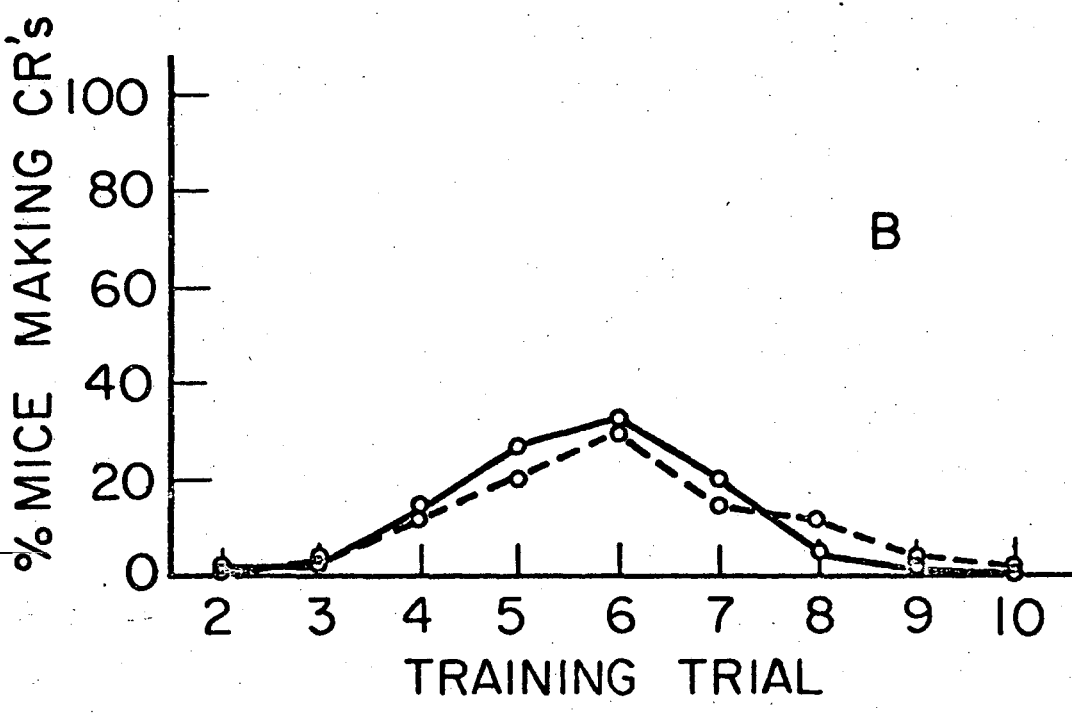
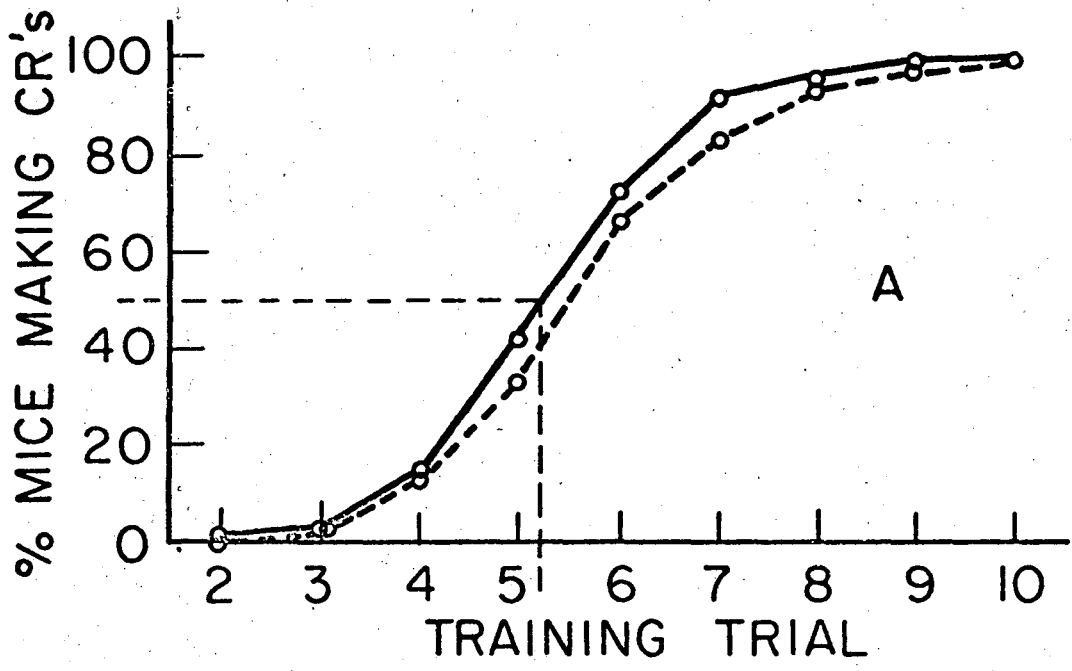
Experiment 16

Design

The purposes of this experiment were to test if Ani would cause amnesia for weak training (only 5 trials) in active avoidance 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,

Figure 24. The figures that follow show the acquisition curves for two groups of subjects being trained to avoid footshock. In Figure 24-A, 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 17. 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 24-B, the 1st avoidance response is plotted in terms of what percent of the subjects 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 number 5, 6, or 7.

