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Temporal Analysis of the Retention of a Food-Aversive Conditioning in *Limax flavus*

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ABSTRACT—In order to understand the mechanisms of learning and memory, early process of the food-aversive conditioning in a terrestrial slug, *Limax flavus*, was investigated. Basic properties of the conditioning were much similar to those in *Limax maximus*. Temporal analysis of the learning process indicated that the slugs indicated that the slugs retained stimulus trace for 1 min and showed short-term and long-term memory phase. The life time of short-term memory obtained with two independent experimental strategies, namely, observation of memory retention and cooling-induced amnesia, was about 1 min. The mechanisms of simple learning in *Limax* were compared with those of insects or mammals.

INTRODUCTION

The molluscs have provided useful model neural systems for the studies on cellular or molecular mechanisms of learning and memory because of their simple neural networks and their large cell sizes. Among these were *Aplysia, Hermissenda, Helix, Pleurobranchaea,* and so on. In the studies using such animals, one must take into account whether the mechanisms of leaning and memory of these animals are same as those of mammals. In other words, how can one apply knowledges obtained from the studies on molluscs to mammals?

Gelperin and his colleagues studied food-aversive leaning in *Limax maximus* and it was found that the animal showed fairly high learning abilities comparable to those of mammals. The slug showed first-order conditioning, second-order conditioning, blocking [1] or extinction of memory [2]. The slugs also showed one-trial associative learning [3]. They mentioned that the slug could be a model system in the study of the mechanisms of learning and memory [2]. However, the early processes of memory formation in *Limax* have not been studied adequately.

It is well known that established memory does not take its final form immediately after the training trial. It takes time to develop. During this time, the memory changes its properties. Whether one accept the notion of short-term or long-term memory or not, it is fact that there are different qualities of memory at various times after learning [4]. Thus, to use *Limax* as a model system on the mechanisms of learning or memory, temporal analysis of the retention of simple conditioned response is needed.

Here, we studied the early learning process of food-aversive conditioning in *Limax flavus* based on the behavioral works in honeybees [5, 6] or in rats [7] and clarified the temporal properties of memory formation and retention in the mollusc.

MATERIALS AND METHODS

Animals

Specimens of *Limax flavus* were cultured in the laboratory on frog chow (Oriental Yeast Co. Ltd.) with a light-dark cycle of 14 hr : 10 hr at 19°C. Animals of 1.5–2.0 g weight were used in the experiments. Prior to the experiments, the animals were housed in a plastic container (350×255×

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62 mm) lined with river sand about 40 slugs/container and were allowed to continuous access to the diet. One week before the start of the training, the animals were placed individually into separate containers (113×105×28 mm) lined with moistened filter paper and were starved until the start of the experiments.

Food-aversive conditioning

The procedure for food-aversive conditioning was basically the same as that of first-order conditioning (FOC) [1, 3]. The animals were conditioned to avoid odors paired with toxic stimulus. In our study, the conditioned stimuli (CSs) were carrot juice or cucumber juice made in our laboratory with a blender and unconditioned stimulus (US) was saturated solution of quinidine sulfate (1 g/90 ml). In case of carrot juice-quinidine pairing, the slugs were transferred with tweezers to a plastic container whose floor was moistened with carrot juice (Ca). Although the slugs could sense both taste and odor of carrot in the container, it was insured that the measure of conditioning was same between the slugs conditioned with the taste and the odor of CS and those conditioned with the odor only. After 2 min of exposure to the carrot juice, the slugs were directly transferred with tweezers to another plastic container lined with filter paper thoroughly moistened with quinidine sulfate (Q) and trapped in contact with the drug for 1 min. Then they were rinsed with saline (in mM: 52.9 NaCl, 4.0 KCl, 7.0 $CaCl_2$, 4.6 $MgCl_2$, 0.2 KH_2PO_4, 2.5 $NaHCO_3$, 5 dextrose, pH 7.6) and were returned to their individual container. This paired presentation of CS and US were repeated 1–3 times with 2 hr-intertrial interval. The FOC procedure was abbreviated as (CaQ)k (k: number of training trials). The same conditioning proceudre was employed when cucumber juice (Cu) was used as CS instead of carrot juice.

