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α-Amanitin Inhibition of Mouse Brain Form II Ribonucleic Acid Polymerase and Passive Avoidance Retention

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SUMMARY

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Injection of α-amanitin into a cerebral ventricle reduced the ability of male mice to retain a passive avoidance response without affecting spontaneous locomotor activity or performance of a previously learned task. α-Amanitin inhibited the brain form II DNA-dependent RNA polymerase in a dose-dependent manner up to 10 μg, at which dose a maximum of 98% inhibition was observed as determined by assay of brain nuclei at the time of training. The effect observed on passive avoidance retention is only seen at maximal (98%) inhibition. Furthermore, the inhibition of brain form II polymerase is transient, indicating that α-amanitin is effective *in vivo* only when virtually 100% inhibition of this enzyme is attained. The liver form II polymerase was also inhibited after cerebroventricular injection, indicating that a significant amount of the α-amanitin reached peripheral circulation. A 50% inhibition of liver form II polymerase was measured within 15 min. However, intraperitoneal injection of 10 μg of α-amanitin did not produce significant inhibition of brain form II polymerase, and retention of a passive avoidance response was not affected.

INTRODUCTION

Drugs and chemicals known to alter RNA and protein synthesis have been administered to animals in attempts to correlate these processes with memory formation. These studies have recently been reviewed (1–4). If the molecular basis of memory consolidation involves the synthesis of specific proteins (or peptides), the first step in this process is synthesis of specific messenger RNA. Recently α-amanitin has been described as a specific inhibitor of the form II DNA-dependent RNA polymerase (EC 2.7.7.6) in eukaryotes (5–7), and four reports suggest that α-amanitin inhibits mRNA synthesis in different organisms *in vivo* (8–11). Since there is no apparent crossover of the form I (nucleolar) polymerase to transcribe the genome of the form II polymerase, the compound therefore becomes specific for the inhibition of new mRNA synthesis.

This paper describes the cerebroventricular injection of α-amanitin to inhibit the brain form II RNA polymerase *in vivo* and

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its effect on the retention of passive avoidance. The results suggest that inhibition of the form II RNA polymerase interferes with the retention of passive avoidance.

METHODS

Animal training procedure. Animals employed were adult male mice (55–70 days of age) of the HaM/ICR strain, obtained from Charles River Laboratories. Mice were housed four per cage for at least 1 week prior to use. The training apparatus was a covered Plexiglas box (30.5 × 20.5 × 22.2 cm) with an electrifiable grid floor. At an appropriate time after injection of α-amanitin or 0.15 M NaCl, a mouse was placed on a platform (8.5 × 11.5 cm) 5.3 cm above the grid floor. When the animal stepped off the platform onto the grid, a 5-sec scrambled shock (0.8 mamp) was delivered. The time required for the mouse to step off the platform was recorded as "training latency." The animal was removed from the test chamber after the shock and returned to its cage. Animals stepping off in less than 5 sec or after 20 min were excluded from the experiment. Mice which escaped the full shock period by returning to the platform were not significantly different on retest from those which received the full 5-sec shock. Animals were retested 4 or 6 hr after the original training. The time required for the step-off response in the second test session was recorded as the "retest latency." Animals not responding within 20 min were given the maximum retest latency score of 1200 sec. Each animal's training latency served as the control for its retest latency, and Student's *t* value was calculated by paired comparisons. In one experiment the ratio of retest to training latency served as a measure of learning.

Locomotor activity. Spontaneous locomotor activity was measured in doughnut-shaped cages, which were 30.5 cm in diameter, with a 7.5-cm-wide circular runway (12). As a mouse traversed the runway, four equally spaced floor panels were alternately depressed, thereby activating microswitches connected to electromechanical counters. The animals were placed one to a cage at various times after cerebroventricular treatment with 0.15 M NaCl or α-amanitin. Spontaneous locomotor activity was monitored at successive 15-min intervals for 2 hr by photographing the counters every 15 min automatically.

