Associative Training of *Hermissenda*

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ABSTRACT   Reflex behavior of *Hermissenda* in response to visual and rotational stimuli is described. It is shown that repeated association of light with rotation modifies the subsequent responses of the animals to light. This modification does not occur after the same period of light or rotation alone. The effect of the associative training is strongly dependent on the amount of daily light with which the animals are maintained.

INTRODUCTION

Because of the complexity and number of neural interactions in central nervous systems, cellular mechanisms underlying more developed forms of behavioral modification have defied careful analysis. Recently several investigators (Bruner and Tauc, 1964; Kandel and Tauc, 1964, 1965 *a, b*) have turned to the somewhat simple nervous system of the opisthobranch mollusk, *Aplysia*. Two types of short-term modification have been studied in this nervous system: habituation and heterosynaptic facilitation. Although the neural circuitry and correlated behavior of habituation are now fairly well defined (Castellucci et al., 1970), the relationship of habituation, involving a single sensory input, to associative training, involving at least two distinct sensory inputs, is tenuous. The second type of short-term modification observed in the *Aplysia* nervous system, heterosynaptic facilitation (as well as "dishabituation") does involve the interaction of two distinct sensory inputs. The underlying neural circuitry, however, for both heterosynaptic facilitation and dishabituation remains obscure.

It therefore seemed important to find a preparation in which it would be possible to clearly define the cellular connections responsible for the interaction between two sensory pathways and then to analyze electrophysiologic and behavioral changes resulting from repeated paired stimulation of these two pathways. In the nudibranch mollusk, *Hermissenda crassicornis*, considerable progress has been made in determining the synaptic interactions within and between the visual and statocyst pathways (Alkon and Fuortes, 1972; Alkon, 1973 *a, b*; Alkon and Bak, 1973; Detwiler and Alkon, 1973). In this study, behavioral experiments will be described which demonstrate that repeated tem-

poral association of visual and rotational stimuli to *Hermissenda* changes the animal's response to subsequent stimulation of the visual pathway. In another study (Alkon, in preparation)[1] experiments will be described demonstrating changes in the synaptic responses of hair cells to stimulation of the visual pathway in animals whose behavior had been modified by the associative training.

METHODS

Maintenance

Animals were maintained in "Instant Ocean" artificial seawater at 13°C. Light cycles (1.2 × 10³ ergs/cm²-s on aquarium floor) were produced with a single 60-W incandescent light at a distance of 2 feet from the animals. Unless otherwise specified the animals were trained and tested at the end of the dark interval after 3, 4, and 5 days of 6½ h of light per day (Fig. 3). For one type of experiment (Fig. 11), an 18-h daily period of illumination was used.

Training

Animals were placed in a black container (with open top) of dimensions: 28 cm long, 15 cm wide, 12 cm high (cf. Fig. 1). Within each container was 350 cc of "Instant Ocean" artificial water. Minor departures from the following standard training procedures were occasionally made and will be described with the results. Animals were trained and tested in groups of six, or five when photographic records (see below) were made. The containers were then placed on a rotating platform (Arthur H. Thomas Co., Philadelphia, Pa.) within an incubator kept at 15°C. The apparatus rotated at 130 rpm for 25-s periods alternating with 105-s rest periods. (This will be referred to as "training.") When rotation was associated with illumination, a diffuse light (5.0 × 10³ ergs/cm²-s on container floor) was turned on for 45 s before and then during each 25-s rotation cycle. (This will be referred to as "associative training", Fig. 3.)

Testing

After 3 h of the above training, the rotation (and light) cycles were stopped. A 3-cm diameter light spot (intensity: 4.5 × 10³ ergs/cm²-s on container floor) was then projected onto the bottom of the black container (cf. Fig. 1). The edge of this spot was always positioned to be 6 cm (measured at the beginning of the test period) from the nearest animal. The other test animals were always more than 6 cm but not more than 25 cm away (measured at the beginning of the test period) from the spot's edge.