Cooling

In the experiments concerning the early learning phase, the conditioned slugs were cooled to about 1°C. In this case each animal was kept in the individual case and was left for 5 min in a freezer-compartment of a refrigerator. Within 3 min, the animal lost its motor activity and its body temperature changed to about 1°C (measured with thermocouple). This cooling procedure is abbreviated as "F".

Measure of Conditioning

The test apparatus which was used to measure odor preferences of the slug is shown in Figure 1. The apparatus consisted of 3 rooms. The food odor sources (carrot or cucumber juice) and frog chow were separately placed on each of the two side rooms. The food odor was generated with a filter paper moistened with food juice and that of frog chow with gel containing its powder. These odor sources were lined on each floor. Individual slug was placed in the center room and the room was covered with a plastic board. As walls of the room were perforated, the animal could access the odors but could not eat the sources. On the floor of center room, line was drawn to divide the room into "carrot side" (or "cucumber side") and "chow side".

The measure of conditioning was designated as

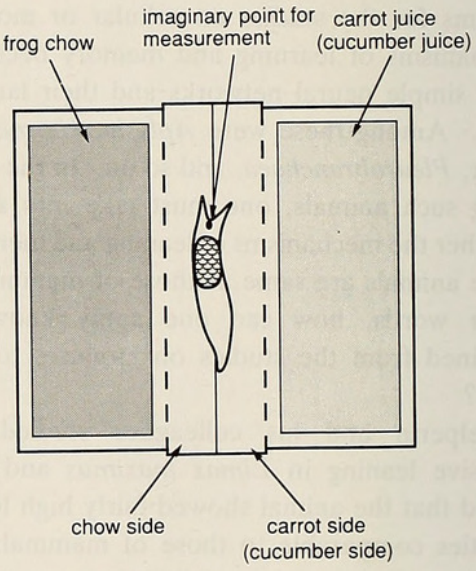

FIG. 1. Rough sketch of testing apparatus. The apparatus consisted of 3 rooms. Two side room were for odor source of CSs and frog chow. The slugs were put on the floor of the center room inbetween them, whose walls were perforated. The center line divided the center room into chow side and carrot (cucumber) side. The mark shown on the slug's head was imaginary, which was used to measure carrot time or cucumber time. See text for the definition or measurement of these measures of conditioning.

"carrot time" (or "cucumber time"), which was the percentage of time each slug's head spent over the carrot side in the carrot odor versus frog chow odor test trial. This measure was obtained by dividing the total time each slug's head spent over carrot side (cucumber side) by total measured time (120 sec/single measurement×3 measurements= 360 sec). Thus "carrot time" was obtained by following equation;

$$[\text{carrot time } (\%)] = \{[\text{total time over carrot side}] / [\text{total measured time } (120 \text{ sec} \times 3)]\} \times 100$$

The smaller carrot time means the better conditioning of the animal. The "cucumber time" was defined in the same way as "carrot time". A small portion of head between tentacles indicated by a small dot was used as a marker for the measurement. The start of measurement was the first time each slug's head crossed the center line. Most slugs showed choice behaviors, such as waving their head or tentacles before selecting a side. Some slugs ran through the center room and some crawled up to a cover board. In the measurement, however, we did not take into acount whether slugs showed choice behaviors or not. Although this contributed to the larger deviation in "carrot time", we wanted to exclude any judgement done by experimenters. Measurements were carried out with 2 hr-interval. The experimenters did not know the experimental treatments experienced by the slugs being tested.

RESULTS

(1) *Associative learning in* Limax flavus

Before analyzing the early process of associative learning in *Limax flavus*, we studied the basic characteristics of the learning behaviors in the slug and compared them with those of *Limax maximus* as reported by Gelprin and his co-workers [1–3].