Cerebroventricular injections. Freehand cerebroventricular injections of α-amanitin or 0.15 M NaCl were made in the left cerebral ventricle of conscious mice as described by Haley and McCormick (13). India ink was injected in preliminary experiments to verify the site of injection. α-Amanitin was dissolved in 0.15 M NaCl at a concentration of 1 mg/ml. Injection volumes were 10 μl unless otherwise specified.

Isolation of mouse brain and liver nuclei. Brain nuclei were isolated by a modified procedure of Blobel and Potter (14). Tissue was homogenized in 1 volume (w/v) of 0.25 M sucrose in 10 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$, pH 7.5, and diluted with 2 volumes of 2.3 M sucrose in the same buffer. Homogenates were underlaid with a 1.9 M sucrose–buffer cushion and centrifuged for 20 min at 70,000 × *g* (25,000 rpm) in a Beckman SW 50.1 rotor. Liver nuclei were isolated as described above, except that a 2.3 M sucrose–buffer underlay was used. Nuclear pellets were resuspended in 1 ml of 1 M sucrose, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 10 mM Tris-HCl, pH 7.5.

RNA polymerase assay in isolated brain and liver nuclei. The standard reaction mixture for form II RNA polymerase activity contained, in a final volume of 125 μl, 7 μmoles of Tris-HCl (pH 7.9), 0.75 μmole of NaF, 0.4 μmole of dithiothreitol, 0.075 μmole each of GTP, CTP, and ATP, 0.0125 μmole of unlabeled UTP (all nucleotides purchased from P-L Biochemicals), 1 μCi of [5-^{3}H]UTP (22.2 Ci/mmole; New England Nuclear), 0.2 μmole of $MnCl_2$, 28.8 μmoles of $(NH_4)_2SO_4$, and 50 μl of the respective nuclear suspension. Control assays were performed under the same conditions, except that 0.1 μg of α-amanitin was included in the individual assays. Nucleolar form I activity was determined under identical conditions, except that all assays contained 0.1 μg of α-amanitin and 6.26 μmoles of $(NH_4)_2SO_4$. All reactions (in triplicate) were incubated for 5 min at 30°. Under these conditions the incorporation was linear with

respect to time. Reactions were stopped by pipetting 100-μl aliquots onto Whatman DE-81 filter paper discs (2.3 cm in diameter). The discs were then washed and counted as described by Lindell *et al.* (5). Form II polymerase activity was determined by subtracting the assays with α-amanitin from those without α-amanitin. DNA was determined by the method of Burton (15) on triplicate 50-μl aliquots of each nuclear sample. RNA polymerase activities are expressed as picomoles of UMP incorporated per minute per milligram of DNA.

Isolation of labeled polysomes and labeled polysomal RNA. Mice, 16/group, received 10 μg of α-amanitin cerebroventricularly. At 2 and 12 hr, they were given 10 μCi (10 μl) of [5,6-^{3}H]uridine (40.4 Ci/mmole; New England Nuclear) cerebroventricularly and killed 30 min later. Controls received labeled uridine alone. Polysomes were isolated and suspended as described by Weiss *et al.* (16), except that the homogenization solution contained 0.5% 20-cetyl ether (Brij 58, Sigma) as suggested by Uphouse *et al.* (17). The yield of ribosomes was 2–3 mg as measured by the absorbance at 260 nm, based on 1 mg of polyribosomes per milliliter = 18 A_{260} units.

Polyribosomes were incubated in 0.5% sodium dodecyl sulfate with Pronase (10 μg/ml, Calbiochem) that had been digested for 30 min at 37°, and RNA was extracted twice with equal volumes of phenol saturated with 0.15 M NaCl and 0.015 M sodium citrate. RNA was precipitated by adding 2 volumes of 95% ethanol to the aqueous phase, containing 0.1 M NaCl. Samples were left overnight at −20° and centrifuged at 16,300 × *g* (10,000 rpm) in a Sorvall HB-4 rotor. RNA precipitates were dissolved in 0.05 M Tris-HCl–0.1 M NaCl, pH 7.5, and reprecipitated twice more at −20°. RNA samples were dissolved in 100 μl of 0.05 M Tris-HCl–0.1 M NaCl, pH 7.5, and 5-μl samples were counted in a Nuclear-Chicago Isocap/300 for 100 min in 10 ml of Aquasol (New England Nuclear) and corrected for the background of Aquasol alone.

