The siphon withdrawal reflex of *Aplysia* undergoes differential classical conditioning with coaneous stimulation of the siphon or mantle shell as the discriminative conditioned stimuli (CS) and shock to the tail as the unconditioned stimulus (US). The reflex has proved to be useful for analyzing the neural mechanisms of conditioning. To test the generality of this experimental system, we have begun to compare the properties of conditioning in *Aplysia* with those of conditioning in vertebrates. We first examined the effect of the interstimulus interval (ISI) by varymg the time between presentation of the CS and US in different groups of animals. Significant differential conditioning was obtained when the onset of the CS preceded the onset of the US by 0.5 sec, and marginal conditioning was obtained when the ISI was 1.0 sec. By contrast, no significant conditioning occurred when the CS preceded the US by 2.5, or 10 sec, when the onsets of the stimuli were simultaneous, or when US onset preceded the CS by 0.5, 1.0, or 1.5 sec (feedback conditioning).

Next examined the effect of contingency by giving one group of animals normal differential conditioning, and a second group the same training but with additional USs interspers between the paired trials. Presentation of these additional USs reduced the degree in which the US was contingent on the CS, but did not change the number of pairings. Animals receiving normal training again showed significant conditioning, whereas animals receiving additional USs showed no conditioning.

The results of these experiments indicate that conditioning of the *Aplysia* siphon withdrawal reflex is similar to conditioning in vertebrates in two major respects: the effects of interstimulus interval and contingency. It may therefore be possible to begin to analyze the neural mechanisms of these features of conditioning in *Aplysia*.

The *Aplysia* gill and siphon withdrawal reflex is proved to be a useful system for studying the neural mechanisms of several types of learning (see Kandel and Schwartz, 1982, for a review). Recently classical conditioning of that reflex has been demonstrated (Carew et al., 1981, 1984), and the mechanism of the conditioning has been analyzed on the cellular level (Auriana et al., 1983; Hawkins et al., 1983; Kandel et al., 1983). In this paper we describe the experiments aimed at achieving a better characterization of conditioning of the reflex via the behavioral level.

It is important for two reasons: First, a further characterization of the behavioral phenomena can place constraints on possible cellular mechanisms of the learning. Second, this characterization can aid the generality of our experimental system. The more similar conditioning in *Aplysia* and in vertebrates is on the behavioral level, the more likely it would seem that basically similar neural mechanisms might be involved in these different species.

Studies of learning in vertebrates have revealed two basic features of conditioning that parallel it from nonassociative learning such as habituation and sensitization. The first of these is contiguity, which refers to the temporal pairing of stimuli. Thus, conditioning of many discrete skeletal responses, such as the rabbit retinoide membrane response, is optimal if the conditioned stimulus (CS) is paired with and precedes the unconditioned stimulus (US) by approximately 0.5 sec, whereas fewer or no conditioning occurs if the two stimuli are presented separately in time or if the US precedes the CS (for reviews, see Gormezano, 1972; Kimble, 1961; Mucknath, 1974). Previous experiments have shown that relative conditioning of the *Aplysia* siphon withdrawal reflex occurs if the CS is weak cutaneous stimulus to the siphon or mantle precedes the US (tax shock) by 0.5 sec (Carew et al., 1981, 1983). In the experiments described in the first part of this paper, we investigated the effect of temporal contiguity by systematically varying the interval between presentation of the CS and the US (the interstimulus interval).

A second basic feature of vertebrate conditioning is contingency, which refers to the degree to which one stimulus predicts another (Lashley, 1949; Rescorla, 1967). One way in which the effect of contingency has been demonstrated is to present extra, unexpected, or unproductive USs during training. This procedure decreases the degree to which the US is contingent on the CS, and decreases conditioning (Rescorla, 1968). In the experiments described in the second part of this paper, we investigated how this effect also occurs in conditioning of the *Aplysia* siphon withdrawal reflex.

Preliminary results from some of these experiments have been reported previously (Hawkins et al., 1983; Kandel et al., 1983).

