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Cycloheximide Induced Amnesia and Recovery as A Function of Training Parameters

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QUINTON, E. *Cycloheximide induced amnesia and recovery as a function of training parameters.* PHARMAC. BIOCHEM. BEHAV. 2(2) 173–180, 1974. – Mice were given 2 or 3 training trials in a passive avoidance task following an injection of cycloheximide or saline. They were tested 1, 1.5, 3, 24, or 72 hr after training and tested again 72 hr after the first test trial. All the cycloheximide groups except the 1 hr groups were inferior to saline controls on the first test trial, and there was no suggestion of spontaneous recovery over the intervals tested. Test 2 performance was generally inferior to Test 1 performance for all groups, but the cycloheximide groups showed the greatest drop in performance. A second experiment extended train/test intervals to 144, 146, 148, and 192 hr. Spontaneous or test induced recovery again did not occur. The discussion attempts to reconcile these results with prior reports of recovery in terms of differential conditioning of different components of passive avoidance memory by the different training procedures. This results in partial sparing of some components of passive avoidance memory by cycloheximide, which has the appearance of recovery under certain test conditions.

Passive avoidance Cycloheximide Memory Specific avoidance response General fear response

SEVERAL studies have reported that amnesia develops over a period of time following training if the animal is treated before or shortly after training with sufficient antibiotic to inhibit cerebral protein synthesis 85% or more [2]. Typically, the animal shows no amnesia when tested shortly after training, but some amnesia is apparent 1.5 hours after passive avoidance (PA) training, and is well developed 5 or 6 hr after either PA or maze training [4,21]. The rate of development of amnesia is usually referred to as the amnesia gradient.

However, Quartermain and his co-workers did not find evidence of a cycloheximide (cyc) induced amnesia gradient following PA training in two recent studies [20,23]. They reported that the appearance of amnesia after training in cyc treated animals is dependent upon the intensity of the training trial foot shock. If the animal was given a "low" foot shock (0.16mA), amnesia was apparent when the animal was tested one minute after training and all subsequent test intervals. If it was given a "high" foot shock (1.6mA), a transient amnesia appeared 24 hr after training, but not at earlier or later test intervals. No amnesia gradient was observable after either training conditions [20]. This group has also reported that recovery from amnesia in the "low" shock group can be induced by giving the animals one or more test trials in the apparatus, or one test trial followed 1 hr later by a non-contingent "reminder" shock [19].

It is apparent from the performance of the saline injected animals in the Quartermain and McEwen [20] study that the "high" foot shock was a more effective conditioning stimulus than the "low" foot shock. It seems plausible to suggest that conditioning was stronger in the "high" foot shock group than in the "low" foot shock group, and therefore the "high" foot shock group may have been the PA equivalent of the "overtraining" groups in the early maze studies [3,6] where it was found that overtraining protected against the amnestic effects of the antibiotic.

The first experiment was intended to investigate the generality of the findings of the Quartermain group by increasing the number of training trials in a PA task as a means of increasing the strength of conditioning, rather than increasing the intensity of the training trial foot shock. The PA task used in this study reveals a well-defined time-gradient of amnesia and no spontaneous recovery of memory within 72 hr of training when cyc treated mice are given one training trial [21]. In the present study, mice were given two or three training trials and then tested at various intervals out to 72 hr after training to determine whether the additional training would eliminate the amnesia gradient and/or in-

duce spontaneous recovery. The mice were given a second test trial 72 hr after the first one to determine whether the first test trial had induced a recovery of memory.

EXPERIMENT 1

METHOD

Animals

The animals used in this study were male C57 BL/6j mice (Jackson Laboratories), 10–12 weeks old. They were housed 6 per cage, given food and water ad lib, and maintained on a 12 hour light cycle. They were adapted to the laboratory and vivarium for at least two weeks before being used in the experiment.

Apparatus

The step-through passive avoidance apparatus used in this task was basically similar to the ones described elsewhere [9,15]. It consisted of a panel (12 × 22 in.) to which a rectangular box (6 in. w × 9-1/2 in. d × 10 in. l) was attached to one side and a metal platform (2-1/2 in. l × 1-1/2 in. w) was attached to the other side. Between the box and the platform was a rectangular hole (1-1/2 × 2 in.) in the panel, the bottom of which was flush with the upper surface of the platform but 1/2 in. above the metal floor of the box. The platform was illuminated by a 7 W light bulb (with reflector) located 7 in. above the platform. The interior of the box was not illuminated. The platform and floor of the box were connected in series to a power supply set to deliver 2.0 mA A.C. constant current.