Selectivity of the leanining

In the first experiment, the slugs were grouped into four representing the treatment conditions to be compared. Slugs in group CaQ (n=8), group CaS (n=8), group CuQ (n=8) and group CuS (n=8) were exposed to 3 carrot-quinidine pairings, 3 carrot-saline pairings, 3 cucumber-quinidine pairings and 3 cucumber-saline pairings, respectively. Approximately 24 hr after the training, carrot time and cucumber time were measured for each slug. From the results of the carrot odor versus frog chow odor preference test (Fig. 2, left), it is evident that the slugs in group CaQ showed much less carrot time than the slugs in group CaS, CuQ and CuS. Analysis of variance showed differences among the groups, $F(3, 28)=5.56$, $P<0.005$. Post hoc individual comparisons (Newman-Keuls test [8]) indicated that group CaQ was significantly different ($P<0.025$) from groups CaS, CuQ and CuS. Similar results was obtained for the cucumber odor versus frog chow odor preference test (Fig. 2, right). There were differences among the groups, $F(3, 28)=6.14$, $P<0.005$. The individual comparisons indicated that group CuQ was significantly different ($P<0.025$) from groups CaQ, CaS and CuS.

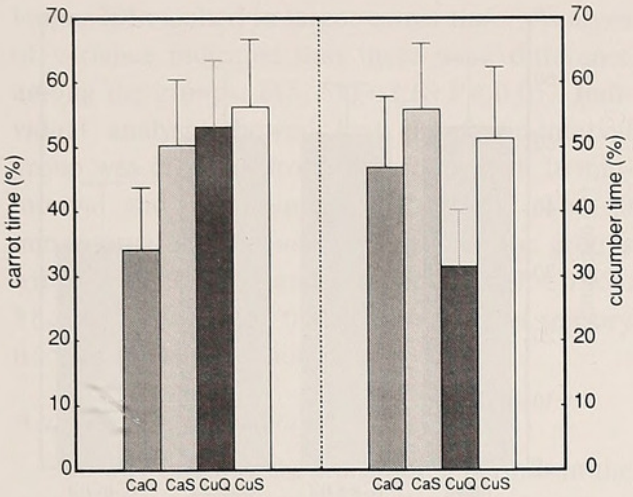

FIG. 2. Associative learning in *Limax flavus*. Carrot time and cucumber time of slugs in 4 experimental groups (n=8, for each group) were compared. Conditioned stimuli (CSs) were 2 min-exposure to carrot juice (Ca) or cucumber jiuce (Cu). Unconditioned stimuli (US) were 1 min-exposure to saturated quinidine sulfate solution (Q) or Limax saline (S). Slugs in each group received 3 pairs of CS·US with 2 hr-intertrial interval. Bars: standard deviations.

The reduced carrot time shown by group CaQ, as compared with other groups, suggests that the slugs in group CaQ associated carrot odor with quinidine sulfate. In the same way, the slugs in group CuQ associated cucumber odor with quinidine.

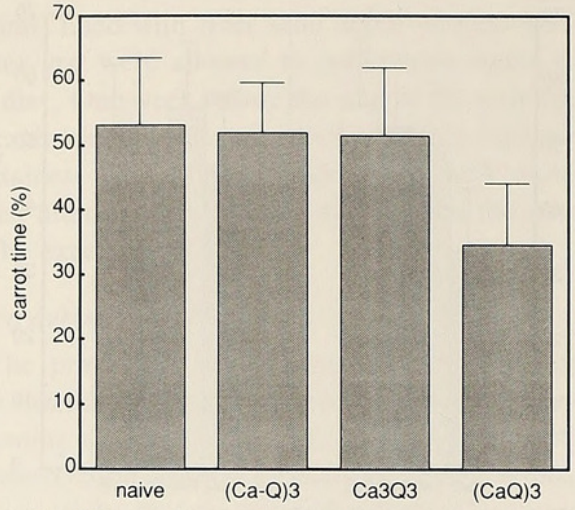

FIG. 3. Effect of quinidine exposure on carrot odor preference of the slugs. Slugs in group (Ca-Q)3 (n=8), Ca3Q3 (n=8) and (CaQ)3 (n=8) were exposed 3 times to carrot juice and then to quinidine sulfate. The time schedules of each group were different (see text). Slugs in group naive did not receive any treatments. Bars: standard deviations.

