Memory Impairment after Subcutaneous Injection of Acetoxycycloheximide

Abstract. *Subcutaneous injection of 240 micrograms of acetoxycycloheximide in mice rapidly produces marked inhibition of cerebral protein synthesis. Treated mice were trained to escape shock by choosing the lighted limb of a T-maze. When trained five or more minutes after injection, they had a normal capacity to learn. They remembered normally 3 hours after training, but 6 hours after training they had markedly impaired retention. Amnesia persisted thereafter. Injections immediately after training had a less marked but significant amnesic effect. These studies suggest that protein synthesis is not necessary for learning or for memory for 3 hours after training but that it is required for long-term memory. The protein synthesis which appears to be necessary for long-term memory occurs during training, or within minutes after training, or both.*

Intracerebral (*1*, *2*) or intracranial (*3*) administration of inhibitors of protein synthesis interferes with the establishment of long-term memory. Subcutaneous injections of these drugs were avoided in these studies because administration of puromycin by this route is relatively ineffective in inhibiting cerebral protein synthesis (*4*). Intracerebral administration is certainly advantageous since it permits marked inhibition of cerebral protein synthesis with a relatively small dose and therefore minimizes systemic toxicity. However, it has several limitations including the production of increased intracranial pressure, the formation of small brain lesions at the sites of injection, and the introduction of very high concentrations of the drug at these sites. Because of these limitations, we studied the effects of subcutaneous administration of large doses of acetoxycycloheximide.

Male Swiss albino mice (*5*) were injected subcutaneously on the back with 240 μg of acetoxycycloheximide (*6*) in 40 μl of 0.15*M* NaCl before or after being trained to escape shock by choosing the lighted limb of a T-maze (*2*, *7*) to a criterion of five out of six consecutive correct responses. The incorporation of valine-1-C^{14} into cerebral protein was inhibited by approximately 90 percent within 10 to 15 minutes after administration of the drug (Table 1). The onset of generalized inhibition was far more rapid than it was after intracerebral injections (*1*) since the subcutaneous route obviates the need for lengthy diffusion from localized intracerebral sites of application. The mice developed diarrhea 3 to 4 hours after administration of the drug; this condition persisted for a number of hours. However, for at least the first 2 hours after injection, they were not distinguishable from mice injected with saline. Seven percent of the mice injected with this dose died within 24 hours. The survivors showed no evidence of illness 2 to 3 days later. Because of the rapid onset of inhibition of cerebral protein synthesis, it was possible to train the mice shortly after injection when they appeared completely well and to test them when they had completely recovered from the drug.

Mice injected with acetoxycycloheximide before being trained learned as well as uninjected mice or those injected with saline. The average time of training was 8 minutes. All groups required an average of approximately 13 trials to reach criterion (acetoxycycloheximide, 12.9; saline, 13.1), and the acquisition curves of these two groups could be superimposed, as had been found in studies after intracerebral injections (*1*, *2*). Mice which had been injected intracerebrally 5 hours before training had required approximately 15 trials to reach the same criterion in this task (*2*). Mice injected with saline had an average of 77 percent savings when tested 7 days after training (Fig. 1). Mice injected with acetoxycycloheximide 5 minutes to 5 hours before training had approximately 35 percent savings when tested 7 days later (Fig. 1). When being retrained, more than a third of the mice in these groups required at least as many trials to reach criterion as they required in initial training, whereas all mice injected with saline had some savings. Since all mice which required at least as many trials in retraining as in initial training were scored as having 0 savings (*1*), the savings shown for the mice injected with acetoxycycloheximide may be considered to be an overestimate. When negative savings were included in the calculation, the mice injected 5 minutes or

Table 1. Inhibition of cerebral protein synthesis at different times after injection of acetoxycycloheximide. Mice were injected subcutaneously with 240 μg of acetoxycycloheximide and, at the time indicated, with 5 μc of valine-l-C^{14} (New England Nuclear Corp.). They were decapitated either 5 or 40 minutes after injection with valine as indicated. The cerebral hemispheres were removed and homogenized, and samples were precipitated with trichloroacetic acid (TCA), washed, and counted (*1*). Each determination was made on four mice, and the average is shown. The degree of inhibition indicated for the mice killed 40 minutes after injection of ^{14}C-valine was calculated as described by Flexner *et al.* (*9*) but was found, in this case, to be an overestimate (*10*).