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



○---○ NaCl (N=46) ○—○ Ani (N=169)

NaCl³ which received three successive injections of saline 2 hours apart, and NaCl⁵ which received 5 successive injections of saline 2 hours apart. The experimental groups received either 1, 2, 3, 4, or 5 injections of Ani (Ani, Ani², ... Ani⁵) each series starting 15 min prior to training and subsequent injections at two hour intervals. (See pages 63-65 and 81-82 for a discussion as to why this particular injection schedule was used.) 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+Ani⁵ in which saline was administered prior to training and, starting 2 hours later, 5 successive injections of Ani were given. This group should not differ from the saline controls if (a) Ani⁵ has no permanent debilitating effects and (b) the necessary protein (s) for longterm memory can be synthesized during the 1-3/4 hours after training when inhibition is not present.

Procedures

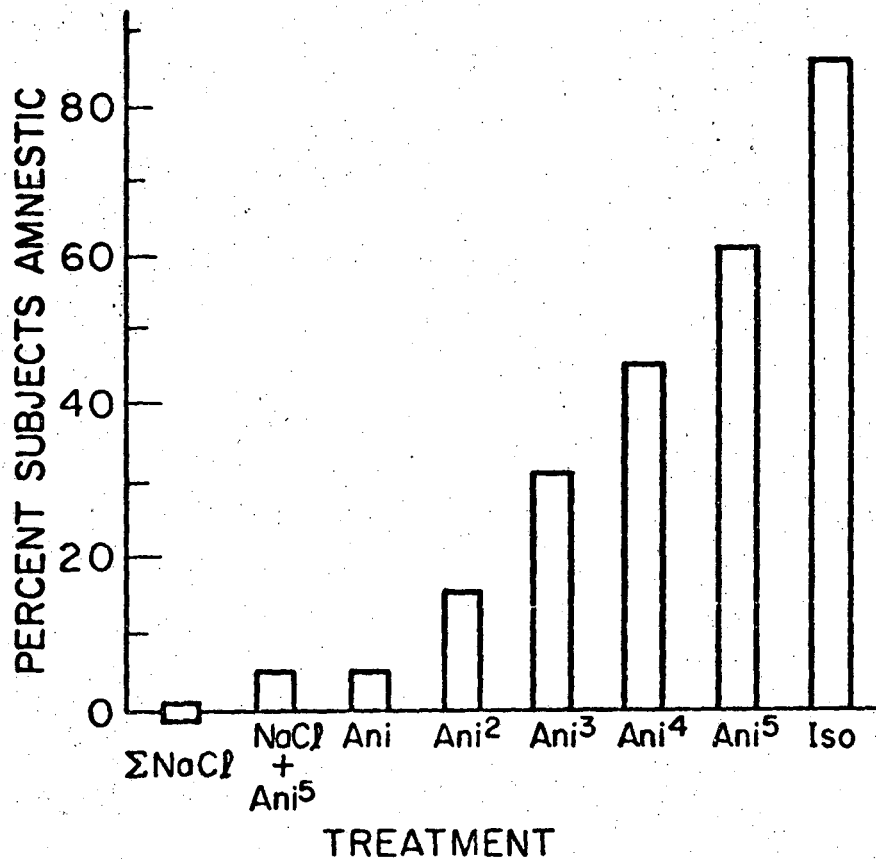
All subjects were given 5 training trials. On the retention test (given 1 week after training), each subject was trained until it made one avoidance response; an avoidance response to the correct side of the T-maze is the conditioned response (CR). Twenty subjects were run for each group. Amnesia for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

Results

Comparing the saline versus the Ani-injected subjects in Figure 25, it will be seen that at least 3 successive injections of Ani (6 hrs of inhibition) were required to cause a significant percent of the mice to become amnesic. However, even after 5 successive injections of Ani (10 hrs of inhibition) the percentage of amnesic subjects is significantly less than 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 Ani⁵. A 15% difference in amnesia exists between Ani² and Ani³, Ani³ and Ani⁴ and also between Ani⁴ and Ani⁵ (Figure 25).

The distribution of the retention scores (Figure 26) shows that as one moves from Ani to Ani⁵ subjects take more and more trials to make their 1st CR on the retention test. In these graphs, it is clear that the combined NaCl groups, NaCl+Ani⁵ and Ani do not differ significantly in shape, yet all differ markedly from the Iso group; there is almost no overlap in the distributions. Ani⁵ is clearly closer to Iso than to the combined NaCl groups.

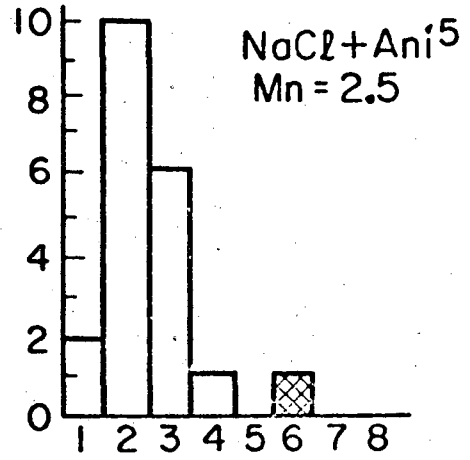
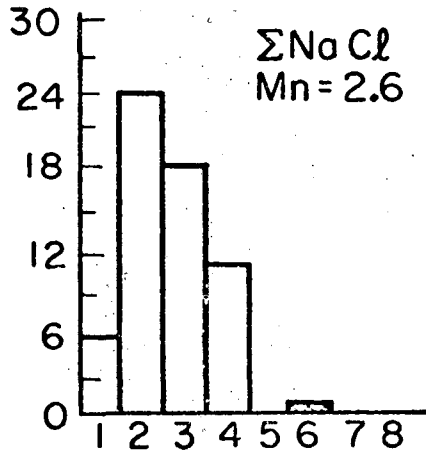
The Ani injections also had a significant effect upon the escape behavior (Table 15). In the Ani⁴ and Ani⁵ groups, significant numbers of subjects made an error by escaping to the wrong side of the T-maze (those mice making an avoidance