Biochemical Procedure

To estimate the degree of inhibition of protein synthesis at the time of training, 3 pairs of mice were injected subcutaneously with 33 μc of leucine–4, 5–3H (Schwartz/Mann, sp. act 65 c/m mole) 30 min after saline or cycloheximide injection, then briefly anesthetized 30 min later with CO_2 and decapitated. The midpoint of the incorporation interval was thus 45 min after the cyc/saline injection, the time at which behavioral training was begun.

Each cerebrum (whole brain minus olfactory lobes, cerebellum, and lower brain stem) was quickly removed and homogenized in 5 ml 0.1 N NaOH (4° C). A 1 ml aliquot of this homogenate was then added to 3 ml of 4° C 10% trichloroacetic acid (TCA), vortexed vigorously and placed in an ice bath for 1 hr. The precipitate was centrifuged and the supernatant decanted; then the precipitate was resuspended in 2 ml 10% TCA and heated at 90° C for 15 min, centrifuged, decanted, washed once more with 1 ml of 5% TCA, decanted, and the final pellet solubolized in 3.5 ml 0.1 N NaOH. The supernatants from the above procedure were pooled to estimate the free leucine.

Three aliquots of 100 μl each from both the TCA insoluble (protein) and TCA soluble (free leucine) fractions of each brain were added individually to vials containing 10 ml of a PPO-POPOP-Toluene-Triton scintillation cocktail and counted in a Beckman LS100 scintillation counter to 0.7% standard error. The CPMS were corrected for quench, averaged, and converted to DPM.

Behavioral Procedure

Injection. The mice were injected subcutaneously between the shoulders with a 0.4 ml of 0.15 M saline (sal) or 0.4 ml or a cycloheximide (actidione, Nutritional Biochemicals Corp.) solution (10 mg cyc/ml in 0.15 M saline) 45 min before training. The cyc solution was made shortly before use.

Training. A mouse was trained by placing it on the platform and permitting it to step spontaneously through the hole in the panel into the box. When the front feet of the mouse touched the floor of the box its rear feet were still on the platform. It thus completed the shock circuit, received the 2 mA shock foot shock and jumped further into the chamber. The duration of shock was very brief (less than 0.3 sec.) because the mouse received the shock only when it bridged the platform and floor of the chamber.

The mouse remained in the chamber for 10 sec and was then returned to the platform for the second training trial. If a mouse did not step into the chamber within 10 sec on the second training trial, it was gently pushed into the chamber where it remained for another 10 sec. The third training trial was a repetition of the second trial, and the mouse was returned to its cage after the final 10 sec detention in the chamber.

Test. On test trials the animal was placed on the platform and allowed to step spontaneously into the chamber (no foot shock on test trials). Step-through latency (STL) was measured as the time the animal took to enter into the chamber with all 4 feet. If it stepped into the chamber, it was immediately removed and returned to a holding cage. If it did not step into the chamber within 60 sec, it was removed from the platform and returned to its cage and was assigned a STL of 60 sec. One group of cyc injected and 1 group of saline injected mice were given the first test trial 1, 1.5, 3, 5, 14, or 72 hr after the training trial and the second test trial 72 hr after the first test trial (12 independent groups, N = 18/group).

Four additional control groups (N = 18/group) were later added to this experiment. All 4 groups were given 3 training trials but only 1 test trial. Three of the groups were given the test trial 24 hr after training and the other group was tested 72 hr after training. One of the 24 hr test groups was injected with cyc 1.75 hr before the test trial, another group was injected with cyc 5.75 hr before test trial, and the third group was injected with cyc 23 hr before the test trial. The group tested 72 hr after training was injected with cyc 1 hr after training.

RESULTS

The degree of inhibition of protein synthesis was determined by the following formulae:

$$\left[1 - \frac{X_c}{Y_c} \Big/ \frac{X_s}{Y_s}\right] \times 100$$

where X_c = TCA-insoluble radioactivity (DPM) from cyc created animals, and Y_c = TCA-soluble radioactivity from cyc treated animals; X_s and Y_s are the corresponding numbers from the saline treated animals [3]. Cerebral protein synthesis was found to be inhibited by an average of about 94% during the incorporation interval.

There was no difference in initial training trial STLs between cyc and sal group ($p>0.1$, all groups, matched pair t test). Cyc had no apparent effect on training or short term memory since there was no difference between the cyc and sal groups in the number of animals that had to

be forced into the shock chamber on the second or third training trials (χ^2, $p>0.05$). In both the cyc and sal groups more animals had to be forced into the chamber on the third training trial than on the second training trial (χ^2, $p<0.05$).