**Materials and Methods**

*Aplysia* coeniggena weighing 150-300 g were obtained from Pacific Marine (Vancouver, CA), Sea Life Supply (San Diego, CA), or Marine Specimens Unisited (Pacific Palisades, CA). Animals had their parapodia surgically removed to permit visualization of the siphon 1 or more after axotomy, and they were housed in individual perforated circular pans in 350 gallon aquaria for at least 3 more 6 hours before experiments were begun (for details, see Clovis et al., 1983). A differential conditioning procedure based on that described by Carew et al. (1983) was used. For each experimental session, one CS was a 4 sec tactile stimulus applied to the siphon with a styrofoam brush, and the other CS was a 5 sec electric shock (150 ma, 60 Hz AC, for 0.5 sec applied to the mantle shell with bipolar capillary electrodes. The US was a stronger electric shock (13-sec, 60 Hz AC, for 1.5 sec) applied to the sial with capillary


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Effects of Interstimulus Interval and Contingency on Classical Conditioning of the Aplysia Siphon Withdrawal Reflex

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Figure 1. Differential conditioning procedure. A. Dorsal view of an Aplysia. Epicuticular two sites used to deliver conditioned stimuli: the siphon and the mantle shell. The unconditioned stimulus (US) was an electric shock delivered to the tail. For illustrative purposes, the papillae are shown intact and rotated. However, the behavioral studies were all carried out with freely moving animals whose papillae were surgically removed. B. Differential conditioning paradigm. One group (siphon) received the siphon CS (CS+) paired with the US and the mantle CS (CS-) specifically unpaired with the US. The other group (mantle) received the mantle stimulus as the CS+ and the siphon stimulus as the CS-.

Figure 2. Comparison of forward and backward conditioning. A. Diagram illustrating the timing of the CS+ and the CS during 0.5 sec forward and 1.0 sec backward conditioning. B. Results of an experiment in which one group of animals received 4 sec forward conditioning and another group received 1.0 sec backward conditioning. Testing was carried out before (PRE) and 15 min after (TEST) 7 training trials. The animals receiving forward conditioning showed a small increase in responding to the CS+ and a significantly greater increase in responding to the CS-. The comparison of forward conditioning showed no difference in responding to the CS+ and the CS- following training. Statistical analysis was performed using ANOVA with a Bonferroni/Dunn test. All other figures are expressed as means ± SEM.
**Results**

Effect of intraexcitatory interval on conditioning of the siphon withdrawal reflex

We investigated the effect of the interstimulus interval by training different groups of animals with different CS-US intervals and measuring the strength of conditioning. Our first experiment tested backward conditioning. Half of the animals in this experiment were trained with a 1.0 sec backward CS-US interval (US onset preceded CS onset by 1.0 sec) and half the animals were trained with a standard 0.5 sec forward interval (CS onset preceded US onset by 0.5 sec). Each group also received a CS 2.5 min after the US (i.e., specifically unpaired). As expected, the animals receiving 0.5 sec forward training showed a small increase in responding to the CS, which we attribute to associative conditioning, and a significantly greater increase in responding to the CS (Fig. 2). This difference between the increase in responding to the CS and the CS-US interval (ΔCS-US = 0.5 sec) is our measure of associative learning. Using this measure, a within-group comparison showed that animals receiving the 0.5 sec forward training exhibited significantly associative learning (mean conditioning score = 12.2 ± 3.5 SEM, t₁₀ = 3.47, p < 0.01). By contrast, animals receiving the 2.0 sec backward training did not show evidence of associative learning (mean learning score = 0.4 ± 2.3, t₁₀ = 0.17, not significant). Moreover, a between-groups comparison showed that conditioning for the 0.5 sec forward group was significantly greater than that for the 1.0 sec backward group (ΔΔCS = 7.47, p < 0.05). Since animals were randomly assigned to the two groups and were treated the same in all other respects, we conclude that training with a 1.0 sec backward CS-US interval produces little or no conditioning of the siphon withdrawal reflex.

We then performed a series of experiments similar to the one illustrated in Figure 2, but with different CS-US intervals (Fig. 3, Table 3). Of the intervals tested, the best conditioning was obtained with the standard 0.5 sec forward training (Fig. 3 and Table 3 show the average results from all replications of this condition). Marginal conditioning occurred with a 1.0 sec forward interval (ΔCS-US = 2.00, p < 0.05; one-tailed), while there was no evidence of conditioning with forward intervals of 2.0, 5.0, or 10.0 sec, or backward intervals of 1.0 or 1.5 sec. The same pattern of results was obtained when animals were tested 15 min after training and when they were retested 24 hr after training (Table 3). Control animals trained with a 0.5 sec forward CS-US interval did show significant differential conditioning both as 15 min and 24 hr in each of these experiments (p < 0.05; one-tail or joker in each case).