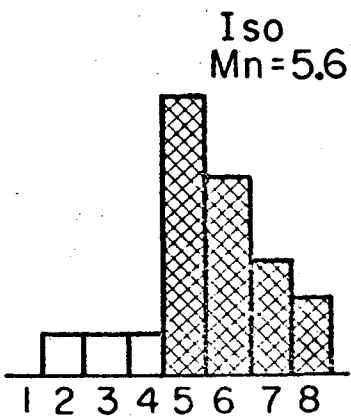
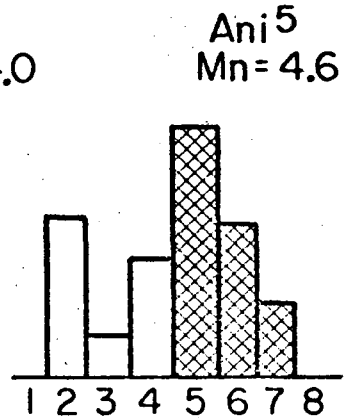
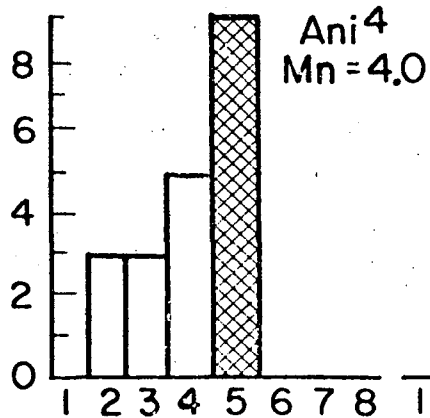
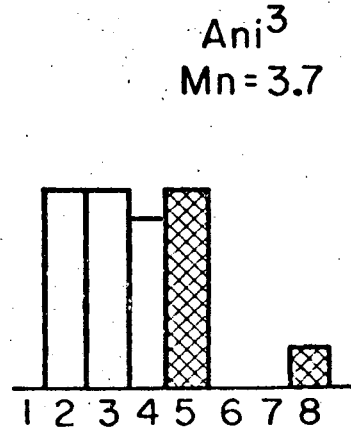
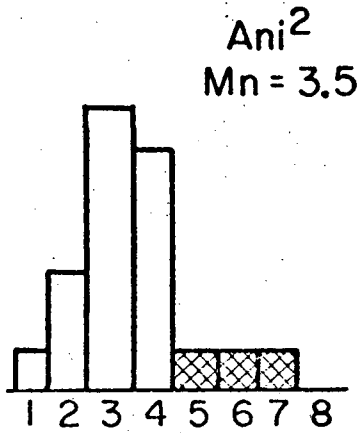
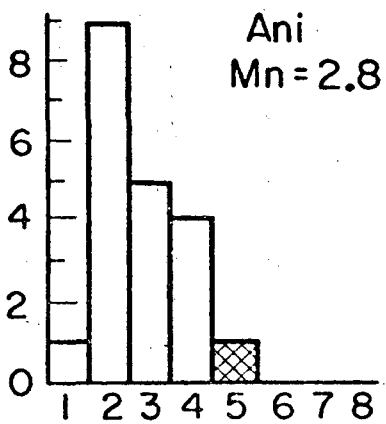
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Figure 25. The effect of the duration of inhibition of protein synthesis by Ani on memory for footshock avoidance training.

Figure 26. The distribution of retention scores (the number of trials to make the 1st CR). The area of the combined saline groups (Σ 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 amnesic (i.e., first CR on trial 5 or later). Note that across the Ani groups (Ani to Ani⁵) the shaded area is increasing, and the means are shifting toward the amnesic 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.



NUMBER OF SUBJECTS



RETENTION TRIAL

Table 15

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

Σ NaCl	NaCl+Ani ⁵	Ani	Ani ²	Ani ³	Ani ⁴	Ani ⁵
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 Ani⁵ group may be completely amnesic for the escape response portion of this training task. All the groups have N's = 20 except Σ NaCl (N = 60).

on the first retention trial were not included in these calculations). Few Ani, NaCl+Ani⁵ or NaCl injected subjects made discrimination errors.

Experiment 17

Design

In Experiment 16, subjects received only marginal training (5 trials). In this experiment, 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 hrs. In addition, subjects were classified as to how many trials it took before they made their 1st avoidance response (CR). Other conditions of shock and training were as in Experiment 16. The table below gives the schedule of injections and method by which each duration of inhibition was obtained (Table 16).

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 27). A comparison of the saline and combined 8, 10, 12, and 14 hour inhibition groups showed that 0% of the saline subjects were amnestic while 59% of those subjects in the

Table 16

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

The groups used in Experiment 17, 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").