Figure 1 shows the median STLs for all groups on the first training trial and the first test trial. All cyc groups had shorter STLs on the first test trial than the respective sal control groups (Mann-Whitney U, $p<0.04$, two-tail), except for the groups tested one hr after training ($p>0.3$). Comparisons between STLs of the cyc groups given 2 training trials (2T) or 3 training trials (3T) at each test interval revealed that only the groups tested 72 hr after training were significantly different ($p = 0.05$, Mann-Whitney U, two-tail). Similar comparisons between the 2T and 3T sal groups revealed that only the sal groups tested 1 hr after training were significantly different ($p = 0.04$).

To determine whether an amnesia gradient developed within either of the cyc/training conditions, the STLs of the 1 hr test group (Fig. 1) were compared with the STLs of the other groups within each training condition. None of the comparisons between the 2T cyc groups were significant. For the cyc groups given 3 training trials, the group tested 1 hr after training had a significantly longer median STL than any of the other groups ($p<0.05$, Mann-Whitney U, one-tail) except for the group tested 72 hr after training ($p>0.1$).

The median STL of the 72 hr test group in each cyc/training condition was compared (Mann-Whitney U, one-tail) with the STLs of the other groups within the same training condition to determine whether spontaneous recovery occurred. None of these comparisons were significant for the 2T groups, but all comparisons (except with the 1 hr group) were significant for the 3T groups ($p<0.05$).

The test performance of the control groups which had been injected with cyc after training indicate that the apparent amnesia gradient and recovery suggested by the above difference between the 3T cyc groups may instead reflect onset and recovery from some residual effects of cyc. When cyc was injected 1.75 hr before a test trial given 24 hr after training, or 71 hr before a test trial given 72 hr after training, there was no performance decrement on the test trial (median STL for both groups was 60 sec). However, when cyc was injected 5.75 hr or 23 hr before the test trial given 24 hr after training, performance was significantly impaired (median STL of 33.5 sec and 43.5 sec, respectively; $p<0.05$, both groups compared with the 3T 24 hr sal group)).

Inspection of Fig. 1 suggested that the performance of the 2T sal groups improved as the training-test interval