Brain perfusion and washing. Animals received 10 μg of α-amanitin in 10 μl of 0.15 M NaCl and 2 hr later were anesthetized with ether. When perfusion *in situ* was performed, the abdomen was opened and the descending aorta was clamped, a cut was made in the right ventricle, and 30 ml of 0.15 M NaCl were injected into the left ventricle. Washing involved removal of the brain from the skull, splitting the brain open to expose the ventricular space, and washing the ventricular space and the surface of the cortex with NaCl from a wash bottle. Some brains were minced with a razor blade (approximately 3 mm^3) after perfusion, and the mince was washed with NaCl from a wash bottle. Nuclei were then isolated as described above. Data represent an average of four brains as a percentage of control form II polymerase ± the standard error of the mean.

RESULTS AND DISCUSSION

The time between the original punished response and the retest is an important factor in determining the response latency in the second trial. Retest latencies of control mice were significantly longer than the training latencies when training-retest intervals were 0.5, 1, 2, 4, 8, or 24 hr (Fig. 1). The training-retest interval of 4 hr resulted in the greatest statistical significance in increase of retest

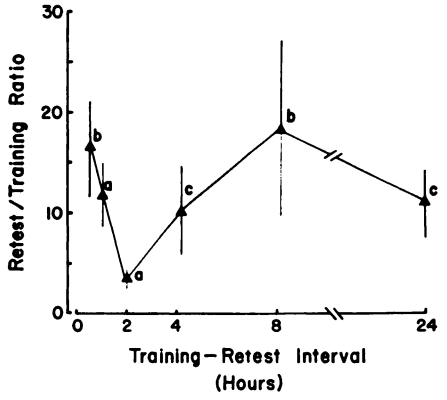

FIGURE 1. *Passive avoidance retention of uninjected mice as a function of time between training and retest trials*

Each point represents the mean ± standard error of the individual retest-to-training ratio of the platform step-off latencies for groups of 12 mice. The significance of the increases of the retest latencies over the training latencies was calculated by paired comparisons: *a*, $p < 0.05$; *b*, $p < 0.01$; *c*, $p < 0.001$.

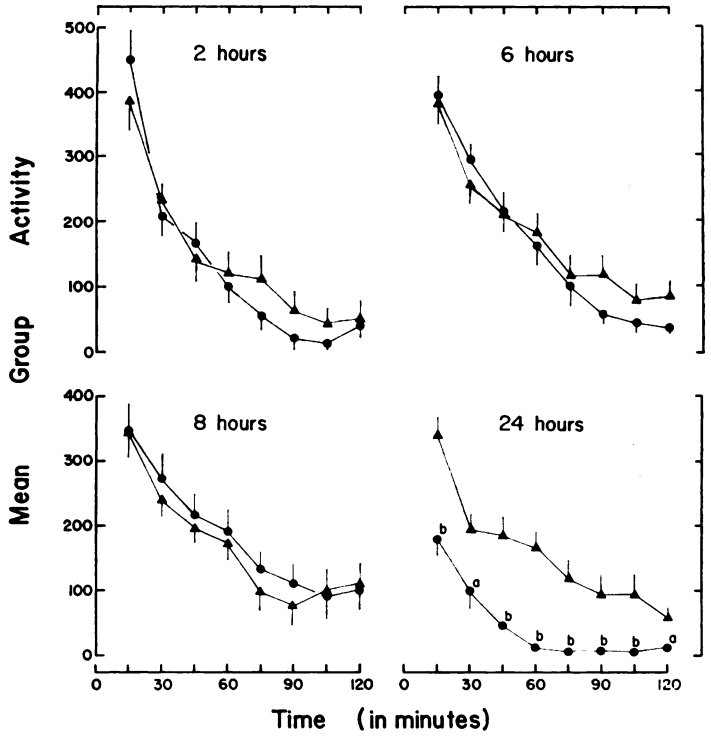

FIG. 2. *Effects of α-amanitin on spontaneous locomotor activity of mice*
Animals received a cerebroventricular injection of 0.15 *M* NaCl (▲) or α-amanitin, 10 μg (●), at the times indicated, before being placed in the activity chamber. Probabilities of points being significantly different from scores of NaCl-treated subjects: *a*, $p < 0.05$; *b*, $p < 0.01$.

