Title

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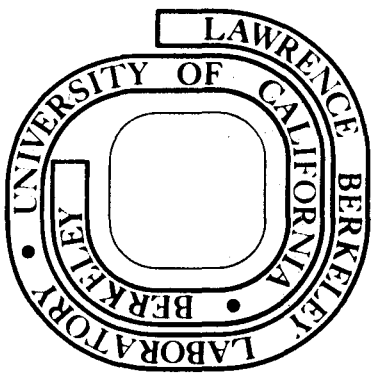

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DISCLAIMER

The Relation of Memory Formation to Controlled Amounts of Brain Protein Synthesis

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Running head: Duration of Protein Synthesis and Memory

ABSTRACT

The Relation of Memory Formation to
Controlled Amounts of Brain Protein Synthesis

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Anisomycin, an inhibitor of brain protein synthesis, was used to control the time and duration of protein synthesis occurring in mice, after they were trained on a one-trial passive avoidance task. It was found that if synthesis was stronly inhibited for 6 - 8 hours, a high percentage of the subjects were amnestic. However, if small amounts of protein synthesis were allowed to occur by permitting some limited recovery of protein synthesis, then memory was established. The longer the duration of this controlled synthesis and the closer it occurred to training, the greater the percentage of subjects remembering the training.

Mice, Passive Avoidance, Memory, Anisomcyin, Inhibition of Protein Synthesis, Amnesia

This study carries forward our research (4,5,6) and that of others (e.g., 1,3,7) in which inhibitors of protein synthesis are employed in order to elucidate biochemical processes involved in longterm memory. A brief recapitulation of some earlier research will provide necessary background for the questions to be taken up in the present study.

While the agents Cycloheximide (2,4,7,8,9) and Acetoxycycloheximide (1,3) have frequently been employed in such studies, we have found Anisomycin (5,6) to be particularly useful for two reasons: (a) the duration of inhibition of protein synthesis in mouse brain can be controlled by giving successive injections of Anisomycin (Ani) at 2-hr intervals (5), and (b) an injection that produces about 2 hrs of inhibition at 80% or greater -- or even a series of such injections -- is far below the lethal dose of the drug (5). The degree of amnesia has been found to depend upon both the duration of inhibition and several parameters of the passive-avoidance training situation (shock intensity and latencies to enter and to escape the training box).

As strength of single-trial training was increased, a single pre-training injection of Ani was found to become less effective in causing amnesia. This was also true when multiple injections were employed. With constant conditions of training, increased durations of protein synthesis inhibition caused greater amnesia. The greater the duration of inhibition, the greater the amnesia. Control experiments demonstrated that this greater effectiveness could not be attributed to either the multiple injection procedure itself or to the greater dose of Ani, per se, that was used in the multiple injection groups. Within practical limits

of increasing training strength and the duration of inhibition of brain protein synthesis, it has in principle been demonstrated that for any increase in training strength that blocks amnesia, a duration of inhibition exists that will reestablish the amnesia. Similarly for any duration of inhibition that blocks memory, a greater training strength exists that will block the amnesia (5,6).

From our previous studies we 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 studies that follow, we 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 ach.eve a high level of amnesia.

INHIBITION OF BRAIN PROTEIN SYNTHESIS

From the work that has been published (5) 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 1.

BEHAVIORAL EFFECTS

Materials and Procedures

The subjects used in the experiments were C57Bl/Jf female mice about 60 days of age (18-20 gm). The colony, maintained at the Dept. of Psychology, Univ. of Calif., Berkeley, was in its 29-34th generation of inbreeding. The training, testing and apparatus have been previously described (4,5). In brief, subjects were trained in a one-trial passive avoidance apparatus which consisted of a black start compartment joined to a white shock compartment by a partion containing a mousehole. Subjects were permitted to enter the white compartment through the mousehole whereupon the received a foot-shock until they returned to the black compartment. On the retention test given 1 week after training, the mice were placed into the black compartment and the time required for the subject to enter the white compartment was taken as a measure of retention. A latency-to-enter the white shock compartment on the test day of 20 sec or less was defined as amnesia. 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 1

Design

In all the experiments previously reported, inhibition of protein synthesis 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 + 40 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 1 graphically illustrates the design of the experiment. Ani+Ani was included (a) to show that under the training conditions employed the third injection 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 moderatly 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. (The training latency is the time the mouse takes to step from the black start box into the white compartment on the training day. The escape latency is the time from shock onset in the white compartment until the mouse returns to the black compartment.)

Results

As can be seen in Figure 1, 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 subjects that were not amnestic 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 amnestic 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 did not differ significantly between Ani+Ani and Ani+Ani-90-Ani.

