Table 1. Average scores of mice injected subcutaneously with pentylenetetrazol (50 mg/kg) at various time intervals after intracerebral injections. Mice were observed for 5 minutes after injection of pentylenetetrazol and scored for overt seizure activity by an observer who had no knowledge of the nature of the material that was injected intracerebrally. The scoring system used was: 0, no seizure activity; 1, occasional twitching of body or jerky elevation of tail; 2, frequent twitching of body or jerky elevation of tail; 3, prominent seizure which involved only a portion of the body; 4, generalized major seizure. Numbers in parentheses refer to the number of mice in each group. Statistical comparisons were made with the Mann-Whitney U test. The mice injected with puromycin differed significantly from every other group at the .02 level of significance or better.

Substance injected intracerebrally	Total dose (μg)	Time after injections		
		1 hour	5 hours	11 hours
Puromycin	200	2.6 (19)	2.5 (22)	
Saline	0	0.4 (15)	0.9 (10)	
Acetoxycycloheximide	20	0.4 (15)	0.6 (10)	0.9 (11)
Puromycin and cycloheximide	200 of each	0.7 (15)	1.6 (19)	
Hydrolyzed puromycin	200		1.5 (20)	

cin into a growing polypeptide chain (*4*). Since cycloheximide might block the formation of peptidyl-puromycin (*5*), this may be the mechanism by which it antagonizes the effect of puromycin on seizure susceptibility and memory.

When pentylenetetrazol was administered 24 hours after intracerebral injection, all mice were extremely prone to develop seizures, and there was no significant difference found between puromycin-, acetoxycycloheximide-, or saline-injected animals. This delayed change in irritability could be due to the beginning of scar formation in the injection tract.

Since it seemed likely that the puromycin effect on memory 3 hours after training was related to the abnormalities of electrical activity which it produced, the effect of the anticonvulsant agent diphenylhydantoin on memory in puromycin-injected animals was studied. Mice were injected intracerebrally with a total of 200 μg of puromycin. Immediately thereafter they were injected intraperitoneally with diphenylhydantoin (35 mg/kg) or with saline. Five hours after injection they were trained to choose the correct limb of a T-maze to a criterion of three out of four consecutive correct responses (*6*). Three hours after training they were tested for retention (*6*). Thirty-one mice injected with diphenylhydantoin had an average of 57 percent savings, whereas 19 puromycin-injected mice that did not receive the anticonvulsant had an average of 24 percent savings. Diphenylhydantoin treatment significantly improved retention ($P < .02$, Mann-Whitney U test). This confirms our impression that the amnesia noted in puromycin-injected animals 3 hours after training may be related to seizure activity.

We have recently found that mice injected intracerebrally with acetoxycycloheximide learned a one-choice maze normally, to a criterion of three out of four consecutive correct responses, and remembered normally 3 hours after injection, but had markedly impaired savings 6 hours after injection and thereafter (*6*). Because of the finding that the puromycin effect on memory was related to abnormalities in electrical activity we have evaluated the possibility that acetoxycycloheximide might also be acting in this manner. As shown in Table 1, acetoxycycloheximide-injected mice are no more susceptible to pentylenetetrazol-induced seizures than are saline-injected mice. This was true not only 5 hours after intracerebral injection, the time when the mice were usually trained, but also 11 hours after injection, a time when acetoxycycloheximide-injected mice have forgotten what they learned 6 hours previously (*6*). The effect of diphenylhydantoin on acetoxycycloheximide-induced amnesia was also determined. Eleven mice were injected with diphenylhydantoin immediately after intracerebral injection of 20 μg of acetoxycycloheximide. They were trained 5 hours later and tested 6 hours after training. They had an average of 27 percent savings 6 hours after training. Therefore, diphenylhydantoin does not apparently prevent the acetoxycycloheximide-induced amnesia.