Responses of the animals to the test spot were monitored in two ways. The first method consisted of automatically photographing the container of animals (with test spot) at 3-min intervals using a 35 mm motor-driven camera (Nikkon, Japan). With this method an 8-s dim red light coincided with a 1-s opening of the camera shutter. An analysis of the animals' responses was then made subsequently from the developed film. One advantage of a photographic record of the animals' behavior is that it entirely re-

[1] Alkon, D. L. 1974. Neural correlates of associative training in *Hermissenda*. (Manuscript in preparation.)

moves the observer from the test situation. A second advantage is the possibility of following each animal's movements with time since the movement is usually slow enough to allow fairly accurate tracking. The outstanding disadvantage of the photographic record is that the animals can interact with or attack each other, thus introducing an additional source of stimulation. To minimize such stimulation a second technique of monitoring the animals' response to the test spot was used. The animals were directly observed at 3-min intervals during the 2-h test period. With this method a dim red background light was used. When the animals reached the test spot they were removed from the container. When an active animal approached a second animal, interaction was prevented by gentle obstruction of the active animal's path with a closed curved forceps. Such obstruction almost always resulted in an avoidance response and a redirection of the animal's movement. Although the touched animal received some stimulation, stimulation of the second animal was prevented. Stimulation of the active animal was also minimized since interaction between *Hermissenda* usually involved elaborate vigorous agonistic behavior.

With either the photographic record or direct observation (with prevention of interaction) an animal was considered to have reached the test spot of light if it reached the spot's edge (i.e. if the spot covered any part of the animal). Almost invariably, however, if an animal reached the edge of the light spot it would proceed further so as to be fully within the spot's perimeter. Thus, little judgement was necessary in deciding whether or not the animal had reached the spot.

Statistical Methods

Since for all groups with the exception of one, the experiments were terminated before every animal reached the test spot, the data contain censored (specifically, right-censored) observations. Therefore, the statistical methods employed must be able to take into account this censoring. Procedures due to Gehan (1965) and Breslow (1970) were used to ascertain whether or not differences exist among groups.

The Gehan statistic provides a distribution-free test of the equality of two cumulative distribution functions when the samples are subject to arbitrary right censorship. This statistic is related to the Wilcoxon Test statistic for two populations with uncensored, untied observations.

Breslow extends Gehan's test to allow for the comparison of K populations in two situations. In the first, the distributions of the censoring variables vary for different groups and in the second, as in Gehan's two-population test, the censoring distributions are considered to be equal. Both statistics are asymptotically distributed as chi-square with K-1 degrees of freedom under the null hypotheses. The Breslow statistic for equal censoring has as a lower bound a statistic which is equal to $(N + 1)/N$ times the Kruskal-Wallis Test statistic for K populations with uncensored, untied observations where N is the total number of observations.

The Gehan-Breslow procedure, rather than the Kruskal-Wallis test statistic, was used for these data in order to accommodate the censored observations in group comparisons. For all comparisons made among groups which were censored at the same time, the appropriate Gehan-Breslow test statistic was relatively close to the lower-valued Kruskal-Wallis statistic computed as though the censored observations were

tied for last place. When the overall Gehan-Breslow test of significance of whether K groups differ was found to be statistically significant, further examination of the data was aided by pairwise comparisons among the groups, subject to the usual caveats with regard to multiple comparisons.

A permutation test was applied to the data of Fig. 11 since one group consisted only of censored observations. A Fisher Exact Test was applied to the data of Fig. 9.

RESULTS

Reflex Behavior

VISUAL   Hundreds of animals exposed to $6\frac{1}{2}$ h of daily light were directly observed during the light and dark periods. By the third day of exposure to this light cycle a clear difference in the behavior of animals during the dark and light periods was observed. During the light period animals moved at velocities of up to 0.3 cm/s. During the dark period the animals were most frequently immobile. This diurnal rhythm is not unlike that observed for other gastropod mollusks (Strumwasser et. al., 1966; Strumwasser, 1967; Kupferman, 1968; Jacklet, 1972). In addition to this activation effect of light, the animals are reflexly attracted to light (Fig. 1). When a group of *Hermissenda*, maintained as just described, are placed in a container of seawater (see Methods) and, after a 10-min interval in darkness, a light spot (intensity: 4.5 $\times$ 10^3 ergs/cm²-s) is projected onto the container's floor, 75% of the animals (labeled "pretest") proceed toward and enter the spot within 40 min (Fig. 4). Animals, once having entered the spot, tend to remain close by or within its perimeter.