Effects of quinidine on the odor preference

The next experiment was designed to examine the influences of quinidine to the changes in odor preference. The slugs were grouped into four representing the treatment conditions to be compared. Slugs in group Ca3Q3 were exposed 3 times to carrot juice for 2 min with 2 hr-interval, then they were exposed 3 times to quinidine solution for 1 min with 2 hr-interval. Slugs in group (Ca-Q)3 were first exposed to carrot juice for 2 min and to quinidine for 1 min. But the inter-stimulus interval was 30 min. The training trial was repeated 3 times with 2 hr-interval. The training procedures for slugs in group (CaQ)3 was the same as those for group CaQ described in the preceeding section. No treatment was applied to slugs in group naive. The results are shown in Figure 3. The carrot odor versus the frog chow odor test indicates that carrot time was reduced only in the slugs in group (CaQ)3. Analysis of variance showed that there were differences among the groups, $F(3, 28)=4.97$, $P<0.01$. Individual comparisons showed that group (CaQ)3 was different from the other groups ($P<0.025$). The carrot times were the same among groups Ca3Q3, (Ca-Q)3 and naive ($P>0.25$)

The experimental results described above clearly indicate that exposure to quinidine *per se* is not sufficient condition to reduce the slugs' preference for the odors and that the reduction was a result of the association by the slugs between the attractive food odors and aversive exposure to quinidine. This strongly demonstrated that *Limax flavus* is capable of associative learning.

Effects of number of training trials

The effects of number of training trials on the learning process of *Limax flavus* was investigated. The training trials were increased from 1 to 4 times and changes in carrot odor preference were examined. Sixty slugs were divided into 5 groups. The slugs in group (CaQ)1 (n=12), (CaQ)2 (n=12), (CaQ)3 (n=12) and (CaQ)4 (n=12) were exposed to carrot-quinidine pairings for 1, 2, 3 and 4 times, respectively, with 2 hr-intertrial interval. The slugs in the control group (n=12) were treated in the same way as slugs in group (Ca-Q)3 in Figure 3. As is shown in Figure 4, the slugs learned to avoid carrot odor after one training trial, which was in good agreement with the result of a similar study in *Limax maximus* [3]. Analysis of variance indicates that there were differences among the groups, $F(4, 55)=9.11$, $P<0.001$. Post hoc individual comparisons revealed that group (CaQ)1, (CaQ)2, (CaQ)3 and (CaQ)4 differed ($P<0.005$) from the control group and that no significant difference was observed among groups

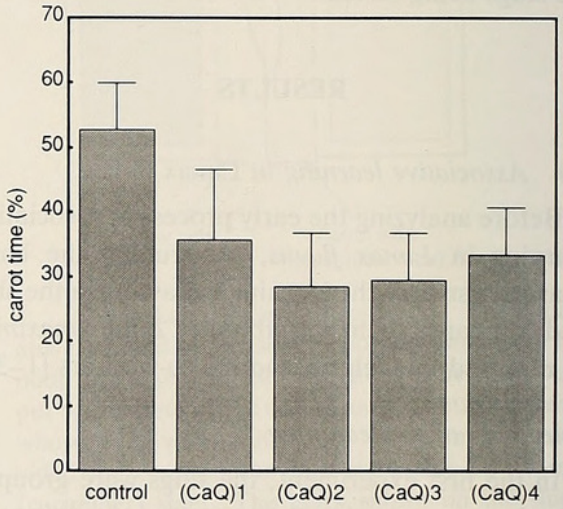

FIG. 4. Effect of number of training trials on the conditioning. Slugs in control group received the same treatment as those in group (Ca-Q)3 in Fig. 3. Bars: standard deviations.

(CaQ)1, (CaQ)2, (CaQ)3 and (CaQ)4 ($P>0.25$). The results indicate that the slugs are adequately conditioned to avoid carrot odor even with one training trial.

These characteristics were fairly in good agrement with those obtained by Gelperin and his colleages in *Limax maximus* [1–3]. Thus we could proceed to the analysis of the early process of the odor-aversive learning in *Limax flavus*.