We conclude from these experiments that, because of its effect on cerebral electrical activity, puromycin is not a useful drug for studying the hypothesis that memory storage is based on the synthesis of protein which facilitates synaptic connections, and that the mechanism of the amnesic effect of puromycin in our experiments (*1, 2*) and those of others (*7*) is uncertain. However, on the basis of the present evidence, acetoxycycloheximide does not produce general cerebral abnormalities, and it remains possible that it exerts its effect on memory (*6*) by preventing the synthesis of protein which facilitates synaptic connections.

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Amnesic Effects of Small Bilateral Brain Puncture in the Mouse

Abstract. *A small acute brain puncture produced retrograde amnesia in a passive avoidance learning situation in mice. If injury to the hippocampus was inflicted either immediately, 1 hour after the learning, or 1 hour before the learning, the animals showed a retention deficit; the degree of this deficit was related to the time interval. No effect of this injury was observed on retest performance when the animals were treated as long as 6 hours before or after the learning trial.*

Retrograde amnesia has been noted in animals after treatments such as electroconvulsive shock (ECS), anesthesia, spreading cortical depression (*1*), and intracerebral injection of certain antibiotics (*2*). However, brain injury or ablation alone has never hitherto been shown to produce a memory loss that depended on the retention interval. Simply inserting a needle or just touching the brain may sometimes produce profound changes in function (*3*). In view of the fact that many investigators

are now injecting substances directly into the brain, we wanted to determine whether simple needle puncture alone might have any amnestic effects in a passive avoidance situation. Brain lesions produced by needle puncture are usually so small that their possible influence upon the animal's behavior may not be revealed by insensitive behavioral procedures such as active avoidance or simple mazes. Lashley's law of mass action required fairly extensive brain damage before performance in a maze was affected (*4*). We wanted to see whether there is some temporal relation between production of these acute lesions and learning, and to investigate whether this brain injury could have some proactive effects on the behavior of untrained animals.

Female albino mice, CF1 (18 to 20 g), were used (*5*). The mice were lightly anesthetized with ether 1 or 2 days before the learning trial, and their scalps were incised and retracted so that bregma and sutures were easily visualized.

A step-through apparatus described by Kopp *et al.* (*6*) was used in our study. The animal was placed on the outer illuminated smaller compartment facing the doorway, and the timer was started. When the mouse stepped into the larger darkened compartment and reached the third pair of plates, the timer stopped, and the mouse received a punishing shock (800 volts a-c through 2 megohms in series causing approximately 320 μa root mean square $\pm$ 20 percent to flow through the animal either for 0.8 second or permanently). The animals received 1 to 3 shocks, and sometimes more, in this situation. After this procedure, the mice were put back into their home cages or exposed to treatment.

Table 1. Effect on retest latencies of acute cerebral puncture performed immediately after learning trial (one punishing shock). There is no significant difference between needle and saline treatments. Numbers in parentheses in first column represent numbers in treatment groups.

Treatment	Trial 2, median step-through latency (sec)	Inter-quartile range (sec)	Significance as opposed to control group
None (32)	189.1	80.0–278.0	
Needle only (32)	68.6	40.9–140.3	$P < 0.005$
Needle plus saline* (25)	86.2	34.6–117.2	$P < 0.005$

* One microliter was injected into each hemisphere.

All injections were done freehand in unanesthetized mice. A 26-gauge needle, shortened to the length of 2.0 mm by a metal stopper, was inserted through the skull successively into both hemispheres in the region above the hippocampus (2.0 mm caudally and 2.0 mm laterally from the bregma) (*7*) at different time intervals before (6 hours or 1 hour) and after (immediately or 1, 6, or 18 hours) the learning trial. A 50-μl Hamilton syringe also was used to inject 1 μl of saline immediately, 1, or 6 hours after the learning trial. We incised the skulls of the control animals and handled them in the same way as the experimental group, except that the needle was not inserted through the exposed skull.

Retention was tested 24 hours after the learning trial by placing each mouse on the outer platform and recording the time required to step into the dark compartment. The upper cutoff point was arbitrarily set at 300 seconds. After the completion of retest, the animals were killed by decapitation, and their heads were fixed in 10 percent formalin. Gross evaluation of the needle tract indicated that the needle penetrated the hippocampus. Figure 1 shows a section of the brain in which trypan blue was injected for better visualization of the needle tracts.