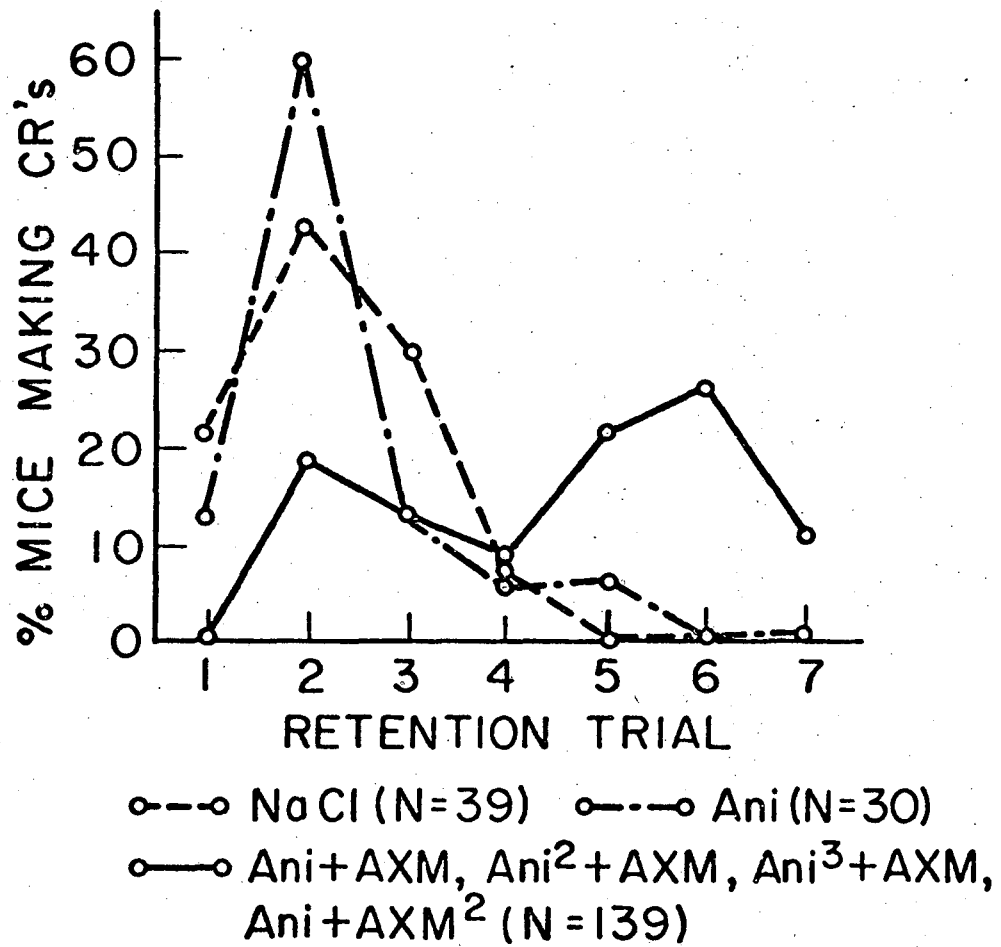
long duration of inhibition groups were amnesic. The subjects receiving a single Ani injection prior to training did not differ significantly in the percent amnesia from the saline controls (Figure 27).

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 important this variability effected the amnesia induced by inhibition of protein synthesis. In the following paragraphs some performance variables 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 amnesic treatment. The faster the rate of learning the less effective the amnesic treatment (Table 17).

The fact that the rate of acquiring the avoidance habit affected retention raises a question as to what should be defined as memory loss. If we use a fixed criterion of memory loss, this implies that the rate of acquisition at training and testing have no relation; that is those subjects

Figure 27. 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 amnesic on a fixed criterion bases (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 amnesic. None of the NaCl subjects were amnesic.



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Table 17

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)

The effect of the rate of acquisition on the percent amnesia.
As the rate of acquisition increases the percent amnesia decreases.

Table 18

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

The effect of the number of training trials on the percent amnesia in mice. The more trials a subject is given the lower the probability that it will be amnestic when retrained.

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

learning quickly do not always learn the task quickly. If we use a savings measure to define amnesia (less than 30% savings) then this implies that quick learners if they are truly amnesic will relearn quickly. The central problems are (a) we must make one or the other assumption since we cannot test if a given subject would always have learned quickly or slowly. (b) If we do not assume that fast learners are usually fast, and in fact they are, then by the fixed criterion definition of amnesia used in Experiment 16, we could never show that fast learners suffered a memory loss. (c) By the same reasoning slow learners with a slight memory loss would in most cases be classed as amnesic. I feel that the sliding scale provided by defining amnesia as less than 30% savings on relearning is the best criterion since it would be a serious handicap to use a criterion of memory loss that might make it impossible to demonstrate memory loss. In this experiment and in Experiment 18 amnesia is defined as a savings score of less than 30% on the retention test.

The number of training trials (6, 8, or 10) seemed to have had some effect upon the amnesia (Table 18). 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 19 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 amnesic and those subjects making discrimination errors and having high rates of learning to be the least likely to be amnesic. As the number of errors increases the amount of shock a subject received increased. It maybe that, to some extent, the more shock a subject received at training the less likely the subject would be amnesic at retraining.

The longer the duration of inhibition of brain protein synthesis, the higher the percentage of amnesia (Table 20). This table 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+AXM² 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 hrs of training.

The duration of inhibition and the number of training trials both effect amnesia (Table 21), such that those subjects with the most training and the shortest duration of inhibition

Table 19

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 effect of rate of acquisition and number of discrimination errors on the percent amnesia. The percent amnesia is defined by a savings score of less than 30%. From the table above it appears as if those subjects that made more errors at the training session were less likely to be amnesic when tested one week after training. Across the subjects making no errors 70% were amnesic, while 55% of the subjects making 1 error were amnesic. None of the comparisons were significant; however, large N's might confirm a weak trend.