(2) An analysis of the retention
Interstimulus intervals (ISI)

In the classical conditioning, temporal relationship between CS and US is important because it will show us one of the learning processes, that is, sensory trace, involved in the animal. Fifty-seven slugs were divided into 6 groups according to Ca-Q time interval, T (in minutes) as follows: group 0 (n=10), 0.5 (n=11), 1 (n=11), 5 (n=10), 10 (n=5) and 30 (n=10). The slugs were exposed to 3 Ca-Q pairings with 2 hr-intertrial interval. Since our conditioning procedure corresponded to that of delay conditioning, the ISI was difined as the interval from the end of CS presentation to the onset of US. Thus, the onset of the US was delayed by 2 min from the onset of the CS in group 0. The results shown in Figure 5 indicate that longer ISI resulted in larger carrot time. Analysis of variance indicated that there were difference among the groups, $F(5, 51)=2.68$ $P<0.05$. Individual analysis showed that the 0 min-interval group was different from the groups with 10 min-interval and 30 min-interval ($P<0.05$). The 30 min-interval group was different from the groups with 0 min-, 0.5 min- and 1 min-interval ($P<0.05$). The results indicates that the life time of sensory trace in the slug is about 1 min.

Acquisition and retention

One way to study the transitional periods in the memory trace is to test its retention at various time after the training trial. Twenty seven slugs were divided into 6 groups. The slugs in group T [T= 0.5 (n=4), 1 (n=5), 2 (n=5), 5 (n=4), 10 (n=5) and 60 (n=4)] were conditioned with one CaQ pair and their carrot odor preferences were tested at time T (in min) after the end of training. Thus each slug experienced only one testing trial on the conditioning day. The slugs in group 60, however, were further used and their carrot odor preferences were measured until 60 days after the conditioning. The results are shown inf Figure 6. Note that the time (in sec) shown in the abscissa is in logarithmic scale. Retention increased for the

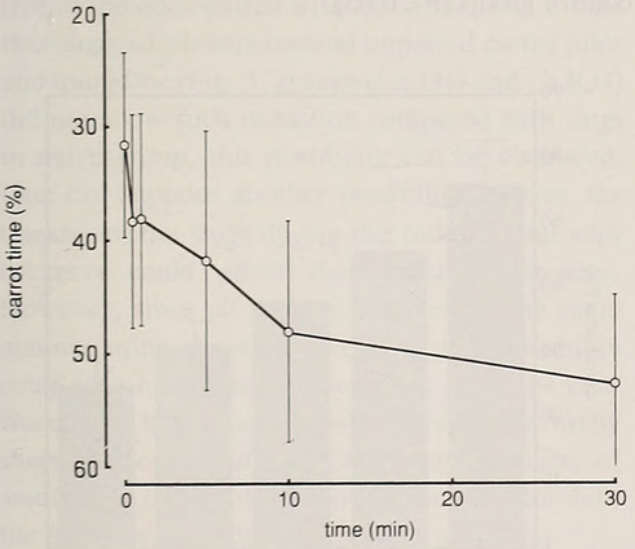

FIG. 5. Interstimulus interval (ISI)-dependence. The time intervals between CS (carrot juice) and US were as follows: 0, 0.5, 1, 5, 10 and 30 min. Note that our conditioning proceudre corresponded to that of "delay conditioning". Thus the onset of US delayed by 2 min from the onset of CS when ISI=0. Bars: standard deviations.

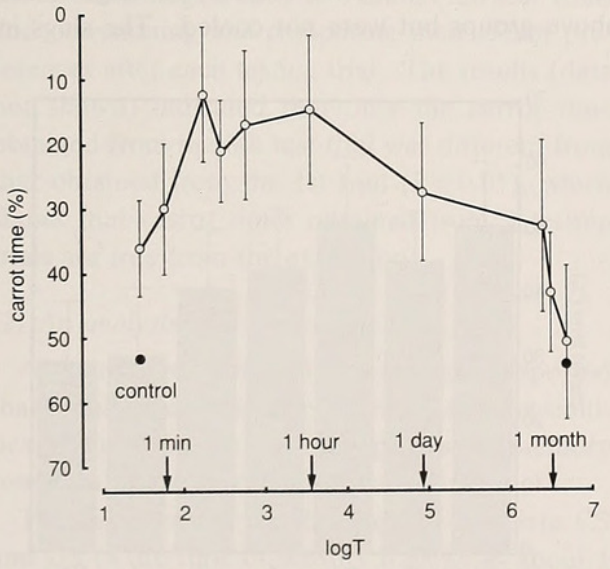

FIG. 6. Time dependence of retention of memory on *Limax flavus* trained once to avoid carrot odor with one CaQ pair (open circles). Note that the time (sec) shown in the abscissa is in the logarithmic scale. Closed circles refer to the carrot time of the contol group. Bars: standard deviations.

first 2 min and was on the level for at least 1 hr after the conditioning. After that, it gradually decreased and settled down to control level in one month. In the early process of memory retention (<1 hr), differences were observed among the groups by the analysis of variance, $F(5, 21)=3.06$, $P<0.05$. Individual analysis revealed that group 0.5 was different from group 2, 5, 10 and 30 ($P<0.05$) and that group 1 was different from group 2 ($P<0.05$).