Since stimuli were delivered by hand in these experiments, the CS-US interval was only accurate to within about 0.25 sec. This margin of error should not be important for shorter intervals but could be a problem for intervals longer than 10 sec. In addition, training by hand allowed some possibility of experimenter bias. We therefore performed a second series of experiments in which we investigated the effect of conditioning with two additional CS-US intervals (0.5 sec backward and simultaneous presentation of the CS and US) with both CSs and the US delivered under electronic control through implanted electrodes. In addition, we trained half of the animals in these experiments with a 0.5 sec forward CS-US interval, which again produced significant differential conditioning (mean conditioning score = 12.9 ± 4.0, t₁₀ = 3.22, p < 0.05). By contrast, training with a 0.5 sec backward interval produced no evidence of conditioning.
while training with simultaneous presentation of the CS' and US produced a negation of conditioning that was not, however, statistically significant (Fig. 5, Table 1).

In experiments with either manual or implanted stimulation, the CS- pathway was the siphon for half of the animals and the mantle for the other half of the animals in each group. We have combined these conditions in the analysis thus far, since previous experiments had shown that conditioning with a 0.5 sec forward CS-US interval is approximately equal in both cases (Carew et al., 1983). However, it is possible that the CS-US interval producing optimal conditioning depends on whether the siphon or mantle is the CS. This seemed particularly likely in experiments with manual stimulation, where the stimuli to the siphon and mantle had different physical characteristics (i.e., brief tactile stimuli to the siphon and 0.5 sec electrical stimuli to the mantle). In order to examine this question, we reanalyzed the data, comparing the siphon scores for the Siphon animals to the siphon scores for the Mantle animals (in which siphon stimulation was the CS) to obtain an estimate of siphon conditioning. Similarly, we compared the mantle scores for the Siphon animals to get an estimate of mantle conditioning for each CS-US interval. This analysis shows significant conditioning for both the siphon and the mantle with 0.5 sec forward training and not with any other CS-US interval (Table 2). Thus, the interval-dependent interval function does not depend on whether the siphon or mantle is the CS; within our limits of resolution.

The results of these experiments demonstrate an effect of temporal pairing or contiguity in conditioning of the Aplysia siphon withdrawal reflex similar to that seen in many instances of vertebrate conditioning, such as conditioning of the rabbit auditory stimulus-response (Gormezano, 1972).

**Table 1. Interstimulus interval (ISI) function (within-group conditioning scores)**

<table>
<thead>
<tr>
<th>ISI (sec)</th>
<th>Manual stimulation</th>
<th>Implanted electrodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>2.0</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>5.0</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>10.0</td>
<td></td>
<td>0.5</td>
</tr>
</tbody>
</table>

*p < 0.01

*p < 0.05

*p = 0.06, one-tailed.

Effect of contingency on conditioning of the siphon withdrawal reflex

We investigated the effect of contingency by adding extra, unpaired or unpredicted USs during training. The basic experimental design is illustrated in Figure 4b. One group of animals received five trials of normal differential conditioning with a 0.5 sec interval interval. A second group of animals (randomly selected from the same population as the first group) received exactly the same training, but in addition received five unpredicted USs interspersed among the training trials. Thus, both groups received the same number of pairings of the CS' and the US (the same amount of contiguity), but for the second group the US was less contingent on the CS' than it had been to occur.

The results of our first experiment with this design are shown in Figure 4b. This figure and Fig. 5 show the data from the 24 hr posttest, at which time the CS' and US strengths have recovered from any habituation during training (see below); the data from the 15 min posttest were qualitatively similar, but were somewhat more variable. As expected, the animals receiving normal training showed good differential conditioning (mean conditioning score = 15.6 ± 3.0, p < 0.001). By contrast, animals receiving additional, unpredicted USs did not show significant conditioning (mean conditioning score = 6.0 ± 2.6, p < 0.001). We have replicated this basic effect in three additional experiments with the same design but with different intertrial intervals or numbers of training trials (see below and Table 2). In each case the result was the same: Animals receiving normal training showed significant conditioning, while animals receiving additional, unpredicted USs did not. When the data from all of these experiments are pooled, animals that received unpredicted USs

**Table 2. ISI function (between-group conditioning scores)**

<table>
<thead>
<tr>
<th>ISI (sec)</th>
<th>Manual stimulation</th>
<th>Implanted electrodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>2.0</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>5.0</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>10.0</td>
<td></td>
<td>0.5</td>
</tr>
</tbody>
</table>