Table 20

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

The effect of the duration of inhibition on the percent amnesia. As the duration of inhibition increases, the probability increases that a subject will be amnestic at retraining. Within the groups given 8-14 hrs of inhibition, the trend does not quite reach significance; however, it is generally consistent with trends reported in other experiments in this thesis.

Table 21

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)

The effect of the number of training trials and the duration of inhibition on the percent amnesia. The table shows that 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 amnesic 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 amnesic.

are the least likely to be amnesic when retested and that those subjects given the fewest number of trials and the longest duration of inhibition are most likely to be amnesic when retested.

Thus it seems probable that several of these factors effect 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 18

Design

The reader maybe asking why we did 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 (p. 64) - 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. In this experiment, we will compare the effects of a 500 μ g dose against that of a 2500 μ g dose. The groups used were: 5Ani+Ani - in this group the subjects received a 2500 μ g dose 15 min prior to training and 2 hrs later received the standard 500 μ g dose. The second group received Ani+5Ani (500 μ g dose followed 2 hrs later by the 2500 μ g dose).

The third group, Ani³, received three successive 500 µg injections of Ani at 2 hr intervals. The last group, Ani⁶, 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 it clear why these various groups were employed. The duration of inhibition at 80% or greater is approximately as follows: Ani⁶ = 12 hrs, Ani³ = 6 hrs, Ani+5Ani = 5 hrs and 5Ani+Ani = 4 hrs. In addition, the total amount of drug given to the subjects in the Ani⁶, 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 reponse 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. Ani³, Ani+5Ani and 5Ani+Ani caused about the same duration of inhibition of protein synthesis. Ani³ caused 10% amnesia, Ani+5Ani caused 0% amnesia, but 5Ani+Ani caused 80% of the subjects to be classed as amnesic. The second comparison is based upon subjects receiving 3000 µg

of Ani in total. Ani+5Ani, Ani⁶, and 5Ani+Ani all received the same amount of drug. Ani+5Ani caused 0% amnesia, Ani⁶ caused 40% amnesia, but 5Ani+Ani caused 80% of the subjects to become amnesic. 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 hrs) should not have had a significant amnesic effect judging from the results of Experiments 16 and 17. 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 Ani⁶ and Ani+5Ani that total drug received does not necessarily lead to amnesia. A similar experiment using passive avoidance (p.80-81) concluded that duration of inhibition, not the quantity of drug per se influenced amnesia. For 5Ani to cause a high percentage of the subjects to become amnesic, it had to be given prior to training as the 5Ani+Ani and Ani+5Ani comparison shows. If 5Ani does not achieve its amnesic power by either duration of inhibition or by virtue of the total amount of drug administered, then how does 5Ani cause amnesia?

DISCUSSION

The finding of principle importance in this chapter, 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 and active avoidance, reduce the amnesic effect of a given duration of protein synthesis inhibition. However, increasing the duration of the inhibition was observed in both passive and active avoidance to counteract the effect of increasing the training strength.

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 hrs of inhibition) were required to cause 80% to 100% amnesia. This same level of amnesia was obtained with active avoidance but only in the most poorly trained subjects and with 14 hrs of inhibition. 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 to .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, 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 amnesic 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 1st 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 1st CR is usually of a long duration (4 - 4.9 sec - the shock coming on at 5.1 seconds). The 2nd and 3rd avoidance responses tend to show latencies of about 2.5 - 3 seconds 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.

In order to obtain 100% amnesia one would have to cause significant amnesia for both the escape and avoidance learning as well as for the associated habituation to the novelty of footshock and the apparatus itself. While this might be possible, the duration of inhibition required might have to be either impractically or prohibitively long.

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 amnesic 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. 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. Subjects may make escape errors for no apparent reason; some mice will be making several correctly directed responses and then make an error. It is unlikely that the mice forgot or did not know which side was correct since they correct their choice, without fail, on the next trial. Thus 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. One can easily overtrain a subject and not be able to detect it - thus adding variance to the amnestic effect.

It is my opinion 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 1st avoidance response when tested on the avoidance training 1 week after the escape training. Naive subjects took only 5.6 trials to make their 1st 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.

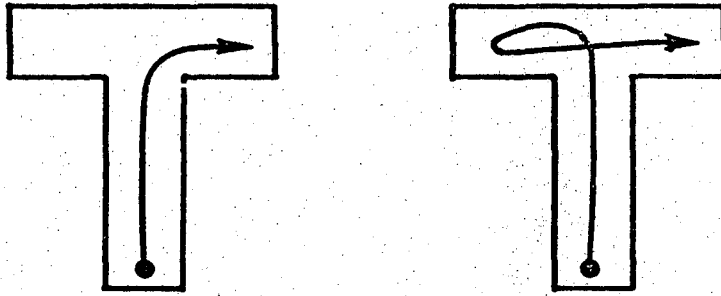
Memory Loss with Three Inhibitors

It was also shown in this chapter 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 amnesic effect. Cyclo had previously been shown to be an effective amnesic agent when administered as the second injection of a series of injections in a passive avoidance experiment (p.76). Thus Ani, Cyclo and AXM have been demonstrated to cause amnesia at a time when they could not have impaired learning.