Table 1 gives data on the effects of needle insertion or saline injection, or both, immediately after learning, into both hippocampal regions on performance of a step-through retest with only a single shock. Both treated groups are significantly lower than controls without brain injections, and they do not differ from one another (*8*).

Therefore, we tried to increase the negative reinforcement of the learning task to see whether the effect of brain puncture might be less pronounced. The animals were exposed to possible shock for 180 seconds (Fig. 2). Animals treated with a needle 16 hours before or after the learning trial were not influenced by this treatment; their median scores were the same as the control values. However, mice injured by the needle 1 hour before the learning trial and either immediately or 1 hour after the trial showed a significant decrease in their retest step-through latencies. Mice punctured by the needle 18 hours after the learning trial and 6 hours before retest did not differ from controls. When the animals were injected with physiological saline in each hemisphere immediately and 1 hour after the learning trial, they showed significant impairment of their retest latencies, whereas a 6-hour group did not differ significantly from the control group.

Table 2 presents proactive disinhibitory effects of this type of acute injury

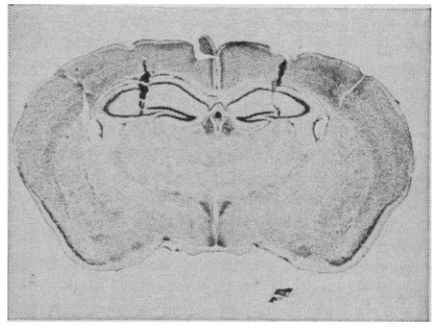

Fig. 1. Section of the brain showing needle tracts penetrating the hippocampus.

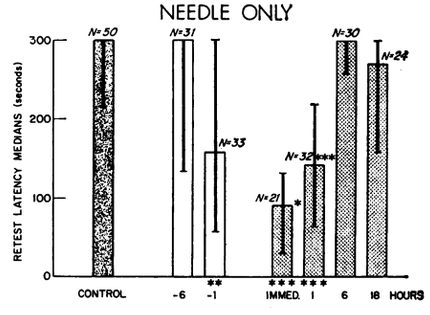

Fig. 2. Effect of acute cerebral puncture performed at different intervals before and after a learning trial on retest latencies. The statistical significance of the difference between each column and the control is indicated under the column. The significance of the difference between two adjacent columns is indicated between the columns as follows: $*P < 0.05$, $** P < 0.01$, $*** P < 0.001$. Animals are given possible shocks for 3 minutes during the training session. The black bars represent the semi-interquartile range (25th to 50th percentile scores).

Table 2. Effect on initial latencies of acute cerebral puncture performed at different intervals before step-through training. Mutual significance of difference between 1-hour and 6-hour groups: $P < 0.005$. Numbers in parentheses in first column represent numbers in treatment groups.

Treatment	Median initial step-through latency (sec)	Inter-quartile range (sec)	Significance as opposed to control group
None (50)	5.9	2.5–14.0	
Needle 1 hour before training (31)	2.8	1.6– 4.9	$P < 0.005$
Needle 6 hours before training (33)	1.2	0.8– 3.4	$P < 0.01$

to the brain on step-through latencies of untrained mice on day 1. This puncture decreased the initial median step-through latencies in both time intervals used (6 and 1 hours before the trial). The decrease in both cases is significant.

Our study showed that a small brain injury in the region of the hippocampus can interfere with a recently learned simple task. However, this effect depends upon proximity in time to the learning trial; groups treated 6 or 18 hours after the original learning trial showed no impairment. When the temporal gradient obtained in this experiment is compared to that obtained when ECS was used to disturb the memory trace (*6*), it is obvious that the brain injury induced by needle is less effective than that induced by ECS. The whole curve is steeper, and the starting median latency is also higher. Needle puncture was most effective in interfering when performed either immediately after or before learning. Extending the time interval in either direction lessened its influence.