ROTATIONAL   When a group of *Hermissenda* are placed in the same container of seawater which is now rotated vigorously (>200 rpm) for 20 s every 2 min, at first all the animals are shaken loose. During the rest period the animals quickly right themselves so that their foot is in full contact with the floor (and occasionally the side) of the container. With successive rotation periods more and more animals are not shaken loose, until, within 5–10 min, most, and usually all animals are not moved by the rotation from their position, foot in contact with the container surface (cf. Fig. 9). Close inspection reveals that during the rotation each animal in fact clings to the surface (Fig. 2), the body musculature contracted and the foot gripping. That the clinging reflex is mediated by the statocyst is suggested by the fact that animals devoid of rhinophores, antennae, and serratae are still able to cling.

Associative Training

In the following experiments standard 3-h training and 2-h testing regimens (see Methods) were used. The animals used in these experiments were trained and then tested at the end of the dark interval after 3, 4, or 5 days of $6\frac{1}{2}$ h of light per day (Fig. 3). Animals observed in the groups of Figs. 4–8 were
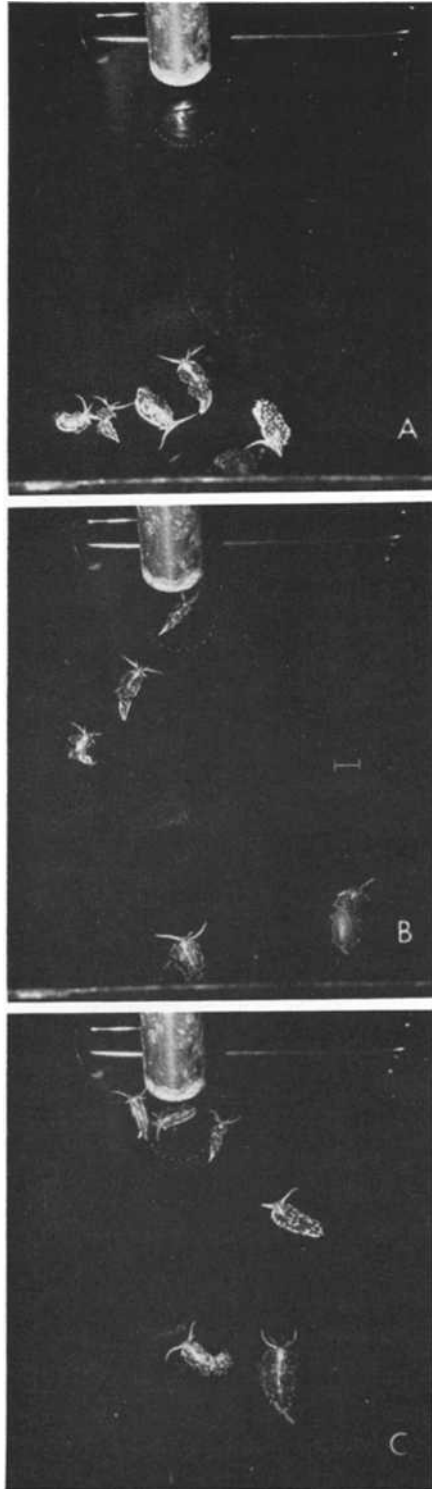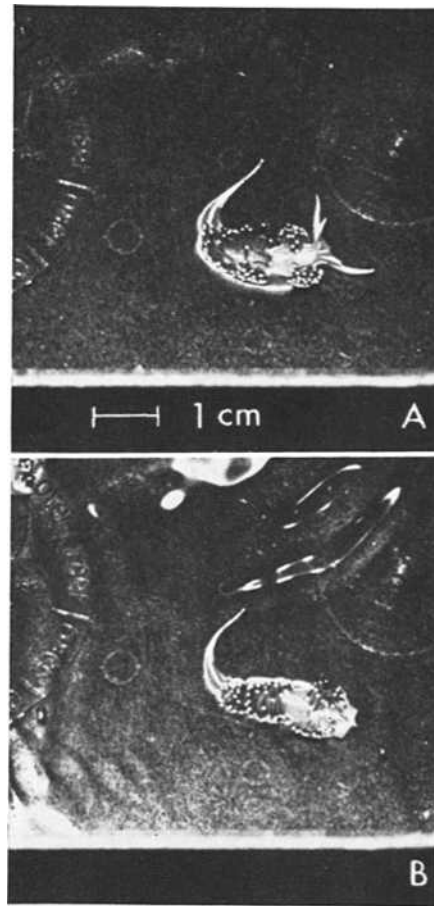
FIGURE 2.   Response of *Hermissenda* to rotation. A, immediately before and B, immediately after 20 s of rotation at 200 rpm. Immediately after and during rotation the entire animal is contracted.