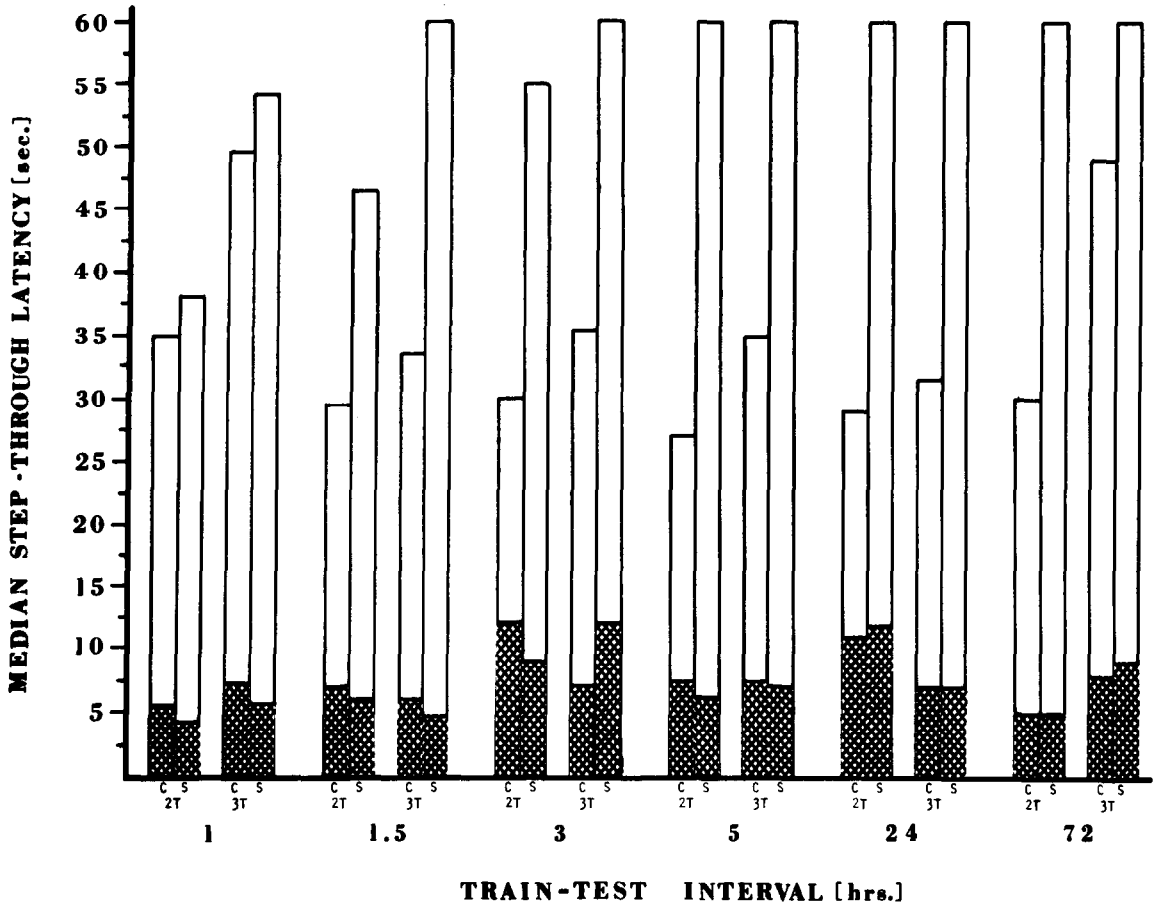

FIG. 1. median step-through latency for all groups on the first test trial, which was given at the times indicated. The shaded portion of each bar graph indicates the median STL on the initial training trial for each group. C= cycloheximide; S= saline; 2T= 2 training trials; 3T= 3 training trials.

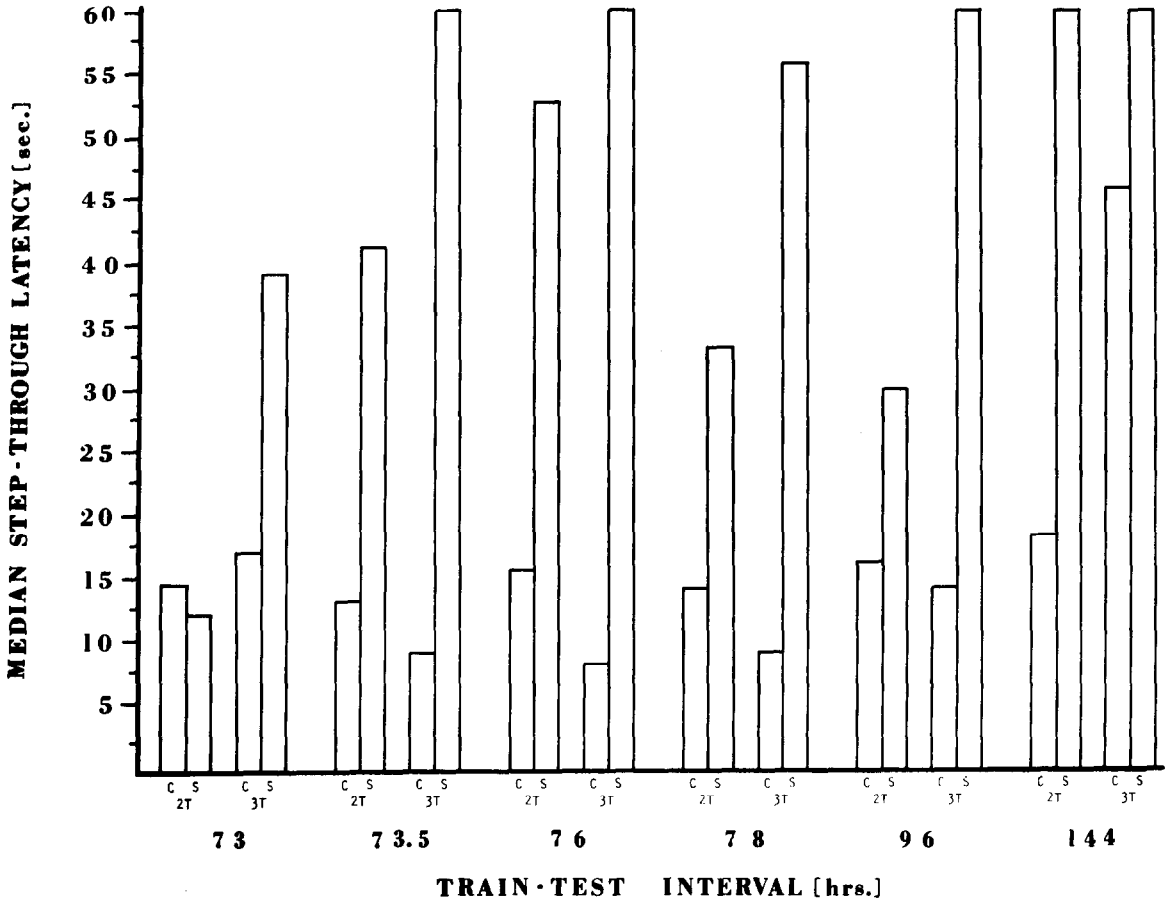

FIG. 2. The median step-through latency for all groups on the second test trial, which was given 72 hr after the first test trial.