Time ^{14}C-valine given (after drug)	Activity (cpm/mg of protein)		Inhibition (%)
	TCA precipitate	TCA supernatant	
Mice killed 5 minutes after receiving ^{14}C-valine			
No drug	18.4	272	
0	8.2	238	49
5 min	4.4	246	74
10 min	2.1	298	90
Mice killed 40 minutes after receiving ^{14}C-valine			
No drug	241	378	
0	21	699	95
1 hr	11	741	98
3 hr	18	535	95
5 hr	35	907	94
8 hr	76	845	86
24 hr	404	546	

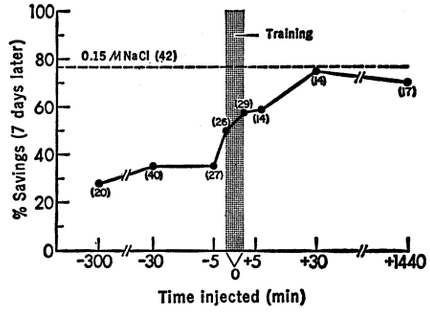

Fig. 1. Effect on memory of subcutaneous administration of acetoxycycloheximide at times before or after training. Mice were injected subcutaneously with 240 μg of acetoxycycloheximide at the indicated time relative to training. Training, designated as 0, took an average of 8 minutes. The mice were tested for retention 7 days after training. Percentage of savings was calculated as described previously (*1*). The numbers in parentheses are the numbers of mice in each group. The 42 controls were injected with saline either 30 minutes before, 5 minutes before, or 5 minutes after training. These groups all had identical memory and were indistinguishable from uninjected mice. The mice injected with acetoxycycloheximide before or within 5 minutes after training all had significantly less savings ($P < .05$, or less, as determined with the Mann-Whitney U test) than the saline controls. The mice injected five or more minutes before training had significantly less savings than those injected immediately after training. Injections of acetoxycycloheximide 30 minutes or more after training had no significant effect on memory.

more before training had an average of approxmiately 20 percent savings, whereas the savings calculated for the saline group was unaffected. Injections immediately before training had a significant effect on memory, but this was less prominent than when injections were given 5 minutes or more before training. Injections given immediately after or 5 minutes after training produced a slight but significant reduction of subsequent savings, whereas injections given 30 minutes or more after training had no effect (Fig. 1).

The time when amnesia first became apparent was determined by testing mice 3 or 6 hours after training. Mice injected 30 minutes before training had normal retention 3 hours after training but markedly impaired retention 6 hours after training (Table 2). Similar results were found in previous experiments in which acetoxycycloheximide was given intracerebrally (*1*, *2*). It seemed possible that the impaired savings observed 6 hours after training was due to the illness which the mice manifested 6½ hours after the drug was given. To evaluate this, we injected mice 30 minutes after training and tested them 6½ hours later. Their savings were not significantly different from those of the controls (Table 2). Therefore, inhibition of cerebral protein synthesis during training or within a short period of time thereafter is correlated with the amnesic effect of acetoxycycloheximide, whereas the subsequent illness which the drug produces is not. Mice injected immediately after training and tested 6½ hours later had slightly impaired retention (Table 2), as was the case with similarly treated mice which were tested 7 days after injection (Fig. 1), long after illness had subsided.

These studies confirm our previous findings (*1*, *2*) that learning, and memory for 3 hours after learning, are normal in mice whose cerebral protein synthesis is markedly inhibited at the time of training and for hours thereafter. As in the previous studies (*1*, *2*), impaired savings was found 6 hours after training and subsequently. Similar impairment was found 6 weeks later in both studies (*1*). Therefore, it appears that protein synthesis is required for long-term memory (somewhat more than 3 hours after training, in this situation). Since diisopropylfluorophosphate and electroconvulsive shock, which may have no significant effect on cerebral protein synthesis, may also impair memory after a latent period (*8*), it remains possible that the acetoxycycloheximide is affecting memory by some unknown mechanism.

