*DELAYED AND SUSTAINED EFFECT OF ACETOXYCYCLOHEXIMIDE ON MEMORY IN MICE**

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It has been suggested that memory may be stored in the nervous system by the synthesis of protein which alters the functional relationship between neurons. This hypothesis has been tested by studying the effect of inhibitors of protein or RNA synthesis on learning and memory. The rationale for this approach and some of its limitations have been evaluated previously.[1, 2]

Puromycin, cycloheximide, and acetoxycycloheximide, all potent inhibitors of cerebral protein synthesis, have been used in this work. Puromycin has been shown to impair memory in mice[3, 4] and goldfish.[5] However, we have recently found that puromycin produces marked abnormalities of cerebral electrical activity, whereas cycloheximide does not.[6] This profound disturbance of brain physiology may be responsible for the effect of puromycin on memory. Therefore, experiments with this inhibitor cannot be interpreted as providing reliable support for the participation of protein synthesis in the memory storage process. Since cycloheximide and acetoxycycloheximide have no apparent effect on general cerebral function, we concluded that interpretable results could be obtained only with these inhibitors.

It has been reported that acetoxycycloheximide-injected goldfish have impaired memory of an avoidance response when tested three days after training.[5] This work supports the hypothesis that protein synthesis is required for memory storage. The studies which have thus far been reported of the effects of acetoxycycloheximide and cycloheximide on memory in mice have produced results which are either equivocal or negative. Flexner *et al.*[7] found that administration of large doses of acetoxycycloheximide produces a transient impairment of performance in mice which were trained to avoid shock in a maze. However, the mice showed signs of severe illness after this large dose of inhibitor was given. Since memory was clearly normal soon after evidence of illness subsided, it is possible that the inhibitor impaired avoidance behavior by making the mice ill rather than by interfering with the synthesis of protein required for memory storage. Certainly a permanent effect on memory was not observed. Our studies[8] of the effects of cycloheximide and acetoxycycloheximide in mice provided no evidence for the requirement of protein synthesis for memory of an escape response in a maze.

In reviewing the studies of the effects of these inhibitors in mice, it was recognized that because of convention, prolonged training was given in both our studies[8] and those of Flexner *et al.*[7] despite the fact that mice learn the solution to a simple maze in a small number of trials. In these studies, the mice were required to make 9 out of 10 consecutive correct responses before training was terminated; they therefore repeated the correct performance of the task many times after they had learned the correct solution. It seemed that this repetition might obscure the effect of inhibition of protein synthesis on memory, particularly if some cerebral protein synthesis escaped inhibition.

The present experiments were designed to evaluate the effects of inhibition of cerebral protein synthesis on memory in mice which received only brief training. The data suggest that cerebral protein synthesis is not required for learning or "short-term" memory but may be necessary for "long-term" memory storage.

Materials and Methods.—Adult male Swiss Albino mice (30–40 gm, Charles River Breeding Co.) were injected intracerebrally in each "temporal" site[3] with 10 μl of 0.15 *M* NaCl containing 0–60 μg of acetoxycycloheximide, as described previously,[4] except that only ether anesthesia was used. Injections were usually made 5 hr before training except as indicated in the text.

Cerebral protein synthesis was estimated at various times after administration of acetoxycycloheximide by a modification of the method of Flexner *et al.*[9] Mice were injected subcutaneously with 2 μc of valine-1-C^{14} (New England Nuclear Corp.) and sacrificed 40 min later. The whole cerebrum was removed, homogenized in 5 ml of 0.1 *N* NaOH, and 3 vol of cold 12% trichloroacetic acid (TCA) were added to aliquots of the homogenate. The TCA precipitate was washed twice with cold 10% TCA, drained, dissolved in 1 ml of 0.1 *N* NaOH, and counted in 10 ml of Bray's solution.[10] Aliquots (0.5 ml) of the TCA supernatant were added to 10 ml of Bray's solution and counted. It has been shown[9, 11] that counts in the TCA-soluble fraction are proportional to the specific activity of cerebral valine and the amount of valine incorporated into cerebral protein was calculated in terms of this. The degree of inhibition produced by acetoxycycloheximide was estimated by comparison with the average incorporation in 10 uninjected mice. Results from studies of the brains of 3–6 mice injected with acetoxycycloheximide were averaged for each determination. There was very little variability in these results.