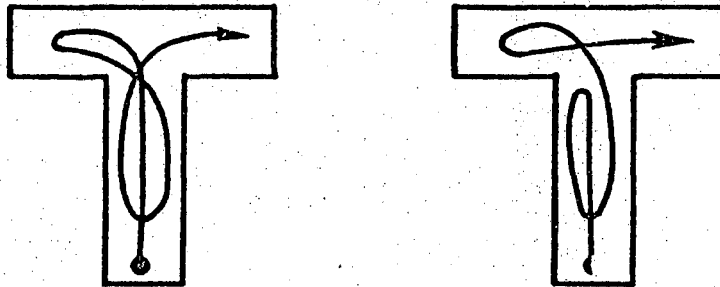
Possible Drug Effect on Acquisition

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 retracing of its previous run through the box. Figure 28 shows some examples of simple and complex escape responses. In Experiment 16, the NaCl- and Ani-injected subjects showed no significant differences in the percent of simple versus

SIMPLE



COMPLEX



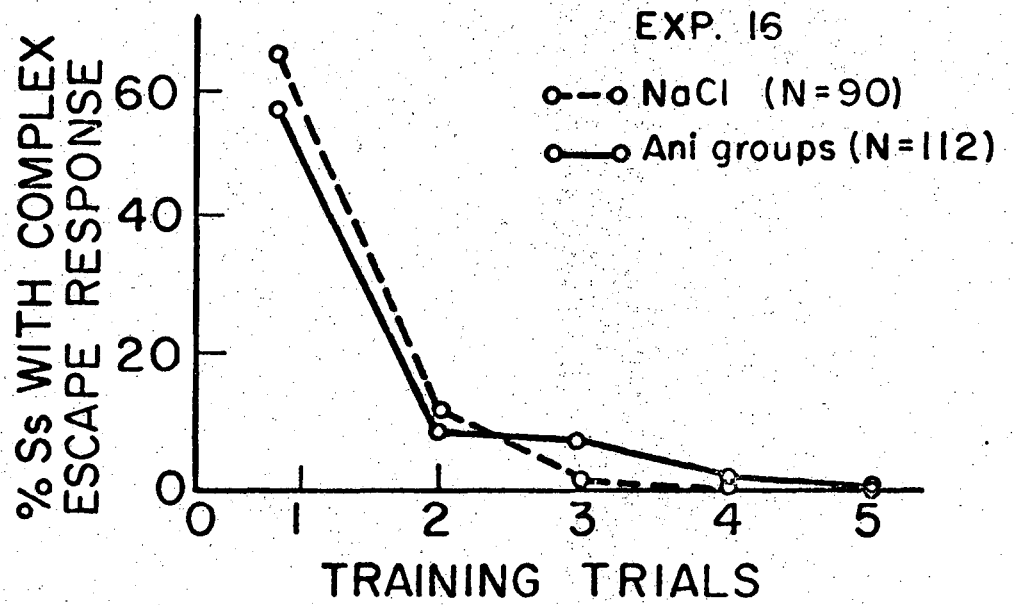
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Figure 28. Examples of simple and complex escape responses for the early training trials of active avoidance.

complex responses. However, in Experiment 17 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 16 and 17 (Figures 29 and 30). 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 per cent 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. In addition, subjects given only a single pretraining injection of Ani did not show significant levels of amnesia (Figures 25 and 27).

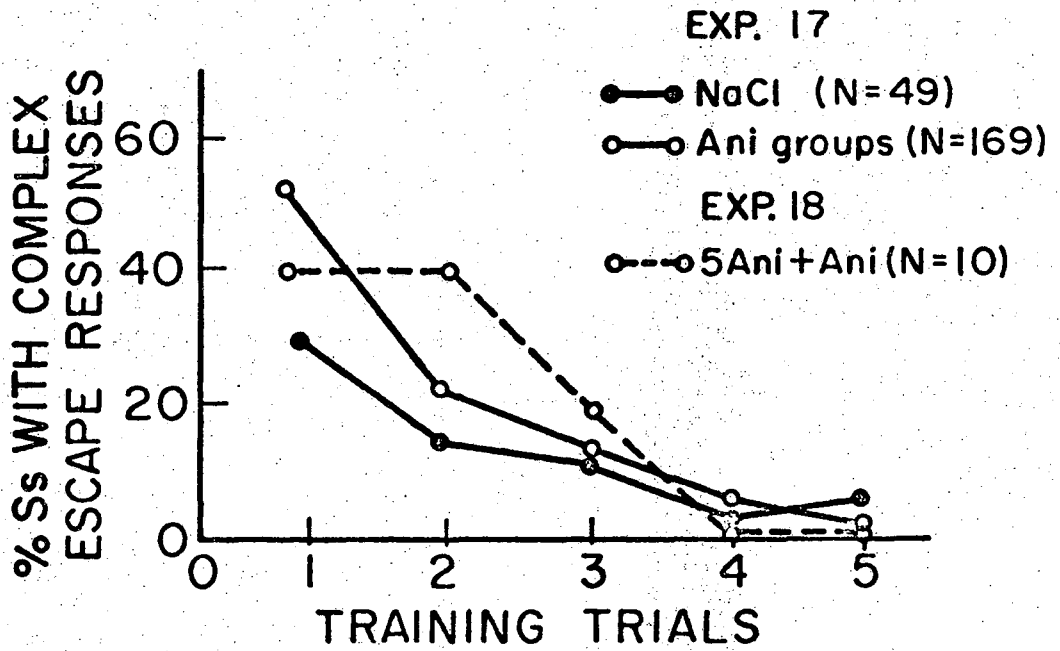
Amount of Drug versus Duration of Inhibition

The results of Experiment 18 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 1st avoidance response, the percent simple versus complex responses (Figure 30) and the duration of shock were within normal limits. Yet, 5Ani+Ani caused highly significant percent of the subjects to become amnesic, while Ani+5Ani did not. How? The 2500 μ g dose of Ani does not significantly alter the duration or extent of inhibition caused by the 500 μ g dose of Ani. Why would



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Figure 29. Distribution of complex escape responses for NaCl- and Ani-injected subjects.