equally distributed across these 3 days. Different animals were used for each experiment with the exception that control groups of Fig. 4 are the same as those of Figs. 5 and 7. The schedule of light intervals used throughout Figs. 4–11 was the same (with and without rotation). During the test period, a time was assigned to each animal as it entered the light spot. The percentage of the total number of animals tested which had reached the light spot was then plotted as a function of time.

Animals exposed to 3 h of 70-s light intervals (see Methods) or 3 h of complete darkness do not take significantly longer to reach a test light spot than animals tested after 10 min of darkness in the experimental chamber (Fig. 4).

FIGURE 1 *opposite.*   Response of animals in experimental chamber to test light spot. A, B, C animals move toward and into light spot within 20 min. Spot (indicated by dashed lines) is obscured by stroboscopic flash in B, C. Calibration: 1 cm.

Only animals exposed to the associative training take a significantly longer time to reach the test light spot than animals given training (rotation alone), animals exposed to 3 h of light intervals or animals exposed to 3 h of darkness (Table I, Figs. 5–8). This was true using the photographic records (Figs. 5, 7)
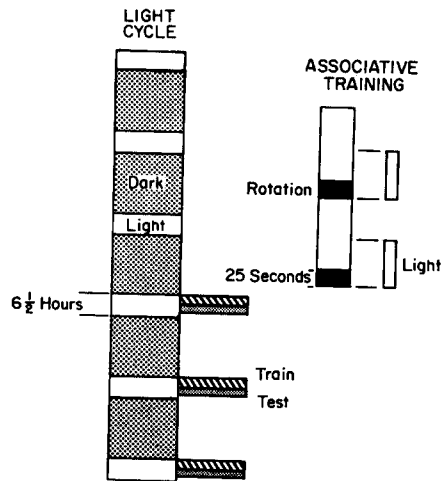

FIGURE 3.   Diagram of maintenance light cycle (left). After at least 3 days exposure to this cycle, animals are given a 3-h training period followed by a 2-h test period. The timing of light and rotation during associative training is illustrated on the right.
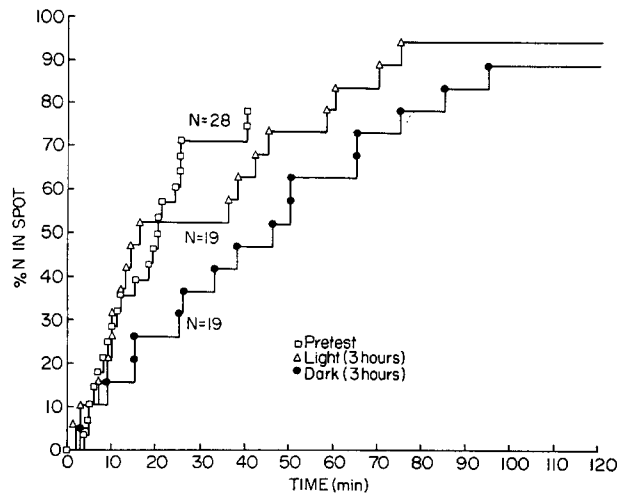

FIGURE 4.   Kaplan-Meier (1958) curves for percent of animals which have reached a test light spot as a function of time. □: Animals tested after 10 min of darkness in experimental chamber. △: Animals tested after 3 h of 70-s light intervals (see Methods). ●: Animals tested after 3 h of complete darkness. These data were obtained from photographic records. Animals were not removed when they reached the test spot nor was interaction between animals prevented. By the Gehan-Breslow test procedure the three groups are not significantly different.

or direct observation (Figs. 6, 8). Animals permitted to interact (Figs. 5, 7) in general tend to take less time to reach the test light spot than animals whose interaction was minimized (Figs. 6, 8).