Mice were trained to escape shock by choosing the left limb of a T-maze as described previously.[4] Either prolonged or brief training was given. Prolonged training was continued until the mouse made 9 out of 10 consecutive correct responses. Brief training was continued until the mouse made 3 out of 4 consecutive correct responses.

Memory was estimated for each mouse at one time after training by determining the number of trials required to reach criterion at that time. Per cent savings is: $[(I - C) - (R - C)/I - C)] \times 100$ where I = total number of trials required to reach criterion on initial training; R = total number of trials required to reach criterion on retraining; C = criterion (3 or 9). Occasionally, mice trained to the criterion of 3 out of 4 consecutive correct responses reached criterion in initial training in only 3 trials. If they reached criterion in 3 trials on retesting they were considered to have 100% savings. There was considerable variability in the savings of mice which received brief training. Therefore, fairly large numbers of animals were used to ensure reliable results. Statistical comparisons were made with t tests and, in some cases, with Mann-Whitney U tests, using significance levels of $P < 0.05$.

Results.—The mice tolerated the intracerebral injections extremely well. Both saline-injected and acetoxycycloheximide-injected animals recovered rapidly from ether anesthesia and were fairly alert within 1 hour after injection. Although diarrhea developed within 3–4 hours of injection of 120 μg of acetoxycycloheximide, this was rarely observed with doses of 20 μg or less.

Acetoxycycloheximide was found to be a highly potent inhibitor of cerebral protein synthesis over a wide concentration range (Fig. 1), and for a substantial period after injection (Fig. 2).

Mice injected with 20 or 120 μg of acetoxycycloheximide 5 hours before training learned the T-maze in the same number of trials as mice injected with saline. When trained to a criterion of 9 out of 10 consecutive correct responses (prolonged training), a total of approximately 16 trials were required to reach criterion. When trained to a criterion of 3 out of 4 consecutive correct responses (brief training), an average of only 6 trials was needed (acetoxycycloheximide: 6.0; NaCl: 6.2).

The effect of acetoxycycloheximide on memory at a number of times after brief training is shown in Figure 2. Injection of 20 μg of acetoxycycloheximide 5 hours

before brief training had no effect on retention 3 hours after training (Fig. 2). Six hours after training retention was, however, markedly impaired. This impairment was sustained over the ensuing week (Fig. 2). Furthermore, 20 acetoxycycloheximide-injected mice tested 6 weeks after training had an average of 27 per cent savings, whereas 15 saline-injected mice had 73 per cent savings at that time.

The effect of a number of other doses of acetoxycycloheximide on cerebral protein synthesis and on memory after brief training is shown in Figure 1. Memory was markedly impaired only by doses which inhibit more than 90 per cent of cerebral protein synthesis.

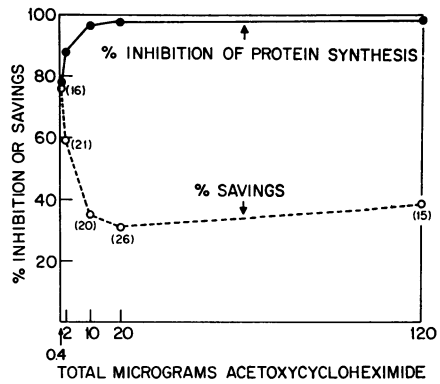

FIG. 1.—Relationship of dose of intracerebral acetoxycycloheximide to inhibition of brain protein synthesis and retention. The indicated doses were administered intracerebrally 5 hr before training in a total volume of 20 μl of 0.15 N NaCl. Inhibition of cerebral protein synthesis was determined in 3–6 mice 5 hr after injection. Mice were trained to a 3 out of 4 criterion. Retention was tested 24 hr after training.