Figure 1 about here

EXPERIMENT 2

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 clsoer 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 and 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 conditions of training (Ani+Ani+Ani = 15%, Ani+Ani+Ani+Ani = 85% amnesia). The procedure 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 1). 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 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%, $P < 0.05$). At the injection interval 2-3, a 20 min delay was not effective, 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 redued 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 percent decrease).

Table 1 about here

EXPERIMENT 3

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 2 and 3. Over this period the capacity to synthesize the memory-related protein(s) appears to be nearly constant, since in Experiment 2 the percent amnesia did not differ significantly between injection intervals 1-2 and 2-3 for the various delay intervals employed (Table 1). The groups used in Experiment 3 were Ani-45-Ani+Ani+Ani, Ani+Ani-45-Ani+Ani, Ani-90-Ani+Ani+Ani, Ani+Ani-90-Ani+Ani, and 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 2 except that only certain combinations of latencies-to-enter and -to-escape were used so as to maximize the amnestic difference between the 45 min and 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 2; thus in Experiment 3 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 2, 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 amnestic subjects (72% vs 28%, $P < .001$, 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 2. 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 amnestic 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 groups did not differ significantly (72% vs 76% amnesia). Apparently, the quantity of protein synthesized per unit time is an important factor in memory formation.

Figure 2 about here

DISCUSSION

In a previous study in which up to 3 succesive injections of Ani were administered, we 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 that will reestablish the amnesia"(5, p.526). Experiment 1 of the present study confirms these earlier results and Experiment 2 extends them by showing that with still stronger training, 4 successive injection of Ani were required to produce amnesia (Table 1, 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 used in Experiment 1 is equivalent to a 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 condition of Experiment 1, 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 2, 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 used in Experiments 1 and 2 is critical to obtaining amnesia in a high percent of the subjects. The results of Experiment 2 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 1 showed that it made very little difference in the percent amnesia whether protein synthesis occurred between injection 1 and 2 or between 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 (s) 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 long enough for the rate of memory-related protein synthesis to decline significantly if memory formation is to be 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 paper and in others (5,6), overtraining has been shown to block amnesia with a given duration of inhibition; however, longer durations of inhibition of 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 amnestic effect than like the single 45 min delay period (Experiment 3). The protein synthesized over two different time periods was not additive and therefore, this does not support the suggestion that protein synthesis can leak for some period of time and thereby establish longterm memory.

Acknowledgments

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Table 1

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 $P < 0.05$.

Figure 1 - The time courses of inhibition of protein synthesis as a function of the injection scedule, 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).

Figure 2 . 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 singledelay 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.