increased (incubation). This observation was confirmed by individual comparisons (Mann-Whitney U, two-tail) between the 2T sal groups. The median STL of the group tested 72 hr after training was significantly larger than the median STL of the groups tested 1 hr ($p<0.02$) or 1.5 hr ($p<0.05$) after training. There were no differences among any of the 3T sal groups.

Figure 2 shows the median STLs of all groups on the second test trial. The performance of the sal groups was again generally superior to the cyc groups. All cyc groups had significantly shorter STLs than their saline controls (Mann-Whitney U, two-tail, $p<0.01$, all groups), with the exception of the 2T group given the test trial 73 hr after training. Comparisons between the sal groups tested at each interval revealed that the 3T sal groups had significantly longer median STLs than the 2T sal groups tested at the same interval (Mann-Whitney U, two-tail, $p<0.05$, all groups), except for the groups tested 76 and 144 hr after training. This indicates that 3 training trials in this task generally produces stronger conditioning (i.e., more resistant to extinction) than 2 training trials. However, this relationship is not apparent in the groups given cyc before training. Only the cyc groups tested 144 hr after training differed significantly ($p<0.01$).

The partial recovery of performance in the 72 hr 3T cyc group (Fig. 1) was maintained on the second test trial (Fig. 2, 144 hr training-test interval). This group had a significantly longer median STL than any other 3T cyc group ($p<0.001$, Mann-Whitney U, two-tail). There were no differences between any of the 2T cyc groups. Similar comparisons within each of the sal-training conditions revealed that the 144 hr 3T group had a significantly longer median STL than the 73 hr 3T group, and the 144 hr 2T sal group had a significantly longer median STL than the 73, 73.5, 78, and 96 hr 2T groups (Mann-Whitney U, two-tail, $p<0.05$, all groups). These differences between the sal groups generally reflected the incubation (improvement in performance as the train-test interval increases) that was noted in the first test trial.

If the first test trial initiated recovery from amnesia, then the performance of some of the cyc groups should have improved on the second test trial. However, there was no suggestion of a test induced recovery; the STLs on the second test trial were significantly shorter than the first test trial STLs for all cyc groups (Matched-pair t-test, two-tail, $p<0.005$, all groups) with the exception of the 3T cyc group tested 72 and 144 hr after training ($p>0.1$). Similar analysis of the difference in STLs between the first and second test trials of the sal groups were not significant for the 2T group tested 3 and 78 hr after training or the 3T sal

group tested 72 and 144 hr after training ($p>0.1$, both groups), but were significant for all other sal groups ($p<0.025$, all groups). These data indicate that the first test trial generally promoted extinction, rather than recovery, of the PA response.

To determine whether the magnitude of the change in performance between the first and second test trials was related to the drug treatment, the number of training trials, or the training–first test interval, the difference scores (Test 1 STL – Test 2 STL) for each group were cast in a 3-way ANOVA (drug x training trials x test interval). A Cochran test for homogeneity of variance [26] was applied to the cell variances and was not significant ($c = 0.1026$, $p>0.05$).

The results of the ANOVA generally indicate that extinction was greater in the cyc groups than the sal groups ($f(1, 408) = 28.9$, $p<0.001$), and was generally greater at the shorter train-test intervals ($F(5,408) = 4.8$, $p<0.01$). The main effects for training trials were not significant, nor were any of the interactions.

DISCUSSION

The data indicate that 2 training trials prevent the development of a cyc induced amnesia gradient which has been reported for this task when only 1 training trial was given [21]. Although the 2T cyc groups tested 1.5 to 72 hr after training was inferior to the respective saline groups, an inspection of Fig. 1 reveals that this was due to an improvement in the performance of the sal groups over time (incubation), rather than a deterioration in performance of the cyc groups. It would seem, therefore, that the amnesic effect of cyc on the 2T groups was related to an attenuation of incubation.