latency ($p < 0.001$). This observation agreed with the results of Kamin (18), who suggested that 4 hr are required for long-term memory formation. We therefore chose 4 hr as the training-retest interval in all our experiments except one, in which the training-retest interval was 6 hr. Longer training-retest intervals were not explored in the present study for several reasons. First, the variability of the data appeared to increase when the interval was greater than 4 hr (Fig. 1). Second, 24 hr after α-amanitin some mice showed signs of toxicity such as ptosis, slight piloerection, and a decrease in spontaneous motor activity (Fig. 2). Finally, all doses of α-amanitin (0.05–10.0 μg cerebroventricularly) employed in this study were lethal to mice within 48 hr. When spontaneous locomotor activity was measured immediately after treatment with α-amanitin, it was observed that 0.5, 1.0, 5, and 10 μg significantly depressed motor activity for the first 45 min of observation (19). Therefore a minimum period of 2 hr after cerebroventricular injection was always employed to avoid early effects of α-amanitin on spontaneous motor activity, and all experiments were completed within 8 hr, during which time no overt signs of toxicity were observed.

Inhibition of whole brain form II RNA polymerase was dependent on the dose of α-amanitin. As seen in Fig. 3, cerebroventricular injection of α-amanitin at various concentrations caused significant inhibition of brain form II polymerase as determined by assay of brain nuclei isolated from mice 2 hr after administration of the inhibitor. The two highest doses employed (5 and 10 μg) inhibited the enzyme activity 92.7% and 98.5%, respectively. Assays of liver nuclei isolated from the same animals indicated that the percentage inhibition of liver form II polymerase activity was some-

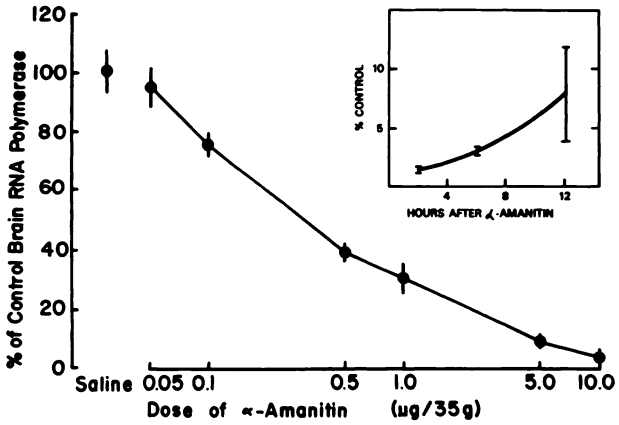

FIG. 3. *Dose-dependent inhibition of brain form II RNA polymerase by α-amanitin injected into left cerebral ventricle*

Mice (three per point) received various concentrations of α-amanitin in 5 µl and were killed 2 hr later. Brain nuclei were isolated and assayed as described in METHODS. Control values were 396 ± 34 pmoles of UMP incorporated per minute per milligram of DNA. α-Amanitin inhibition of form II polymerase at 0.5 and 1.0 µg was significant at $p < 0.001$. Inset: Effect of a 10-µg cerebroventricular dose of α-amanitin. The ordinate is the same as the larger graph: percentage of control brain RNA polymerase.

what less than brain polymerase inhibition at each α-amanitin concentration (5 µg, 62%; 10 µg, 95%). A cerebroventricular dose of 10 µg of α-amanitin rapidly reaches the periphery with an approximate half-time of 15 min, as measured by inhibition of liver form II RNA polymerase at various times (Fig. 4). Liver form II polymerase activity was inhibited more than 90% within 1 hr after injection of 10 µg of α-amanitin.