These results suggest that the retention of memory at the first 1 min is different from that at several minutes after training.

Experimentally induced amnesia

The other way to study the transitions is to induce amnesia in the early phase of retention. Two types of experiments using cooling as an agent to induce retrograde amnesia were carried out.

The procedure employed for the first experiment was based on Erber [5]. Forty four slugs were divided into 6 groups. The slugs in group T [T=0 (n=6), 0.5 (n=7), 1 (n=6), 2 (n=6) and 5 (n=6)] were conditioned to avoid carrot odor with one CaQ pair, after which, they were cooled for 5 min at time T (min) after the end of training trial. The slugs in a separate group CaQ (n=7) were conditioned in the same way as the slugs in the above groups but were not cooled. The slugs in control group (n=6) were exposed to Ca-Q pair with 30 min-interstimulus interval. The results are shown in Figure 7. As the interval between CaQ and F increased, the observed carrot time decreased. Analysis of variance indicated that there were difference among the groups, $F(6, 37)=3.87$, $P<0.01$. Individual analysis revealed that control group, group 0 and group 0.5 were different from group CaQ ($P<0.05$).

The second procedure was based on Hudspeth *et al.* [7]. Twenty-eight slugs were divided into 5 groups. Slugs in group T [T=0 (n=5), 2 (n=6) and 5 (n=6)] were conditioned to avoid carrot odor with 3 CaQ pairs (2 hr-interval) and were cooled at time T (min) after each training trial, (CaQ-F)3. Slugs in (CaQ)3 group (n=6) were conditioned to avoid carrot odor with 3 CaQ pairs (2 hr-interval) without cooling. Slugs in control group (n=5) were exposed to the same stimuli with the same intervals as those in Figure 4. As is shown in Figure 8, longer CaQ-F intervals resulted in the shorter carrot time, which were similar to the results presented in Figure 7. Analysis of variance indicated that there were differences among the groups, $F(4, 23)=8.31$, $P<0.001$. Individual comparison indicated that group (CaQ)3 and group 5 were different from both group 0 and control group ($P<0.05$).

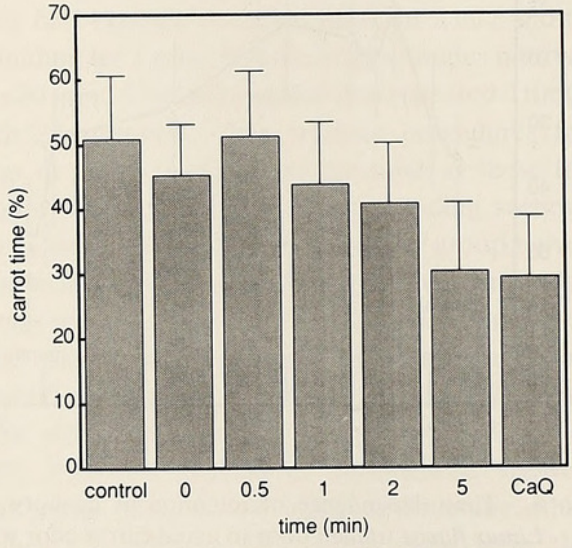

FIG. 7. The time course of retrograde amnesia produced by cooling. The slugs were cooled at different time intervals from the one CaQ training pair (CaQ-F). Bars: standard deviations.