Having established that memory after brief training was markedly impaired by acetoxycycloheximide, we then studied its effects on memory after more prolonged training. In these experiments the largest tolerable dose, 120 μg, was administered. The effect of administration of this dose 5 hours before prolonged training on memory at a number of times after training is shown in Figure 3. Slight impairment of performance was observed 1 day after prolonged training but this is believed to be due to illness which the mice manifested at this time. When illness had subsided,

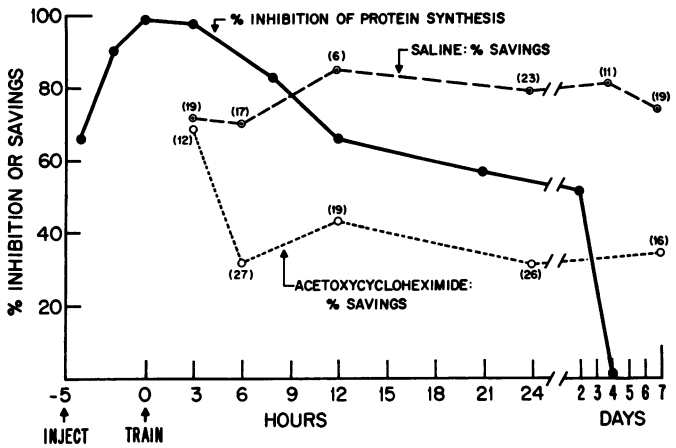

FIG. 2.—Effect of 20 μg of acetoxycycloheximide on cerebral protein synthesis and retention. Inhibition of protein synthesis was determined in 3–6 mice at the indicated times. Inhibition at the time of training was 97%. Training was to a criterion of 3 out of 4 consecutive correct responses. Comparisons of retention in acetoxycycloheximide- and saline-treated mice by the t test: 3 hr, not significant; 6 hr, $P < 0.005$; 12 hr, $P < 0.05$; 24 hr, $P < 0.001$; 7 days, $P < 0.001$.

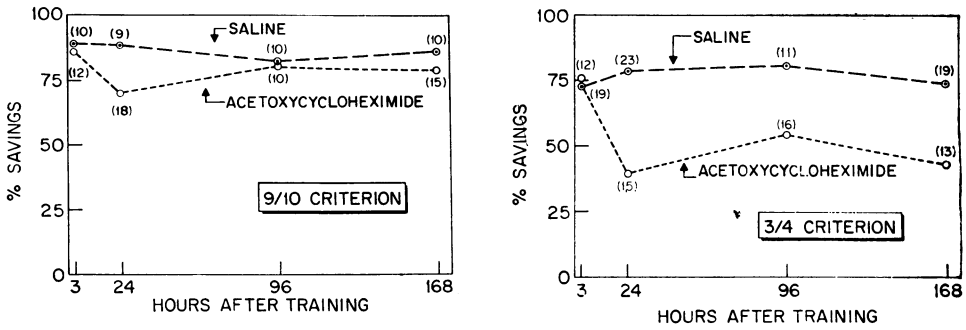

FIG. 3.—Retention after training to a 9 out of 10 or 3 out of 4 criterion. Mice were trained to the indicated criterion 5 hr after injection of either saline alone or with a total of 120 μg of acetoxycycloheximide and tested at the indicated times after training. The number of mice in each group is indicated in parentheses. Acetoxycycloheximide-treated mice did not differ significantly (t test) from saline-treated mice at any time after training when the criterion was 9 out of 10. For 3 out of 4 criterion: 3 hr, not significant; 24 hr, $P < 0.01$; 4 days, $P < 0.1$, > 0.05; 7 days, $P < 0.02$.