From the above figures it is clear that animals exposed only to repeated light intervals reach the spot more quickly than those exposed to the associative training ($P < 0.001$, Figs. 5, 6). Animals exposed only to dark, however, show no significant difference in their response to the light spot when compared to

TABLE I

GROUP COMPARISON STATISTICS

Group comparisons	Gehan-Breslow statistic	Significance
Controls (Fig. 4)		
Pretest (PT)		
3 hours light intervals (CCL)	4.368	N.S.
3 hours dark (CCD)		
Observations with camera (Figs. 5, 7)		
3 hours light intervals (CCL)		
3 hours darkness (CCD)		
3 hours of rotation (CRD)	24.965	$P < 0.001$
3 hours of rotation with light (CRL)		
CRD vs. CCD	0.728	N.S.
CRL vs. CCL	19.341	$P < 0.001$
CRL vs. CRD	12.575	$P < 0.001$
Observations without camera (Figs. 6, 8)		
3 hours light intervals (NCL)		
3 hours darkness (NCD)	35.230	$P < 0.001$
3 hours of rotation (NCRD)		
3 hours of rotation with light (NCRL)		
NCRD vs. NCD	3.658	N.S.
NCRL vs. NCL	22.677	$P < 0.001$
NCRL vs. NCRD	15.319	$P < 0.001$
NCL vs. NCD	0.495	N.S.

animals rotated in darkness (Figs. 7, 8). The fact that animals rotated in darkness for 3 h do not take significantly longer to reach the test spot than animals exposed only to 3 h of darkness indicates that repeated stimulation of the clinging response does not in itself greatly interfere with the animals' ability to subsequently respond to light. Additional observations support the interpretation that during the test period it is the animals' response to light and not the clinging response, which is modified by the associative training. The first such observations consisted of counting the number of animals clinging with each successive rotation interval. Animals do not require fewer rotation intervals to cling when the rotations are associated with light (Fig. 9). Thus the effect of light is not facilitation of the clinging reflex. The subsequent decrease in the
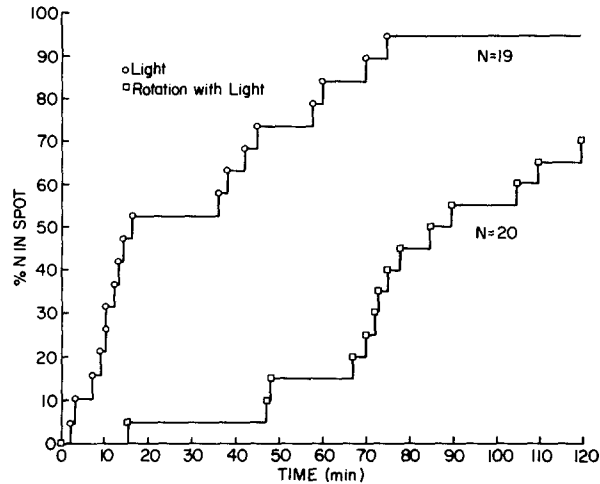
FIGURE 5.   Kaplan-Meier (1958) curves for percent of animals which have reached a test light spot as a function of time. O: Animals tested after 3 h of light intervals (see Methods). □: Animals tested after 3 h of rotation intervals associated with light (see Methods). These data were obtained from photographic records. Animals were not removed when they reached the test spot nor was interaction between animals prevented. The two groups are significantly different (P < 0.001) by the Gehan-Breslow test procedure.
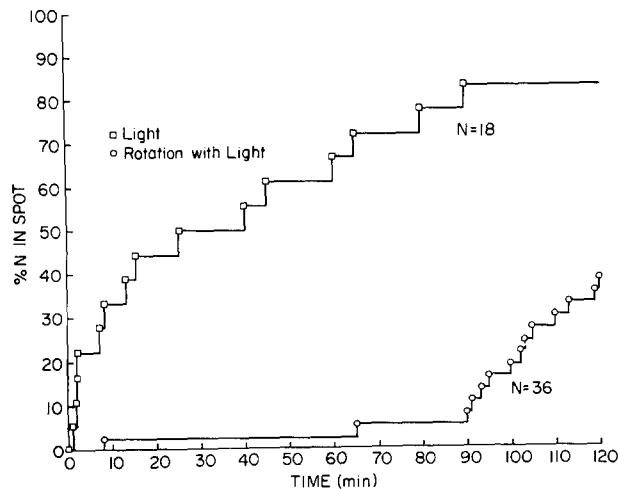