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Figure 30. Distribution of complex escape responses for NaCl-, Ani-, and 5Ani-injected subjects. The data for the 5Ani curve comes from Experiment 18. NaCl and Ani differ by $P < 0.01$ on training trial number 1.

the 2500 μ g 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 principle 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 longterm 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.

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

VII. DISCUSSION

The Research Problems

The thesis research focused on four major problems in the literature which tended not to support the hypothesis that protein synthesis was required for long-term memory formation. The first difficulty was that the criteria of what was memory loss was usually not very rigorous. I feel that the criterion of amnesia used in this thesis for both passive and active avoidance is rigorous enough such that few would doubt that the retention performance was clearly different between those subjects classed as amnesic and not amnesic. It seems that complete amnesia for a group of subjects is possible if training is not too strong and/or inhibition is of long duration.

The second problem upon which this research was focused was that most of the literature reported statistical effects; the magnitude and the degree of effect were generally such that control and experimental groups showed a great deal of overlap. Rarely are more than 60% of the subjects affected. In many of the groups that have been run in the experiments reported here, both the magnitude of the effect and the almost complete lack of overlap between experiments and control subjects made statistical verification that the drugs had had an effect unnecessary.

The third problem is related to the ones above, if not the cause. Amnesic treatments have rarely been reported that affect

most of the subjects. With control over the degree of training that each subject receives, we have been able to obtain quite consistent effects ranging from little or no amnesia in "over-trained" subjects to complete amnesia. The variation in effectiveness of the amnestic agent I have attributed primarily to (a) variation in the training parameters (which were previously uncontrolled and unmeasured) and (b) to uncontrolled sources of variability such as variation in shock sensitivity, arousal, and individual differences in learning, etc. In our experiments, much of the variance has been reduced (particularly in passive avoidance) by recording the parameters of training and grouping subjects according to the degree of training which they actually received.

The last problem was that too much training blocked or reduced the amnestic effect of brain protein synthesis inhibition. This has been demonstrated to be true within and across many of the experiments of this thesis (both for passive and active avoidance). However, it has been shown just as frequently that longer inhibition periods will reestablish the amnestic effect. It is still hard to understand how a small increase in training strength requires a rather large increase in the duration of inhibition of protein synthesis (this is particularly true of active avoidance) to reestablish amnesia. Many of our preconceptions about what seems reasonable in memory formation may have to be set aside until more is known about the processes and their time courses.

Problem of Interpretation

Blocking protein synthesis, like making a brain lesion and then attributing a loss of function to a process or tissue that is not present, offers a long known and expounded logical problem. For this reason, Chapter V offers important support for the hypothesis that long-term memory requires protein synthesis. In Chapter V, amnesia was established by using long durations of inhibition of protein synthesis. In comparison groups, small amounts of protein synthesis were allowed to occur at various times and for various durations of time after training. This controlled, post-training, protein synthesis was shown to lead to memory formation. Two important trends were reported: (a) the longer the period of controlled protein synthesis, the more subjects remembered training, and (b) the closer the protein synthesis occurred to training, the more subjects remembered training. Some pilot studies, thus far not reported, suggest that the length of retention for the training from 24 hrs to 7 weeks after training is directly related to the duration of protein synthesis. The control subjects showed no loss of memory over this same period of time. These experiments showed directly that protein synthesis is necessary for long-term memory.

Training

A great deal of stress has been placed on the parameters of training since (a) better training results in lower levels of amnesia unless longer durations of inhibition are used and (b) even

in a seemingly simple training procedure like that of passive avoidance a number of training parameters exist and variability exists in the values of these parameters that a given subject will have. In passive avoidance, the following parameters were found to control the degree of acquisition and retention: (a) duration of shock, (b) latency to enter the shock compartment, (c) shock strength, and (d) the size of the mouse hole (previously unreported--the larger the diameter of the mouse hole the greater the training strength). Also, the retention period affects the percent amnesia such that the longer the retention period the higher the percentage of amnesic subjects (conditions of training and inhibition being constant).

In active avoidance, the principal problems are: (a) how reliable is the behavioral measure of learning and (b) what criterion should one use to define memory loss. The behavioral measure must reasonably reflect what a subject has learned; otherwise one is unable to determine how much learning a given degree of training actually causes. In our early attempts to deal with active avoidance, it was found that by some training procedures subjects learned in 6 training trials, but by other procedures varying only slightly it seemed to take 10-14 trials. In addition, most of the subjects trained using the latter procedure showed no signs of having learned to make avoidance responses at the time of training, yet at the retention test their performance indicated that they had clearly acquired the task in spite of the