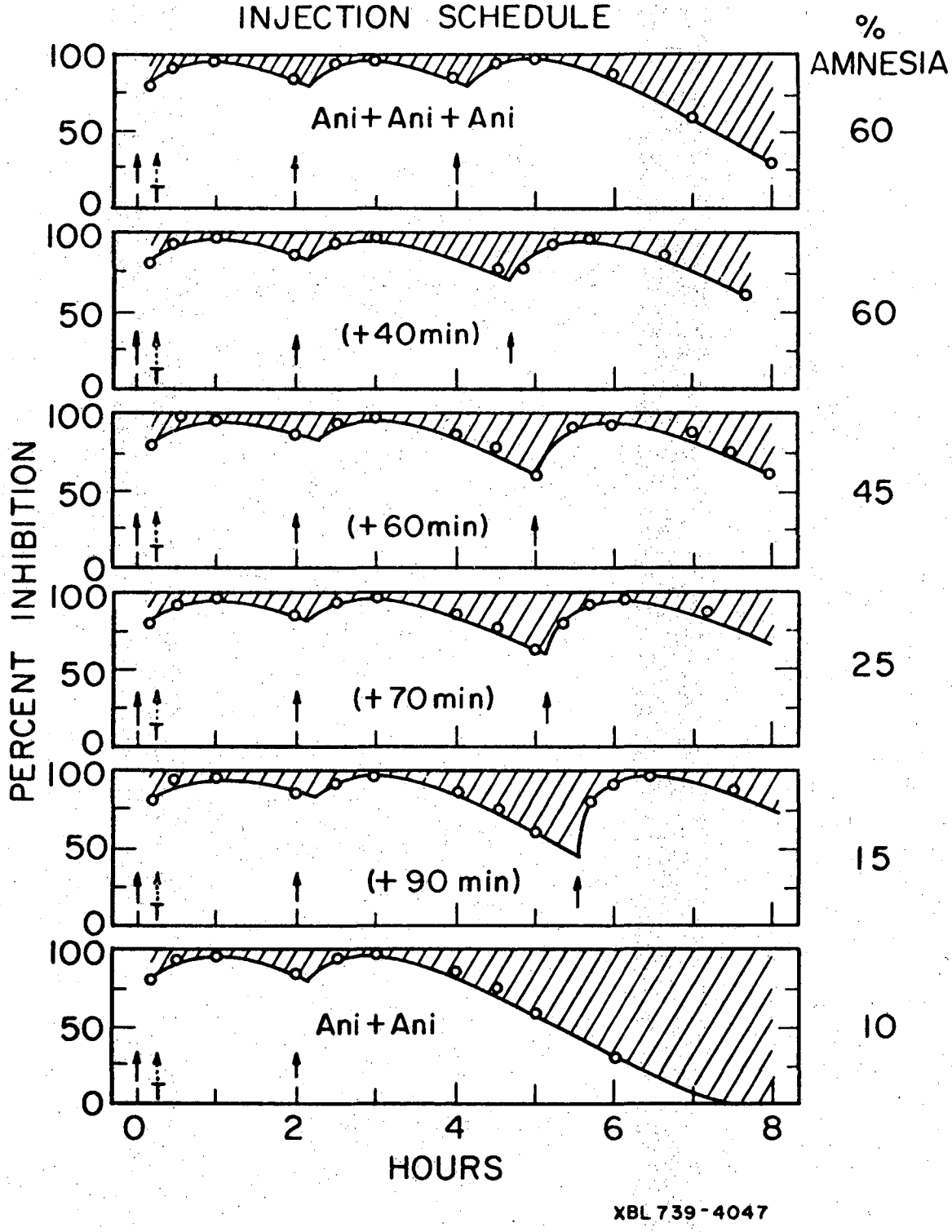
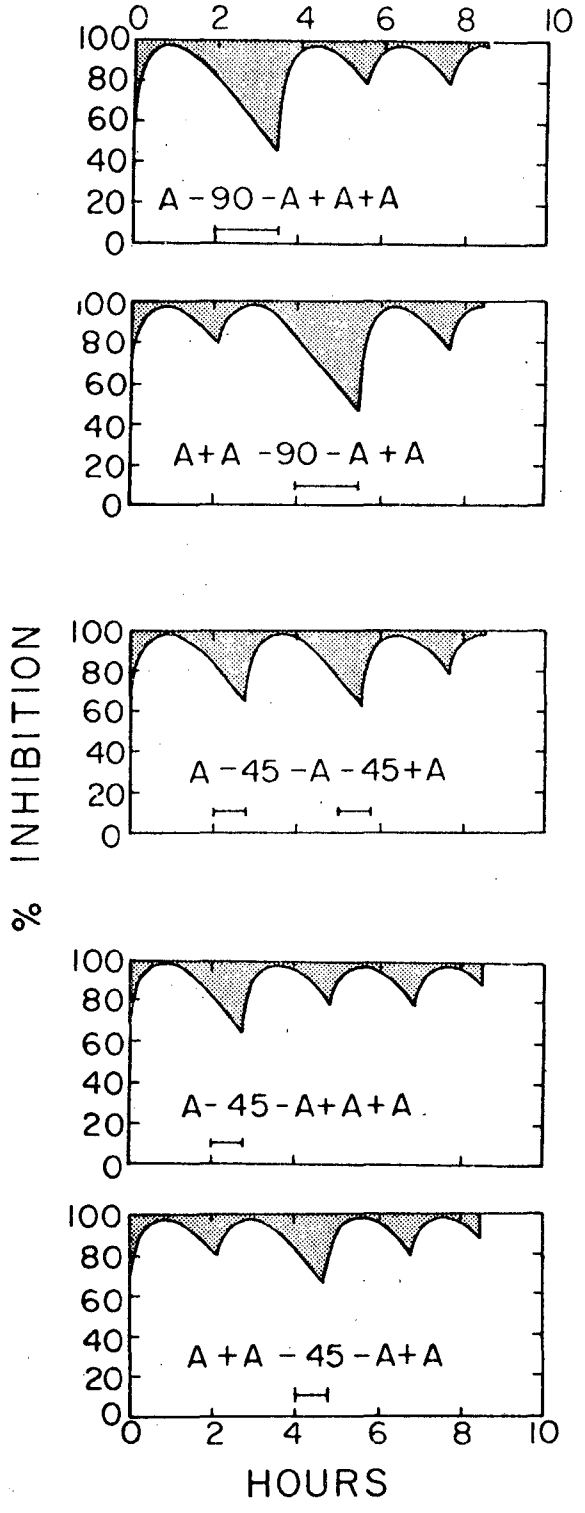

INJECTION SCHEDULE
% AMNESIA
PERCENT INHIBITION
100
50
0
Ani + Ani + Ani
60
(+40min)
60
(+60min)
45
(+70min)
25
(+90 min)
15
Ani + Ani
10
T
0
2
4
6
8
HOURS
XBL 739-4047

Fig. 1



PROTEIN SYNTHESIS (min.)	% AMNESIA
67	28
59	76
48	72

XBL738-3991

Fig. 2

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