The performance of the 3T cyc groups was more complex. Performance did deteriorate after the 1 hr test trial and improved on the 72 hr test trial to the level of the 1 hr test group. These results may indicate the onset of amnesia and later partial recovery. However, since cyc injected 5.75 hr or 23 hr before the test trial impaired test performance while injections 1.75 or 71 hr before testing had no effect on performance, a more likely interpretation would be that the decrease in intermediate test performance reflects a transient impairment of PA performance by cyc, independent of its amnesic effects. Granted this interpretation, then the performance of the 3T cyc groups is quite similar to the performance of the 2T cyc groups in that there was no gradient of amnesia or recovery. The major effect of the additional training trial was to elevate the general level of performance of the cyc treated mice. Amnesia again seemed to be related to an attenuation of incubation.

There was no impairment of performance independent of the amnesic effect in the 2T cyc groups. Furthermore, performance of the intermediate 3T cyc groups was equivalent to that of the respective 2T cyc groups. It seems possible, therefore, that the performance of the 2T cyc groups represents an amnesic baseline for this task. Increasing the number of training trials can elevate test performance in cyc treated mice, but the expression of this additional component of memory may be susceptible to impairment by some transient effect of cyc. This is a topic for additional research.

There was no evidence of a test induced recovery of memory in this study. The mean STL on the second test trial was less than the mean STL on the first test trial for all treatment groups, and this decrease in performance was significant for most of the treatment groups. These data suggest that the first test trial contributed to the general extinction of the response, rather than to reactivation of some residual memory trace. While these results are contrary to the report of Quartermain *et al.* [19], they are consistent with the findings of Geller and Jarvik [11], who used electro-convulsive shock (ECS) as the amnesic agent (Quartermain *et al.* [19] reported that recovery occurred whether ECS or cyc was used as the amnesic agent) and a task similar to that used by the Quartermain group, except that there was no flashing light in the shock chamber and the animals were not detained in the entrance chamber before being permitted to respond on the training and test trials. These are possibly critical differences.

The results of the analysis of variance further indicate that the degree of extinction was generally greater in cyc treated animals, and in those animals which were given the first test trial soon after training. The incubation which seems to be associated with the learning of this task is therefore impaired not only by cyc, but also by an extinction trial given during the early stages of incubation. Particularly note the apparent amnesia that developed between the first and second test trials in the 3T 1 hr cyc and 2T 1 hr sal groups (Figs. 1 and 2). These results are very reminiscent of the findings of Jarvik and his co-workers [13,24] that detention in the entrance chamber of a PA apparatus is an effective amnesic treatment, and the effectiveness is inversely correlated with the training-detention interval. They have also reported that cyc potentiates this detention induced amnesia [12]. Perhaps the act of recall without reinforcement before incubation (or perhaps consolidation) is complete somehow obliterates part of the developing memory trace so that long term memory may never develop to full potential. Cyc may potentiate this effect.

Since the spontaneous partial improvement of performance in the 3T cyc animals did not occur until 72 hr after training, it seemed possible that greater recovery might have occurred in the 3T cyc animals if the test trial had been delayed longer than 72 hr after training. Also, partial recovery might have occurred in the 2T cyc animals if the first test trial had been given at a longer train/test interval than 72 hr. It also seemed possible that test induced recovery might have occurred if more test trials with a shorter interest interval had been given. The second experiment was performed to test these possibilities.

EXPERIMENT 2

METHOD

Four groups of mice (N = 24/group) were injected with cyc or sal before training. One group from each drug condition was given 3 training trials and the other was given 2 training trials, as described in Experiment 1. All animals were given the first test trial 144 hr after training, and additional test trials 146, 148 and 192 after training.

RESULTS AND DICUSSION

The results of this experiment are summarized in Fig. 3. It is apparent that increasing the training test interval to 144 hr did not promote additional recovery in either cyc