FIGURE 6.   Kaplan-Meier (1958) curves for percent of animals which have reached a test light spot as a function of time. These data were obtained by direct observation of animals entering the test light spot. Animals were removed when they reached the test spot and interaction between animals was prevented. □: Animals tested after 3 h of light intervals. O: Animals tested after 3 h of rotation intervals associated with light. The two groups are significantly different (P < 0.001) by the Gehan-Breslow test procedure.
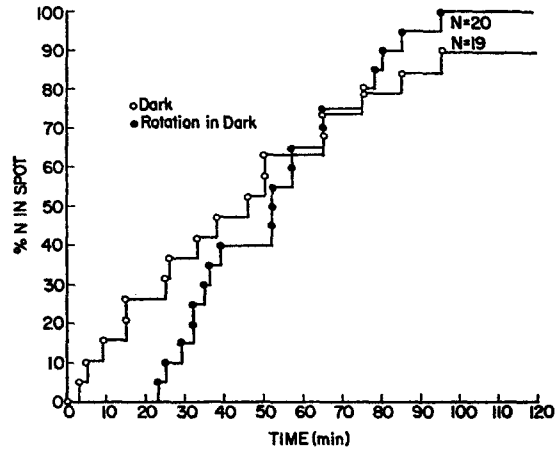
FIGURE 7.   Kaplan-Meier (1958) curves for per cent of animals which have reached a test light spot as a function of time. O: Animals tested after 3 h of complete darkness. ●: Animals tested after 3 h of rotation intervals (see Methods) in complete darkness. These data were obtained from photographic records. Animals were not removed when they reached the test spot nor was interaction between animals prevented. The two groups are not significantly different by the Gehan-Breslow test procedure. Furthermore, the group rotated in darkness is significantly different ($P < 0.001$) from the group rotated with light in Fig. 5.
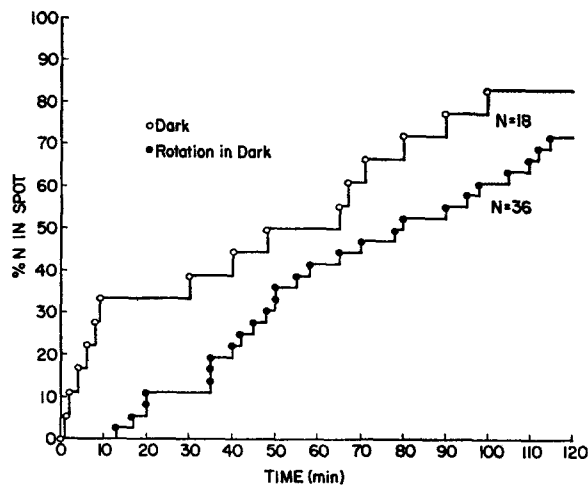

FIGURE 8.   Kaplan-Meier (1958) curves for percent of animals which have reached a test light spot as a function of time. This data was obtained by direct observation of animals entering the test light spot. Animals were removed when they reached the test spot and interaction between animals was prevented. O: Animals tested after 3 h of complete darkness. ●: Animals tested after 3 h of rotation intervals (see Methods) in complete darkness. The two groups are not significantly different by the Gehan-Breslow test procedure. In addition, the group rotated in darkness is significantly different ($P < 0.001$) from the group rotated with light in Fig. 6.

ability of animals (given rotation with light) to reach the light spot during the test period cannot be explained, therefore, by a prolonged facilitation of the clinging reflex.