4 and 7 days after training, memory was essentially normal. In contrast, sustained impairment after brief training was again observed. It should also be noted that memory 3 hours after brief training was again found to be retained despite administration of this very large dose of acetoxycycloheximide. Therefore, memory 3 hours after brief training as well as at all times after prolonged training appears to be refractory to the largest tolerable dose of the drug.

Acetoxycycloheximide produces systemic illness, particularly in large doses. To evaluate the relationship of systemic illness to the amnesic effect of the drug, the effect of subcutaneously administered acetoxycycloheximide on memory after brief training was studied. After administration by this route, one would expect at least as much systemic effect but less cerebral effect than after intracerebral administration of the same dose. Subcutaneous administration of 20 μg of acetoxycycloheximide inhibited 79 per cent of cerebral protein synthesis, measured 5 hours after injection. Mice trained at this time to a criterion of 3 out of 4 consecutive correct responses learned normally. When tested 24 hours later they had 82 per cent savings, whereas mice injected subcutaneously with saline had 76 per cent savings (17 mice in each group). Therefore, the systemic toxicity which acetoxycycloheximide produced is not responsible for the amnesic effect which we observed after intracerebral administration of this dose.

Having established that the cerebral action of acetoxycycloheximide is responsible for its amnesic effect, we attempted to evaluate the possibility that this may be due to a general cerebral abnormality produced by inhibition of cerebral protein synthesis for 5 hours before training, rather than to interference with the synthesis of protein specifically required for memory storage. To test this, we trained mice at a time when cerebral protein synthesis had been inhibited for a long time but was returning to normal. The brains of these mice would be expected to manifest many of the general abnormalities which might be produced by sustained inhibition of cerebral protein synthesis; but inhibition would no longer be extensive enough to prevent the synthesis of sufficient protein to store memory, if indeed this occurs. We trained mice 18 hours after intracerebral injection of 20 μg of acetoxycyclo-

heximide. As shown in Figure 2, this dose produced extensive inhibition for 18 hours, yet, at this time, inhibition has been reduced to a level which was not correlated (Fig. 1) with impairment of memory. We found that mice treated in this manner learned normally and had normal memory 24 hours later (Table 1). Therefore, the impairment of memory by acetoxycycloheximide appears to be correlated with very extensive inhibition of cerebral protein synthesis at the time of training and, perhaps, in the ensuing few hours.

Since the amnesic effect of acetoxycycloheximide does not become manifest until some time between 3 and 6 hours after training, we determined whether injection of the drug immediately after training had any effect. Injection of 20 μg immediately after training had only a slight (not statistically significant) effect when the mice were tested 24 hours later (Table 1). Injection of 120 μg immediately after training had no effect (Table 1) when the mice were tested 4 days later to allow for recovery from the toxic effects of this large dose of drug. Therefore, administration of the drug prior to training is necessary for demonstration of its amnesic effect. This is in contrast with the finding that acetoxycycloheximide impairs memory in goldfish[5] if given immediately after training. It should be noted that maximal inhibition does not develop until some time between 3 and 5 hours after injection of the drug (Fig. 2). This suggests that if protein synthesis is indeed required for memory storage, such protein has probably been synthesized before the drug has diffused sufficiently to interfere. Establishment of maximal inhibition at the time of training or shortly thereafter is apparently of great importance in demonstrating the effect of acetoxycycloheximide on memory in the situation which we studied.

It has been shown that if animals are trained while under the influence of a depressant drug, retention is most apparent when they are tested while under the influence of this drug and may be somewhat impaired when they are tested in its absence.[12] It seemed possible that our mice which were trained while acetoxycycloheximide was present in the brain in high concentration and tested after this drug was eliminated might be performing poorly because of this process of "state-dependent learning." To evaluate this, 17 mice were trained 5 hours after intracerebral injection of 20 μg of acetoxycycloheximide. Four days later they were