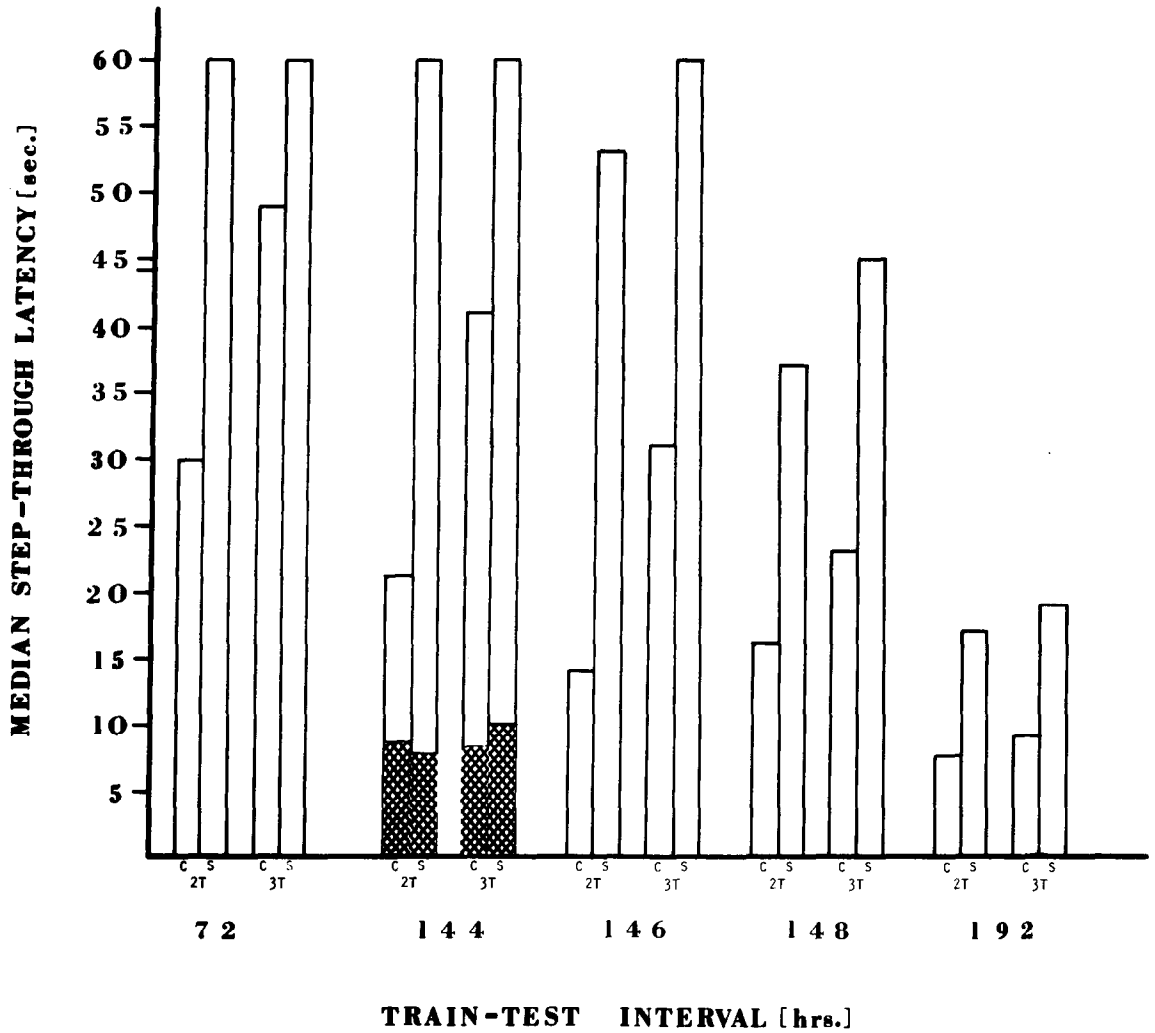

FIG. 3. Median STL for groups given test trials 144, 146, 148 and 196 hr after training. The 72 hr groups are from Experiment 1. The shaded portion of the bar-graphs for the 144 hr train/test interval indicates the initial training trial STL for each group.

group. The median STLs of the cyc groups tested 144 hr after training were significantly shorter than the median STLs of the respective sal groups (Mann-Whitney U, $p<0.05$, both groups), but were not significantly different from the equivalent cyc/training groups in Experiment 1 that were tested 72 hr after training ($p>0.1$, both groups). The multiple test trials also failed to promote recovery of memory. By the fourth test trial (192 hr after training), the median STLs of the cyc groups had deteriorated to naive performance levels (the shaded portion of the 144 hr bar graphs, Fig. 3).

EXPERIMENT 3

The incubation observed in the sal mice of Experiment 1 of this paper was not observed in an earlier study which used only one training trial [21]. However, that study employed a relatively short criterion latency on the test trials which may have resulted in a ceiling effect. Since an amnesia gradient did develop in mice given one training trial in that study, but did not develop in cyc treated mice given two or three training trials in the present study, and since the amnesia in the present study appeared to be related to an attenuation of incubation, it seemed necessary to determine whether incubation does in fact occur when only one training trial is given on this task.

METHOD

Two groups of sal injected mice (19/group) were trained as described in Experiment 1, except that only one training trial (1T) was given. One group was tested one hr. after training and the other group was tested 72 hrs. after training.