Further observation of the animals during the training period reveals that the animals' response to light is modified with successive rotations. For associative training (see Methods) a diffuse light precedes and then is paired with rotation. Animals exposed to this diffuse light initially are very active. Even when the animals cling successfully in response to the rotation they still show the activation effect of light (see above) during the intervals between rotation. With successive rotations, however, this activation effect diminishes and by the end of the first hour of training has been markedly reduced. During the second
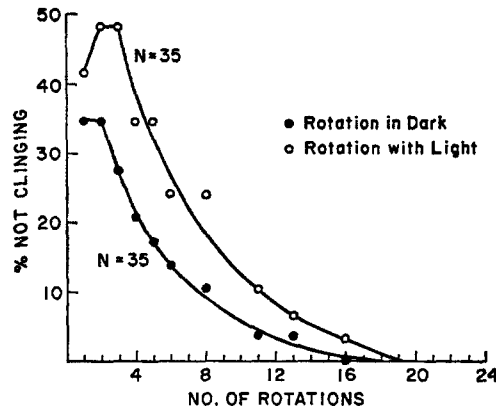

FIGURE 9. Percent of animals not clinging in response to rotation as a function of the number of successive rotations. Rotation at 130 rpm occurred for 25-s periods alternating with 105-s rest periods. O: Animals rotated with light. ●: Animals rotated in darkness. Fisher's Exact Test for two by two tables was used to test differences between the two curves at each point. None of these differences was found to be significant.

half of the training period the diffuse light no longer activates the animals at all. Finally, one additional type of experiment indicates that the effect of the light has been modified. One group of six animals was exposed to training and a second group of six to associative training. During the test period, however, no test light spot was used. The two groups of animals were left, instead, in complete darkness and observed briefly every 15 min for signs of activity. No animals in either group whowed signs of activity for the first 75 min. After this, some of the animals rotated with illumination begin to become active over the next 45 min. Thus, without the activation effect of the test light spots the clear differences observed (Figs. 4–8) during the first 70–90 min do not appear.

Associative Conditions

Although in this study a careful examination of the optimal associative conditions was not conducted, some preliminary experiments were performed. One

group of animals was rotated in darkness for 3 h at the end of the usual 17½-h dark period. A second group was rotated in darkness at the end of the 6½-h light period. No apparent difference in the response to the test light spot was observed (Fig. 10). Thus, light preceding the training period had no effect on the animals subsequently rotated in darkness. This experiment, however, does not measure the effectiveness of the photic training stimulus when given out of association with rotation. In another experiment one particular type of stimulus dissociation did not interfere with the effectiveness of the associative train-
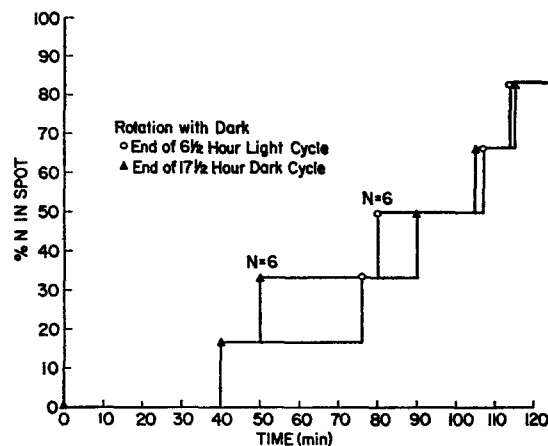

Figure 10. Kaplan-Meier (1958) curves for percent of animals which have reached a test light spot as a function of time. O: Animals tested after 3 h of rotation intervals in darkness; these 3 h were preceded by 6 h of light. ▲: Animals tested after 3 h of rotation intervals in darkness; these 3 h were preceded by the usual dark interval of the maintenance diurnal light rhythm. These data were obtained by direct observation of animals entering the test light spot. The two groups are not significantly different by the Gehan-Breslow test procedure.

ing. In this experiment six animals were exposed to light intervals during the training period which were out of phase with the rotation intervals. As with other experiments the apparatus rotated at 130 rpm for 25-s periods alternating with 105-s rest periods. A 70-s interval of diffuse light (5.0 × 10³ ergs/cm²-s on container floor) followed the rotation interval by 18 s and preceded the next rotation interval by 17 s. Thus, at no time did the light cycle coincide with the rotation cycle. After this training the animals were tested for 2 h. One of the six animals reached the test light spot at 110 min. Using the Gehan-Breslow test previously described, the behavior of these animals was found to be significantly different from that of animals of Fig. 6 given the training (P < 0.005), but not the associative training as described under Methods.