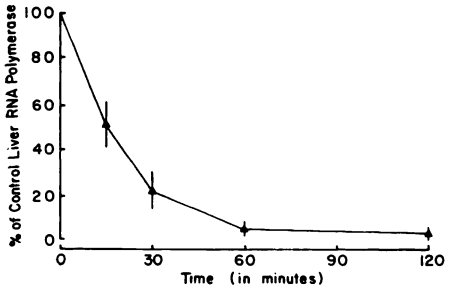

FIG. 4. *Degree of inhibition of liver RNA polymerase II after cerebroventricular administration of α-amanitin*

α-Amanitin (10 µg) was injected into the left cerebral ventricle of mice (two per point), and the animals were killed at the times indicated. Liver nuclei were isolated and assayed as described in METHODS. Control form II polymerase activity was 120 ± 8 pmoles of UMP incorporated per minute per milligram of DNA.

TABLE 1

Specific activity of mouse brain RNA

Each value was derived from 5-µl aliquots of the pooled sample of 16 mouse brains.

Prior treatment	Corrected activity[a]	A_{260}	Specific activity[b]	Control
	cpm			%
Control	47.09	0.054	872	100
2-hr α-Amanitin	3.78	0.285	13.26	1.52
12-hr α-Amanitin	7.46	0.045	165.8	19.0

[a] Corrected for a background of 26.38 cpm. All samples were counted for 100 min.

[b] Specific activity is expressed as the counts per minute per unit of absorbance at 260 nm.

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Since maximum inhibition of brain form II polymerase *in vivo* was produced by 10 µg of α-amanitin, we employed this dose in all animals except where indicated. One-tenth this dose (1 µg) produced 98% inhibition of form II polymerase when added to individual mouse brain homogenates. This observation suggests that low concentrations of α-amanitin in the ventricular space may produce this inhibition only after homogenization. We therefore employed a number of conditions to test this possibility.

Perfusion *in situ*, washing, perfusion and washing, and perfusion, mincing, and washing (in that order) gave 1.5 ± 0.9%, 3.2 ± 0.7%, 4.2 ± 2.4%, and 3.4 ± 1.1% of control form II polymerase activity, respectively. There is no significant difference between any of these treatments and the untreated control given α-amanitin (2.4 ± 1.1%), and we conclude that the inhibition observed (Fig. 3) represents the actual degree of inhibition of the form II polymerase *in vivo*.

We also isolated RNA from brain polysomes after treatment with 10 μg of α-amanitin 2 and 12 hr prior to a 30-min pulse with labeled uridine to attempt to correlate the percentage inhibition of newly synthesized RNA with the degree of form II polymerase inhibition. The specific activities of RNA isolated at these times appear in Table 1. These values are in good agreement with the inset data of Fig. 3, indicating again that brain form II RNA polymerase is inhibited *in vivo*.

When the training-retest interval was 4 hr, significant increases in retest latencies occurred in both NaCl- and α-amanitin-treated animals (Table 2) regardless of the preliminary treatment time (2 or 8 hr) or the route of injection (cerebroventricular or intraperitoneal). However, the increase in retest latency of the group which received α-amanitin 2 hr prior to training was significantly less than in NaCl-treated controls ($p < 0.05$). There is a correlation of the degree of inhibition of brain form II polymerase and an impaired passive avoidance response. In one experiment the training-retest interval was 6 hr and the latency of the retest of α-amanitin-treated mice was not significantly longer than the training latency (Table 2).

Effects of α-amanitin on the retention of a passive avoidance task appear only at the highest dose employed and can be shown only when the brain form II polymerase is more than 98% inhibited. Form II polymerase was inhibited 93% at 5 μg, and passive avoidance was not significantly impaired (Table 2). Likewise, 96% inhibition of brain form II polymerase, 8 hr after α-amanitin, did not significantly impair retention (Table 2). These results are similar to those obtained using actinomycin D (20, 21), where doses which inhibited 80–95% of brain RNA synthesis (messenger and ribosomal)

TABLE 2

Effect of α-amanitin on passive avoidance retention

Each group contained twelve 35-g male mice. Injections were administered cerebroventricularly (c.v.) or intraperitoneally (i.p.).