RESULTS

The median STLs on the test trials are given in Table 1, along with the latencies of the comparable sal groups from Experiment 1. Very little, if any, incubation occurred. The difference in median test STLs of the 1T groups 1 or 72 hr after training was not significant ($U = 165$, $p>0.05$).

GENERAL DISCUSSION

The results of this study generally indicate that

TABLE 1

THE MEDIAN STL (SEC) ON TEST TRIALS GIVEN 1 OR 72 HR AFTER TRAINING FOR GROUPS GIVEN 1, 2, OR 3 TRAINING TRIALS

Training Trials	1 Hour Test	72 Hour Test
1	22	28
2	38	60
3	54	60

additional training prevents the development of an amnesia gradient in cyc treated mice but does not prevent amnesia. However, since the 3T 72 hr cyc group was superior to the 2T 72 hr cyc group, it seems probable that additional training might eventually elevate the performance of cyc treated animals to the level of sal treated animals. This would be difficult to determine since three training trials is a practical upper limit for this task. Nevertheless, the results of this study do support the report by Flood *et al.* [10] that increasing the strength of conditioning by increasing the shock intensity or duration, or both, can significantly reduce the degree of cyc induced amnesia.

There was no evidence of a spontaneous recovery of memory in the present study or in the Flood *et al.* [10] study. The results of Experiment 1 suggest that the apparent "amnesia" and "recovery of memory" reported by Quartermain *et al.* [20] in their high shock group was instead a transient impairment of PA performance by cyc with later recovery. Flood *et al.* [10] reached a similar conclusion.

Test induced recovery of memory also did not occur in the present study. This difference from the results of the Quartermain *et al.* [19] study may be due to differential conditioning of separate components of PA by the different training procedures used in the two studies. Many authors have suggested that the acquisition of PA involves the acquisition of a specific avoidance (IR) and a general fear (CER) component [1, 7, 14, 22, 25]. The relative contribution of these two components to the general PA memory and performance would depend upon training/test parameters. Comparison of the apparatus and training procedures employed by the Quartermain group [19,20] with the apparatus and training procedures reported in the present study suggest that the former establish a CER more strongly than an IR, while the opposite is true for the latter.

It is beyond the scope of this paper to present a detailed analytical comparison between the two training paradigms, but the essential differences can be briefly summarized. In the Quartermain *et al.* [19] study, the shock chamber was illuminated with a flashing light during training and testing. The mouse was given a 2 sec foot shock 18 sec after entering the chamber on the training trial and then removed immediately. The animal was thus subjected to an inescapable punishment in the presence of a highly discriminable CS; conditions most favorable to the establishment of a CER [5,22]. However, conditioning of a specific avoidance response should have been relatively weak due to the 18 sec delay between the response (stepping into the chamber) and punishment [1, 13, 22].

In the present study (and in [21]), the animal was subjected to the general situational cues only during training, and not a specific CS. The foot shock was very brief and administered immediately upon performance of the response to be modified, and the animal terminated the shock by escaping into the chamber. With 1 training trial, these conditions should promote the acquisition of an IR more strongly than a CER [5, 22, 25]. However, with additional training trials one would expect a CER to also develop (note the difference in incubation between the IT and 2T groups in Table 1).

Data have been reported which suggest that CER, as measured by autonomic indices is more resistant to an amnesic agent than IR [8, 16, 17, 18]. It seems plausible to suggest, therefore, that in the Quartermatin *et al.* [19] study cyc induced amnesia of the weakly conditioned IR component of the PA memory, but spared or only partially impaired the CER component. On the first test trial, a CER would not be evoked in the entrance chamber because of the dissimilarity of cues between the entrance and shock chambers, and the mouse would readily enter the shock chamber. With successive test trials, however, temporal and spatial contiguity of the two sets of cues would promote generalization of the CER to the entrance chamber. The animals STL would thus increase over trials and it would appear that memory was recovering. Gold *et al.* [14] have proposed a conceptually similar explanation to account for the apparent recovery of memory following non-contingent foot shock.

With the task used in the present study, 1 training trial is insufficient to overtrain either component and an amnesia gradient would develop following pretraining treatment with cyc [21]. Two and 3 training trials would effectively overtrain the IR and prevent a cyc induced amnesia gradient, but would not effectively overtrain the CER, and incubation of the general fear response would be attenuated (Fig. 1).

The foregoing interpretations of data need to be substantiated by additional research, but they serve the function of unifying seemingly dissimilar data. They also serve to illustrate once again (cf [16]) that memory of a learned event may consist of several components, and amnesic agents may differentially effect these components.

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