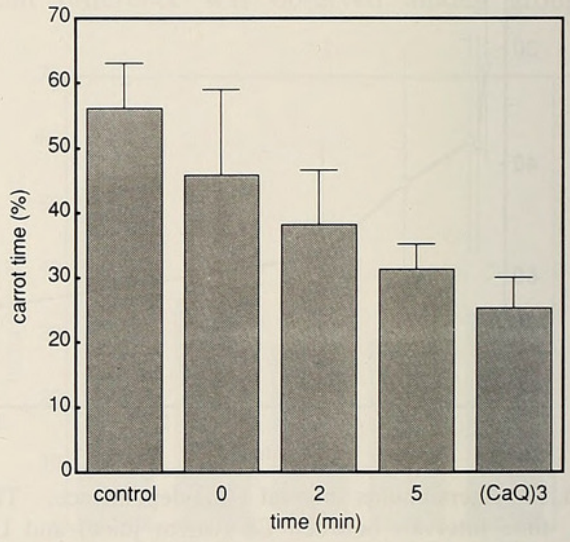

FIG. 8. The time course of retrograde amnesia produced by cooling. The slugs were cooled at different time intervals from each CaQ training pair. This CaQ-F treatments were repeated 3 times. Bars: standard deviations.

These results indicate that memory takes at least two different states, whose transition occurs between 30–60 sec after the training.

DISCUSSION

(1) Associative learning in Limax flavus

Gelperin and his co-workers conducted behavioral studies in *Limax maximus* and found that this terrestrial mollusc displayed associative learning [1–3]. In the present study, we found that *Limax flavus* also showed associative learning on their odor preferences. We used carrot juice and cucumber juice as CSs and saturated solution of quinidine sulfate as US. These stimuli as well as the training procedures are almost the same as those used by Gelperin's group. When carrot juice was paired with quinidine, a preference to carrot odor was reduced (Fig. 2). The reduced carrot odor preference suggests that the slugs in group CaQ in Figure 2 associated carrot odor with the aversive quinidine exposure. There was one possibility that the reduced carrot odor preference was a product of nonspecific or nonassociative consequence of their quinidine experience. However, since the reduction in odor preference was selective to the odor paired with quinidine (Fig. 2) and that slugs which experienced unpaired carrot juice and quinidine (Fig. 3, groups (Ca-Q)3 and Ca3Q3) did not show such reduction compared with slugs in naive group, this possibility can be dismissed. One can suppose another possibility, that is, the transfer of the slugs during the training trial with tweezers could affect their odor preferences. However, since all slugs transferred in the same manner using the same tweezers, this possibility could also be ignored. Therefore, it can be concluded that the reduced odor preferences shown by slugs in groups CaQ or CuQ were products of association of carrot odor or cucumber odor with the aversive quinidine exposure.

As the CSs were in solutions, the slugs could sense both the odors and tastes of CSs when they experienced them. The test, however, was only for odor preferences. Then we carried out experiment where the CS was only carrot odor. The experimental design was as follows: Slugs were placed into a plastic dish with a perforated floor. The dish sat over filter paper evenly moistened with carrot juice. Other procedures as well as the interstimulus interval or stimulus durations were the same as those in Fig. 3. The results obtained showed that the slugs' preference to carrot odor was also reduced (data not shown). Gelperin's group also conditioned *Limax maximus* in two way, (odor+taste)−(quinidine) [3] and (odor)−(quinidine) [1], and got similar results.

Sahley *et al.* [3] reported that *Limax maximus* could associate potato odor with quinidine even with only one exposure to CS-US presentation. Similar result was obtained in case of *Limax flavus*. The carrot odor preference of the slugs was reduced to steady level by one paired presentation of carrot juice and quinidine (Fig. 4). Further reduction in carrot time was not observed even if the number of CaQ pair was increased.

This property, so-called "one-trial conditioning", also reminded us of the possibility that the extinction of memory could occur during odor preference tests, where the odor of food used as CS and the odor of frog chow was applied for 120 sec to the slugs 3 times without aversive stimuli of quinidine sulfate solution. We check the possibility, by extending the number of test trials of the conditioned slugs from 3 to 6 times (120 sec/ trial) and determining and comparing their carrot preferences after each testing trial. The results (data not shown) indicated that only the carrot time obtained from the 6th test trial was different from that obtained from the 1st trial ($P<0.05$), which shows that carrot times obtained from 3 testing trials are free from the extinction.

(2) An analysis of the retention

Although Gelperin and his colleagues reported that *Limax maximus* showed high learning abilities, their studies in the early process of memory retention of the slug were not adequate.