Training-retest interval	Prior treatment time	Treatment	Training latency	Retest latency	p	Activity of form II polymerase at time of training
hr	*hr*		*sec ± SEM*	*sec ± SEM*		*% control*
4	2	None	156 ± 45	527 ± 132	<0.025	
	2	NaCl (c.v.)	185 ± 53	614 ± 135	<0.005	100.0 ± 7.1
	2	α-Amanitin (5 μg c.v.)	194 ± 48	515 ± 148	<0.025	7.3 ± 1.5
	2	α-Amanitin (10 μg c.v.)	189 ± 68	282 ± 49[a]	<0.05	1.5 ± 0.5
	8	NaCl (c.v.)	203 ± 43	550 ± 129	<0.005	ND[b]
	8	α-Amanitin (10 μg c.v.)	164 ± 43	395 ± 86	<0.001	4.3[c]
	2	NaCl (i.p.)	141 ± 37	487 ± 96	<0.005	100.0 ± 8.7
	2	α-Amanitin (10 μg i.p.)	113 ± 25	593 ± 135	<0.001	87.8 ± 9.6[d]
6	2	NaCl (c.v.)	181 ± 60	729 ± 123	<0.001	ND
	2	α-Amanitin	229 ± 61	395 ± 85[a]	NS	ND

[a] Significantly lower than NaCl treatment; $p < 0.05$ by group comparison.

[b] ND = not determined; NS = not significant.

[c] Estimated from the inset in Fig. 3.

[d] Determined after brain perfusion *in situ*.

produced no decrement in 4-hr memory in mice. It would appear that α-amanitin is effective only when there is maximal inhibition of brain form II polymerase.

The observed effect of α-amanitin on passive avoidance retention does not appear to be due to nonspecific behavioral depression. Cerebroventricular injection of NaCl or α-amanitin had no effect on spontaneous locomotor activity 2 hr after administration, at which time testing was begun (Fig. 2). Furthermore, spontaneous motor activity was not altered at either time of retesting, 6 or 8 hr after cerebroventricular injection (Fig. 2).

We also observed no effect of α-amanitin on the ability of mice to recall a previously learned task. Mice (12/group) received one-trial passive avoidance training 24 hr prior to retesting. Six hours prior to the 24-hr retest, the animals received either NaCl or α-amanitin or were left untreated. This preliminary treatment time corresponded to the time before retest when injections were made in the experiments shown in Table 2. Training and retest latencies were: no injection, 71 ± 20 and 497 ± 111 sec; NaCl, 162 ± 43 and 679 ± 134 sec; α-amanitin, 121 ± 33 and 505 ± 122 sec. There were no significant differences among the treatments, and all three groups had significantly longer retest than training latencies. Thus the formation but not the recall of long-term memory appears to require a functional form II polymerase in the brain.

Another interpretation of these results is that reduced mRNA synthesis may interfere with normal neuronal function. Only the synthesis of proteins with rapidly turning over mRNAs would be affected by α-amanitin inhibition of the form II polymerase within the time period employed. However, it has been shown that chronic inhibition of protein synthesis with cycloheximide has no significant effect on general brain function as measured by gross behavior and cerebral electrical activity (22, 23). We also observed no changes in whole brain dopamine, norepinephrine, or serotonin levels 6 or 24 hr after α-amanitin injection (data not shown).

Compounds which act to produce peripheral toxicity may also affect behavior. We do not feel that the peripheral toxicity of α-amanitin significantly affected the results, since intraperitoneal injection of α-amanitin did not impair retention of passive avoidance or produce significant (12%) inhibition of brain form II polymerase. Liver form II polymerase in these animals, was inhibited more than 95%. Therefore it appears that α-amanitin does not reach the brain after intraperitoneal injection.