Our experiments demonstrate that the amnesic effect of acetoxycycloheximide is not related to intracerebral administration of the drug or to inhibition of cerebral protein synthesis for hours before training. They also indicate that some of the protein synthesis apparently required for long-term memory storage occurs within minutes after training since injection of acetoxycycloheximide immediately or 5 minutes after training produces a relatively slight but significant impairment. The ineffectiveness of intracerebral injections of acetoxycycloheximide immediately after training (*1*) is apparently due to the fact that generalized inhibition of cerebral protein synthesis is not achieved until hours after injection by this route (*1*), and to the fact that the protein synthesis apparently required for memory has already been completed by this time. Since there was a lag of 10 or 15 minutes between subcutaneous administration of the drug and very marked inhibition of cerebral protein synthesis, it is possible that a considerable portion of the protein synthesis apparently required for long-term memory occurs within minutes after training although some probably also occurs during the 8 minutes of training. Marked impairment of memory has been observed in the goldfish when acetoxycycloheximide was administered intracranially immediately after training (*3*), which was considerably longer than that in our experiments.

Although the protein synthesis which appears to be required for memory occurs during or within minutes after training (or both), inhibition of protein synthesis during training does not impair memory for at least 3 hours thereafter. This indicates, as suggested previously (*1*, *2*), that a different process is utilized for memory storage during this period and that the absence of a long-term process, which is apparently dependent on cerebral protein synthesis, does not become manifest until the short-term process has decayed sufficiently.

Table 2. Relationship of time of injection and time of testing to the effect of acetoxycycloheximide. Mice were injected subcutaneously with 240 μg of acetoxycycloheximide at the indicated times before training (−) or after training was completed (+). They were tested at the indicated times after training. Each group treated with acetoxycycloheximide was compared with the saline-injected group tested at the same time or approximately the same time after training. *P* values were calculated by means of the Mann-Whitney U test. Numbers in parentheses are numbers of mice in each group; NS, not significant.

Time injected (min)	Time tested (hr)	Savings (%)	*P*
	0.15M NaCl		
−30	3	79 (10)	
−30	6	77 (10)	
	Acetoxycycloheximide		
−30	3	76 (10)	NS
−30	6	29 (20)	<.01
Immediately after	6½	58 (22)	<.05
+30	7	67 (13)	NS

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References and Notes

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5. Mice were obtained from Charles River Laboratories, Inc. They weighed 27 to 32 g at the time of training.
6. Acetoxycycloheximide was the gift of Dr. T. J. McBride, John L. Smith Memorial for Cancer Research, Chas. Pfizer and Co., Maywood, N.J. Its production was supported by NIH contract PH-43-62-50.
7. The mice showed a slight initial preference for the dark limb. In several experiments mice treated with acetoxycycloheximide were trained to choose either the initially preferred or the initially nonpreferred limb. In all cases a marked impairment of memory was found 7 days later.
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10. In calculating the degree of inhibition of protein synthesis, we assumed that the amount of TCA-soluble radioactive material (TCA supernatant) at the time of death reflects the specific activity of ^{14}C-valine in the cerebrum from the time of administration of radioactive amino acid (*7*). In the present studies, the amount of radioactivity in the TCA supernatant and precipitate was determined a number of times between 5 and 40 minutes after injections of ^{14}C-valine which were made at several times after the acetoxycycloheximide had been administered. We found that for 5 to 10 minutes after injection, the TCA-soluble radioactive material in acetoxycycloheximide- and saline-injected mice did not differ markedly but that it became substantially greater in the acetoxycycloheximide-injected mice 10 to 20 minutes after injection and remained at about this level at 40 minutes. Therefore, the TCA-soluble radioactive material in the cerebrum at 40 minutes overestimates the specific activity of ^{14}C-valine in the brain throughout the 40-minute period. The average increment of TCA-soluble radioactive material in the acetoxycycloheximide-injected mice compared to that in the controls is only about 60 to 80 percent as great as that suggested by the 40-minute point. The conventional method for calculating percentage inhibition was used here to make the results comparable with previous work and also because, when inhibition is in the range of 90 percent, the degree of overestimation is relatively small.
11. Supported by PHS grant MH 12773 and career development award K3-MH-18232 (S.H.B.). H.D.C. is an interdisciplinary fellow supported by PHS grant ID-MH-6418. This is communication No. 108 from the Joan and Lester Avnet Institute of Molecular Biology.

13 March 1968