The ISI effective for the slugs to associate CS and US (a life time of sensory trace) was about 1 min. The life times have been reported to be different depending on animals used and on the conditioning procedures, from several hundred milliseconds (rabbit, eyelid response) [9] to more than 24 hr (rat, food aversion) [10]. Most ISI-

measure of conditioning (MC) curves reported were biphasic. As ISI is increased, the measure of conditioning initially increased, and then it gradually decreases. This biphasic pattern of ISI-MC, thus, infers that there is optimal timing to the conditioning. On the other hand, our result (Fig. 5) showed a monophasic curve. The shorter the ISI was, the better conditioning was obtained. Our conditioning procedure corresponded to that of delay conditioning where the CS did not terminate until the US was presented. There is a possibility that the positive ISI-MC relation could have been seen during first 2 min-exposure to the CSs if quinidine sulfate was applied in this period. However, since it would be difficult to remove only carrot juice or only quinidine solution from carrot juice-quinidine mixture, we were not able to observe such ISI-MC relation during this 2-min period. The monophasic ISI-MC curve was obtained by Yeo [11] in rat.

The retention curve of the slug's food-aversive learning was totally monophasic (Fig. 6). But in the very early phase (within 2 min after conditioning), the retention increased. For unknown reasons, the curve was different from those obtained in a honeybee [6] or goldfish [12]. However, it is clear that the origin of the increment is not the aversive quinidine exposure. If quinidine gave some shock to slugs to be insenstive to the external stimuli, the slugs would show carrot time of around 50%, which was shown by slugs in control group. In fact, the carrot time of conditioned slugs at 30 sec or 1 min after the conditioning was about 30%. The value was similar to those obtained on the next day or even after a week later. Thus, the slugs was successfully conditioned to avoid carrot odor even at 30 sec after one conditioning trial. We interpreted these results to mean that the memory in the first 1 min and that in 2–60 min are in different state.

When we cooled the slugs to about 1°C immediately after single conditioning procedure, the slugs failed to avoid carrot odor (Fig. 7). However, the longer the CaQ-F interval was, the better the animal was conditioned. This interval-dependency was also obtained when we repeated the CaQ-F treatments 3 times (Fig. 8). Similar result was reported in mammals [7, 13, 14] and insects [5, 15, 16] mainly using electroconvulsive shock (ECS) instead of cooling. We used cooling to induce retrograde amnesia because the naive slugs avoid the other previously reported treatments such as ECS [7, 14] or CO_2-narcosis [5, 13, 15]. We checked whether the cooling would affect the carrot odor preference of the slugs. This is because there is a possibility that cooling might have some meaning to the slug to brake Ca-Q relation. Eighteen slugs were divided into 3 groups, namely, group control (n=6), (CaQ)3 (n=6) and (CaF)3 (n=6). The slugs in group control and (CaQ)3 were treated with the same procedure as those in Figure 4. On the other hand, the slugs in group (CaF)3 were cooled for 5 min instead of the 2 min-exposure to quinidine sulfate solution as in group (CaQ)3. The results (data not shown) indicated that the cooling did not change the carrot odor preference of the animal. Based on these findings and the fact that the cooling was effective only when it was applied to the slugs within a minute after the conditioning, it was concluded that cooling induced retrograde amnesia in *Limax flavus*.

(3) Short-term memory and long-term memory

Retrograde amnesia is one of the tool to distinguish two memory states, short-term memory (STM) and long-term memory (LTM), in the early process of conditioning [9]. The life time of STM was studied by many workers using various animals and was shown to be several tens seconds [7] or several tens minutes [16]. In the present study, the life time of STM for food-aversive conditioning in *Limax flavus* was shown to be about 1 min (Figs. 7, 8).

The life times of memories are not of importance. Whether one could accept the notation, STM or LTM, or not, it is fact that there are distinguishable memory states in the slug, which were similar to mammals or insects. One is sensitive to some agents (ECS, CO_2 or cooling) and the other is not. Though there are differences in the early phase of the retention curve, the changes in the memory states are much similar to mammals or to insects. Thus *Limax flavus* could provide a useful model system for the study of the memory, not only because of its simpler nervous

system but because of the similarity of the memory states and their transitions to other animals.

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