TABLE 1

EFFECT OF INTRACEREBRAL INJECTION OF ACETOXYCYCLOHEXIMIDE AT TIMES BEFORE AND AFTER TRAINING ON RETENTION

Time injected	Injected (μg)	Time tested	Per cent savings
18 hr before	0	24 hr	79 (10)
	20		71 (14)
5 hr before	0	24 hr	79 (23)
	20		31 (26)
Immediately after	0	24 hr	82 (13)
	20	24 hr	61 (22)
	120	4 days	74 (12)
4 days after	0	24 hr	78 (11)
	20		73 (23)

Mice were injected at the indicated times relative to training, and tested at the indicated times after training to a criterion of 3 out of 4 correct responses. The number of mice in each group is indicated in parentheses. Statistical comparisons of the acetoxycycloheximide-injected mice with appropriate saline controls showed significant differences only in the group injected 5 hr before training.

reinjected intracerebrally with the same dose and tested for retention 5 hours thereafter. These mice had an average of 23 per cent savings. Therefore, the impairment of performance observed in mice trained while under the influence of acetoxycycloheximide is not due to "state-dependent learning" but rather to some effect of the drug on the memory storage process.

We have previously studied the effects of actinomycin D, a potent inhibitor of DNA-dependent RNA synthesis on learning and memory,[13, 14] to evaluate the possible role of RNA synthesis, in memory storage. These studies were limited by the fact that doses of actinomycin which inhibit 95 per cent of cerebral RNA synthesis produce profound and irreversible toxicity within 10 hours of injection, and death within 24 hours. Therefore, we could study the effect of this dose of the drug with some confidence only within 4 hours of training and could study later effects only after administration of a smaller dose. Actinomycin D produced no effect on learning or memory under the conditions in which it was studied. Because the acetoxycycloheximide effect on memory was only found after brief training, it seemed possible that an effect of actinomycin D could be found after brief training. Although we used brief training in our studies of the effects of actinomycin on passive-avoidance training,[13] prolonged training was used in our studies of the effects of this drug on learning and memory of a maze.[14] To further evaluate the effects of actinomycin D, 21 mice were injected in the temporal region with a total dose of 20 μg. This inhibits approximately 75 per cent of whole brain RNA synthesis[13] but the degree of inhibition near the injection site may be substantially higher. Such mice are extremely weak and sluggish 12–24 hours after injection and die within 48–72 hours. They were trained to a criterion of 3 out of 4 correct responses, 5 hours after injection. When tested 24 hours later they had an average of 70 per cent savings despite obvious signs of illness. Therefore, we again could not demonstrate a requirement for RNA synthesis for memory storage. However, a definitive experiment with more extensive inhibition was again not possible because of the profound toxicity of the drug.

Discussion.—The present experiments support our previous finding[3, 8] that learning and "short-term" memory may occur normally despite almost total inhibition of cerebral protein synthesis. Since the acetoxycycloheximide-injected mice learn in the same number of trials and remember as well as saline-injected mice 3 hours after training, the sustained abnormality first observed at 6 hours is apparently due to impairment of a discrete "long-term" memory storage process.

The effect of acetoxycycloheximide on "long-term" memory can only be demonstrated under certain conditions. It is not readily apparent with doses of acetoxycycloheximide which inhibit less than 95 per cent of cerebral protein synthesis. It is only observed after brief training and is almost completely obscured after prolonged training. It is probably not due to systemic illness produced by the drug, nor does it seem to be due to a "state-dependent" learning process. Rather, the data suggest that it may be due to interference with the synthesis of protein which is specificially needed to produce prolonged facilitation of the neuronal relationships which mediate "long-term" memory storage. However, the possibility that this drug is impairing cerebral function in a less specific manner has certainly not been excluded.

The most prominent findings of this study are the delayed effect of acetoxycy-

cloheximide on memory and the requirement for both very extensive inhibition of protein synthesis and brief training to demonstrate this effect. Both the long delay and the relative insensitivity which were found might appear puzzling if "long-term" memory is truly dependent on the synthesis of protein. Although these findings may provoke some reservations in drawing conclusions from these experiments, analysis of the phenomena of "consolidation" and "redundancy" suggests that the data may well be consistent with the requirement for newly synthesized protein for "long-term" memory storage.