appearances of the training record. The training procedure that we used in Chapter VI helped to reduce the discrepancy between what the retention test was telling us and what the training record was showing. However, there are still subjects that show a great discrepancy. In order to use the inhibitors effectively, the measure of learning must be reliable; it must tell us when and how well the subject has learned. In general, it is my conclusion that active avoidance (T-maze) is not well suited to careful analysis of the effects of drugs on memory for three reasons: (a) The measure of learning (i.e., the avoidance response) is not a reliable indicator of what a subject is learning at training. (b) Too many factors seem to or are possibly influencing learning. This means that in a carefully controlled experiment the number of groups would be very large and thus require an enormous number of subjects. (c) The duration of inhibition required to cause amnesia is impractical for regular use. One additional problem is that avoidance conditioning is not a single learning task. Two obvious components can be seen: (a) the subject learns where to direct its response (to the left or right goal box) and (b) to anticipate the shock (avoidance). Subjects being trained to avoid shock are overtrained on the escape portion of the task; thus one would always expect some savings unless the inhibition were very long. Subjects that are just given escape training, without the possibility of making an avoidance, show a significant savings in learning the avoidance part of the task when retrained a week later. Thus the avoidance training situation is a

complex task which across the multiple training trials generates a great deal of variability which must be controlled and measured if careful work on memory is going to be done.

We were able to determine at least some of the parameters that have to be controlled in the active avoidance situation: (a) the rate of acquiring the habit, (b) the amount of practice, and (c) the number of discrimination errors for a given rate of learning. The total amount and distribution of shock very probably modifies learning.

I feel that the point has been demonstrated that retention for passive or active avoidance depends upon protein synthesis occurring within a relatively short period of time after training. Two general trends were demonstrated to be true across the two types of training procedures: (a) increases in training strength block amnesia (or promote better memory processing) and (b) the longer the period of inhibition of protein synthesis, training strength being constant, the greater the disruption of memory (i.e., higher the percentage of amnesic subjects).

While it was desirable to test the effects of inhibition of protein synthesis on memory for active avoidance, I feel that the task is not suitable for careful study of memory, since control over the acquisition is very difficult. Passive avoidance is far easier to control and remains the best training task available to evaluate the early stages of long-term memory formation.

Whatever task one employs, I hope that the points have been made that (a) the behavioral measure of acquisition and retention must reliably reflect the individual subjects learning and (b) the plotting

of an acquisition-retention curve is important so that one can establish how much training a subject has been given. Recently, Squire and Barondes (1972) reported that open-field habituation in mice was unaffected by pretraining injections of cycloheximide. That is, saline- and cyclo-injected mice reduced their activity about the same degree when re-introduced to the open-field at some later time. The "training" session was 10 min long. Is a 10-min exposure period a lot of habituation or very little so far as learning and memory are concerned? If they had plotted acquisition (e.g., decrease in activity) as a function of the number of minutes exposed to the open-field, we would have some idea after how many minutes most of the learning takes place. As it stands, we do not know whether a 10-min session is just enough training to cause a decrease in activity or whether it is 8 or 9 min longer than necessary. The point is that unless we know to what extent the animal has been trained relative to the minimum necessary, we cannot judge what constitutes "overtraining".

The acquisition-retention curve might also help eliminate false reports of strain differences in susceptibility to the amnestic agent. In Chapter V, it was shown that the parameters of training required for different strains to learn passive avoidance differ remarkably. Given that the same relative degree of learning is obtained from each strain, Ani had a similar effect on memory across all strains. If we had used one level of training and did not plot the acquisition-retention curve, we could have falsely

concluded that the BALB/cJ and C57Br/cdJ strains were highly susceptible to Ani while the C57Bl/Jf and CB strains were not affected at all. If each investigator were to show the acquisition-retention curve, it might greatly facilitate the verification of research findings in other laboratories and would facilitate across laboratory comparisons of results.

Permanent Incapacity or Impairment of Memory Formation.

In several of the experiments in this thesis, saline was administered 15 min prior to training and one or more drug injections administered after training. The results have been consistent in that little or no amnesia was caused by inhibition starting 1-3/4 hrs after training. For example in Experiment 8, one group received Ani+Ani+Ani which resulted in 100% amnesia. But another group received a pseudo-injection (I) and then the three successive Ani injections 1-3/4 hrs after training; I+Ani+Ani+Ani resulted in 10% amnesia and Na+Na+Na in 0% amnesia. Thus neither the drug nor the long duration of inhibition (6 hrs at 80% or greater) could have caused a permanent incapacity to account for the amnesia. An even more dramatic case was shown in Experiment 17 in which one group received Ani+AXM² and 6 active avoidance training trials; this group showed 90% amnesia. However, Na+Ani+AXM² group also given only 6 active avoidance training trials yielded 0% amnesia. Thus, it seems

unlikely that either the drugs used or the long duration of inhibition (up to 14 hrs for Ani+AXM²) caused permanent incapacities in general brain function that could account for the amnesia.

In addition, these results indicate that memory-related protein synthesis, sufficient for recall at least 1 week after training, was formed during the 1-3/4 hr period prior to injecting the inhibitors of protein synthesis.

Amnesia by Three Inhibitors

Cycloheximide and AXM had never been shown to be particularly effective amnesic agent when administered after training. Using the multiple injection design, it has been shown in Chapters III-VI that Ani, Cyclo and AXM when administered post-training as the 2nd or later injection can cause significant degrees of amnesia, where a single pretraining injection of Ani does not. Thus all three inhibitors of protein synthesis have been shown to be effective in causing amnesia at a time at which none of the drugs could have impaired learning.

While I feel that the four questions originally set forth as the purpose of these studies have been answered, another major question has been raised: What processes underlie the ability of the CNS to retain the capacity to promote memory-related protein synthesis for hours after training?

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