Effect of Changes in Light Cycle

A group of animals was exposed to 18 h of light daily (rather than the 6½-h periods) and then trained and tested as described above. For animals maintained in this way there was no longer a significant difference between animals trained and those associatively trained (Fig. 11). In general, animals maintained in this way took much longer to get to the light spot than those maintained with 6½-h of daily light.
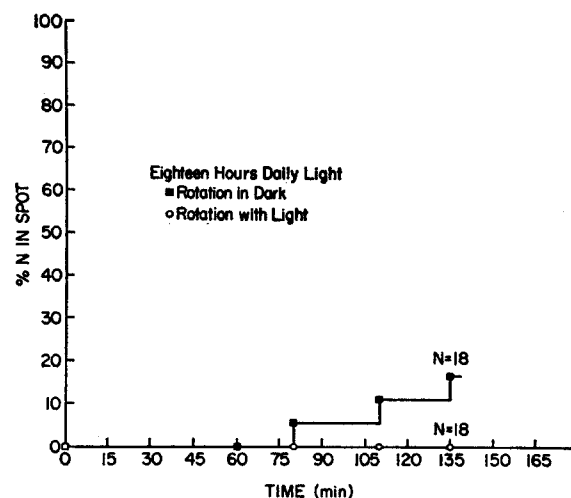

FIGURE 11. Kaplan-Meier (1958) curves for percent of animals which have reached a test light spot as a function of time. Unlike animals described in Figs. 4–10 these animals were exposed to 18 h of light daily. O: Animals tested after 3 h of rotation intervals associated with light. ■: Animals tested after 3 h of rotation intervals in complete darkness. These data were obtained by direct observation. The two groups are not significantly different by the permutation test.

DISCUSSION

Reflex behavior of *Hermissenda* in response to visual and rotational stimuli was described in this and previous reports. It was shown above that repeated temporal association of visual and rotational stimuli results in behavior that does not result from either repeated visual or rotational stimulation alone. This behavioral modification was observed to depend on the daily light cycle.

It was observed that there are two effects of light on the animal: activation or arousal, and attraction. The second effect requires that the animal direct its movement toward a light source i.e., to move in the direction of maximum light intensity. It seems reasonable that the second effect, i.e. attraction toward light, is not possible without the first, activation. The results reported here indicate that the animal's response to light is modified by repeated temporal

association of light with rotation. The method of testing, however, does not reveal whether it is the activation and/or the attraction effect of light which has been modified.

Certainly the behavioral modification described here shares with conditioning an essential feature: its dependency on temporal proximity of repeated stimuli to two distinct sensory pathways. Under the experimental conditions used here, the test light spot no longer attracts *Hermissenda* as it did before repeated pairing with rotation. It is probably not useful, however, to identify this associative training as conditioning. Although the response to light has changed, it has not necessarily become the response evoked by rotation, i.e. clinging (or one similar in function). This would be expected if the behavior were true conditioning in which the conditioned stimulus comes to evoke a response at least similar in function to that originally evoked by an unconditioned stimulus. Further experiments would be helpful in determining what the associative training of *Hermissenda* does and does not have in common with the more familiar conditioning regimens used with vertebrates. For the associative training described, the training parameters could be varied (such as increasing the duration of the rest and/or rotation intervals) to determine the critical associative conditions.

In light of data presented here and the electrophysiologic results previously described, it now seems possible to begin an analysis of changes in neural circuitry (Alkon, in preparation) underlying the observed behavioral modification in *Hermissenda* caused by associated visual and rotational stimulation.

SUMMARY

(*a*) *Hermissenda* are activated by light if they are maintained on a schedule of $6\frac{1}{2}$ h of daily illumination. They also are attracted by light and are able to direct their movements toward and into a light spot.

(*b*) In response to repeated intervals of vigorous rotation, *Hermissenda* right themselves and cling to an available surface with sufficient force to prevent their being shaken free by subsequent rotations.

(*c*) The animals' response to a light spot is modified by repeated association of light with rotation. The response to light is largely unaffected by repeated rotation in darkness.

(*d*) The behavioral modification described varies with changes in the maintenance light schedule.

(*e*) The behavioral data presented, together with electrophysiologic results previously described, now make it possible to begin an analysis of changes in neural circuitry which may underlie behavioral modification in *Hermissenda* caused by associated visual and rotational stimulation.

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