We cannot attribute this effect solely to injury of the hippocampus in view of the fact that adjacent neocortical structure was necessarily damaged too. Neocortical spreading depression has been shown to be an effective method of producing retrograde amnesia (*9*). However, some investigators have shown with different behavioral tests that neocortical influences in most cases are less pronounced than those from the limbic system (*10*). Gardner (*11*) reported that KCl injected through a cannula into the hippocampus immediately after each learning trial of an avoidance response disrupted consolidation of that response. However, KCl injected into the amygdala or septum was without an effect on the same task. Our objective at this point was to demonstrate a temporal gradient resembling that obtained with ECS to reveal a possible common mechanism.

The initial latencies of the group with acute brain injury inflicted 6 hours before their first exposure to the step-through situation were still significantly different from controls (Table 2), whereas the effect of the same injury on their learning ability (measured by their retest latencies) was not pronounced (Fig. 2). The amount of proactive effect of the needle inserted into the hippocampal region 1 hour before the learning trial was approximately the same as that of the corresponding retroactive effect of this treatment.

Apparently, acute brain injury in the region of the hippocampus will produce retrograde amnesia, impair learning, or disinhibit motor activity in a passive avoidance test, depending on the time of the injury.

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Sleep: Effects on Incorporation of Inorganic Phosphate into Brain Fractions

Abstract. *During sleep there is a two- to threefold increase in the incorporation of inorganic orthophosphate-^{32}P into a chemical fraction of the brain of the 20-day-old rat. This increase is not in the lipids or nucleic acids, but is associated with an acid-labile phosphate entity of the tissue residue after extraction of these fractions and phosphorus-containing substances of low molecular weight.*

Little is known about the biochemistry of the nervous system during sleep. Chemical analyses of brain and cerebrospinal fluid from sleeping and waking animals have so far failed to differentiate between the two states (*1*). Tracer techniques have not been exploited to any degree to study the effect of sleep upon brain metabolism (*2*). We report a marked (two- to threefold) increase in the incorporation of inorganic orthophospate into a chemical fraction of whole brain of immature rats during sleep.

Littermates, 20 days old, were kept awake for 90 minutes and then injected with orthophosphate-^{32}P in the tail vein (4 μc per gram of body weight). The litters were then divided into two groups of equal number. One group (S) was placed in quiet cages where the animals fell asleep spontaneously in 2 to 3 minutes. The other group (W) was kept awake by gentle manual stimulation. At the end of 30 minutes the rats were decapitated, a blood sample was taken from the neck, and each brain was removed and homogenized in chloroform-methanol (2 : 1) within 2 minutes post mortem.

The brains were separated into three main chemical fractions: (I) the chloroform-methanol extract, (II) the hot trichloroacetic acid extract, and (III) the sodium hydroxide digest of the tissue residue. The chloroform-methanol extraction followed standard methods for lipid extraction (*3*). The residue was washed three times at room temperature with 95 percent ethanol, once with water at 4°C, and three times with 2.5 percent trichloroacetic acid at 4°C; it was then extracted with 5 percent trichloroacetic acid at 90°C for 30 minutes (*4*). The residue was then washed three times at room temperature with 95 percent ethanol, once with water, and then digested in 0.5*N* NaOH at 37°C for 24 hours. The volumes were 5 ml throughout. The specific activity of each fraction was obtained by standard methods of scintillation counting (5) and by assay of total phosphorus (*6*). Optical density at 260 μ was used to measure nucleic acids in fraction II, and total protein was assayed (*7*) in fraction III. For purposes of comparison, the data from each animal were adjusted to a standard blood level of radioactivity of 1×10^5 count/min per milliliter of blood.

Figure 1 presents the results of a series of seven experiments involving a total of 70 animals. Ten additional experiments slightly modified for a different purpose, including 100 animals, have given essentially the same results. Fraction II, the hot trichloroacetic acid extract, showed a two and a half fold increase in the specific activity of phosphorus during sleep as compared to waking ($P < 0.001$). The specific activities of fraction I and fraction III