Montanaro *et al.* (24) also reported no effect of an intraperitoneal dose (500 μg/kg) of α-amanitin on brain form II polymerase. Our results, with regard to the correlation of form II polymerase inhibition by α-amanitin and the retention of a learning task, are quite different from those of Montanaro *et al.* (24). We could only demonstrate a significant effect of α-amanitin on learning when more than 98% of the form II polymerase was inhibited. From their results [which compare favorably with our dose-inhibition curve (Fig. 3)], a dose of 0.25 μg produces 40% inhibition of whole brain form II polymerase and impairs learning. Our results are supported by a number of other studies which employed inhibitors of protein and RNA synthesis, in that extensive inhibition (greater than 90%) must be attained before behavioral effects are observed (1). We can only suggest that species differences or differences in training apparatus and procedures may be responsible for this discrepancy. For example, it is well known that rats are much more resistant to α-amanitin than mice (25, 26). We have not attempted to duplicate our results in rats. The inhibition we observed with 10 μg of α-amanitin was transient (inset, Fig. 3). Montanaro *et al.* (24) observed a similar effect after a dose of 0.25 μg/rat. This transient effect of α-amanitin *in vivo* was also observed by Tata *et al.* (27) in rat liver.

We have also shown that the same dose of α-amanitin (10 μg cerebroventricularly) affects the ability of mice to retain an active avoidance task. These studies were performed on a Sidman apparatus and have been published elsewhere (19).

This study indicates that α-amanitin inhibition of whole brain form II RNA po-

lymerase has a significant effect on the ability of mice to retain a passive avoidance task. Our results also reveal the critical nature of the degree of inhibition of mRNA synthesis necessary to begin to measure an effect on learning and memory. Mice receiving the highest dose employed (10 μg) still learned significantly, but retention was less than under any other condition of concentration or prior treatment time. Presumably, if 100% inhibition of the brain form II polymerase could be achieved, passive avoidance retention could be totally abolished.

Since α-amanitin has no effect on retention of previously learned behavior (or any other known biochemical effect), these data support the contention that there is a molecular basis for the formation of learning and memory involving the synthesis of new mRNA. α-Amanitin appears to be a more selective inhibitor for these studies than actinomycin D or cycloheximide, since there are fewer immediate toxic consequences than with the other inhibitors. Using α-amanitin as an inhibitor of form II polymerase *in vivo* permits the reduction of mRNA to levels where its availability influences translational expression.

The observation that virtually 100% inhibition of form II polymerase is required to demonstrate the role of mRNA *in vivo* is not limited to the present study. For example, to observe an effect on rapidly turning over rat liver proteins, it is also necessary to achieve this degree of inhibition. This inhibition is also of a transient nature.[2] These observations may limit the use of α-amanitin to systems where complete inhibition of the form II RNA polymerase can be attained. It is therefore necessary to assess the degree of inhibition by assay of the endogenous form II polymerase when α-amanitin inhibition of mRNA synthesis is desired. It is also important to consider the transient nature of inhibition of the form II polymerase *in vivo*. The reason for this time-dependent reversal of inhibition *in vivo* is not yet completely understood.

These results may also indicate that transcription of mRNA in brain is not rate-limiting within cells; rather, the number of cytoplasmic ribosomes or translation is limiting. If ribosomes are rate-limiting in brain tissue, one might expect to find a reduced rate of rRNA synthesis. We therefore measured the activity of the nucleolar form I polymerase (insensitive to α-amanitin inhibition) and found that the level of the form I polymerase activity in untreated animals was the lowest in any tissue yet examined. The activity of the form I polymerase (expressed as picomoles of UMP incorporated per minute per milligram of DNA) is 15.4 ± 7.4, while that of the form II polymerase is 396 ± 34. The ratio of polymerase forms I and II is 0.039, which is the lowest ratio we have seen in any tissue. The level of nucleolar RNA polymerase in a given tissue has been suggested to be indicative of tissue hypertrophy (28), where presumably the demand for rRNA (for new ribosomes) is of paramount importance for increased translation. Alternatively, this result could also suggest that brain ribosomes have a longer half-life than those of liver or other tissues characterized by higher ratios of form I to II RNA polymerase.

In conclusion, these studies lend more quantitative support to the molecular basis of learning and memory retention and the involvement of mRNA. They further reveal the potential of α-amanitin as a tool in the investigation of the role of mRNA in biological systems. The unique nature of the specific inhibition of only the form II DNA-dependent RNA polymerase by α-amanitin allows a more definitive statement of the role of the inhibition of mRNA in long-term memory formation.

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