1. *The phenomenon of "consolidation":* It is generally believed that memory storage may be mediated by two processes, one for "short-term" memory and another for "long-term" memory. Evidence for this comes largely from experiments on the effect of electroconvulsive shock on memory.[15] If seizures are produced shortly after training, amnesia is subsequently found, whereas if they are produced long after training, memory is not affected. At the time when memory is no longer affected by electroconvulsive shock, it is said to have been "consolidated." This time varies with the task studied and with the method of production of the seizure. It has been found to vary from seconds to a number of hours. We have observed that administration of electroconvulsive shock 15 minutes after training has no effect on memory of the task which we studied. This suggests that "consolidation" has occurred during this interval. We were, therefore, initially puzzled when we found that although retention was normal in acetoxycycloheximide-treated mice 3 hours after training, it became markedly impaired subsequently. If "consolidation" had already occurred 15 minutes after training, why was no abnormality of memory observed at 3 hours? This question was based on the assumption that when the "long-term" process has been established by the process called "consolidation," the "short-term" process disappears simultaneously. Indeed, in the descriptive jargon used in such discussions, it is generally stated that the "short-term" process is *converted* to the "long-term" process. On more careful analysis of the phenomena which are observed, it was recognized that this formulation could well be incorrect. Resistance to electroconvulsive shock may indeed indicate that the "long-term" process, which is not disruptable by this treatment, has been established. It need not, however, imply that the "short-term" process has subsided. Rather, it is quite possible that the "short-term" and "long-term" processes may exist simultaneously for some time until the former disappears. This interpretation is suggested by our findings. In the situation which we studied, the "long-term" process has apparently been established within 15 minutes of training. Therefore, electroconvulsive shock administered at this time does not have an amnesic effect. Yet the "short-term" process apparently persists for more than 3 and less than 6 hours. In mice treated with acetoxycycloheximide the impairment of the "long-term" process becomes apparent only at 6 hours after training. Amnesia is said to have developed. In reality, "long-term" amnesia may have been present already for a considerable period of time but may have been masked by the persistence of the "short-term" process. Such an early onset of the "long-term" process, suggested by the early resistance of electroconvulsive shock, is consistent with our finding that inhibition of cerebral protein synthesis must be established at the time of training and that administration of acetoxycycloheximide immediately after training has little or no effect.

2. *The phenomenon of redundancy:* It is well known that functionally identical information may be stored at multiple sites in the nervous system so that the system can function extremely well despite extensive ablation. This property of the nervous system is referred to as "redundancy." Storage of information about maze learning in the mouse may be particularly redundant, since one of his major *modus operandi* is finding his way through tunnels and alleys. Therefore, if one interprets the findings with acetoxycycloheximide to mean that protein synthesis is required for "long-term" memory storage, one could explain the relative inefficacy of doses which inhibit less than 90–95 per cent of cerebral protein synthesis by postulating that the residual protein synthesis is sufficient to store the information. Likewise, a small amount of intact protein-synthesizing capacity might be sufficient to mediate memory storage if prolonged repetition rather than brief training is given. Only slight sparing of a critical process might permit memory of the solution of a maze to which the nervous system of the mouse seems particularly well adapted.

The simplicity of performing maze-learning experiments in the mouse has been a major reason for studying this task. But the adaptation of this species to learn such a task makes demonstration of the amnesic effects of drugs difficult and suggests that considerable additional information will be obtained when different training procedures are used.

Summary.—Mice were trained to escape shock in a T-maze 5 hours after intracerebral injection of acetoxycycloheximide in doses which produce more than 95 per cent inhibition of cerebral protein synthesis. If brief training was given, learning and memory 3 hours after learning were normal; but 6 hours after training a marked impairment of memory was observed which persisted thereafter.

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