*p < 0.01

*p < 0.05

*p = 0.06, one-tailed.
had significantly lower conditioning scores than the animals that received normal training (mean conditioning scores = 0.2 ± 2.2 and 11.3 ± 1.3, respectively, t₁₄ = 4.38, p < 0.01) and, on average, showed no differential conditioning at all (Fig. 3). The lack of differential conditioning in the animals receiving unpredicted USs, compared to animals receiving normal training, was due to two effects: minimally greater responding to the CS+ in the groups tested, to which we would attribute a positive bias created by the extra USs (mean UPs = 10.3 ± 1.7 for animals receiving unpredicted USs and 3 ± 1.5 for animals receiving normal training, t₁₄ = 1.74, p < 0.05; one-tail), and significantly less responding to the CS− (mean UPS = 9.8 ± 1.7 for animals receiving unpredicted USs and 17.7 ± 1.4 for animals receiving normal training, t₁₄ = 3.39, p < 0.01).

In the experimental design illustrated in Figure 4d, we altered the contingency of the US on the CS while holding the number and timing of CS−US pairs constant. In order to do this, we had to alter two other variables: the number of USs and the time between USs. To test whether these alterations, rather than the alteration in contingency, might account for the observed decrease in conditioning, we performed three additional experiments (Table 3). First, we were concerned that animals given unpredicted USs might not have conditioned because they received a higher frequency of USs (one every 2.5 min on average) than animals given normal training (one every 5 min). However, US frequency does not appear to be a critical variable, since (1) animals given normal training with a 2.5 min ITI showed good conditioning (Table 3, Experiment B), and (2) animals given unpredicted USs with a 10 min ITI (thus receiving a US once every 5 min, on average) still did not condition (Table 3, Experiment D). Second, it was possible that animals given unpredicted USs did not condition because they received more USs (109 than animals given normal training (live). However, ani

Table 3. Effect of unpredicted USs (within-group conditioning scores)

<table>
<thead>
<tr>
<th>Group</th>
<th>USs</th>
<th>Trials</th>
<th>ITI (min)</th>
<th>d</th>
<th>SEM</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Normal</td>
<td>5</td>
<td>5</td>
<td>15.6</td>
<td>3.0</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>5</td>
<td>5</td>
<td>6.8</td>
<td>4.5</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>C Normal</td>
<td>5</td>
<td>5</td>
<td>11.3</td>
<td>3.6</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Unpredicted</td>
<td>5</td>
<td>5</td>
<td>6.9</td>
<td>4.2</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>D Normal</td>
<td>10</td>
<td>10</td>
<td>13.8</td>
<td>5.7</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Unpredicted</td>
<td>10</td>
<td>10</td>
<td>7.7</td>
<td>2.2</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>1.6</td>
<td>65</td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05

Figure 2. Posed results from four experiments like the one shown in Figure 4, but with different USs or numbers of training trials (see Table 3). On average, animals receiving normal training showed significantly different conditioning, while animals receiving unpredicted USs showed no differential conditioning.

Dissociation

The results of our experiments indicate that conditioning of the Aplysia siphon withdrawal reflex is similar to conditioning in vertebrates in two major respects: the effects of ISI and contingency. The ISI function for conditioned siphon withdrawal (Fig. 3) is very similar to such functions for discrete skeletal reflexes in vertebrates, such as the rabbit nictitating membrane response (Smit and others, 1969), human Weber's (Bernstein, 1934; McNaughton, 1959), and rat paw withdrawal (Spongso and Kellgren, 1947). In each of these cases, conditioning is optimal when the CS precedes the US by approximately 0.5 sec, and then is
less or no conditioning with longer intervals, or when the US precedes the CS during training (for reviews see Germaine, 1972; Kimmel, 1961, Markuzoh, 1974). The results of our experiments on the effect of contingency air also qualitatively similar to the results of analogous experiments with rats (Rescorla, 1968). Conditioning of the midbranch mollusk Helix pomatia shows a similar effect of contingency (Farley and Kern, in press), and conditioning of another gastropod mollusk, Limax maximus shows three other features of vertebrate conditioning: US-US overlap, US-onset, second order conditioning, and blocking (Saklofske et al., 1981). A form of blocking has also recently been demonstrated in Aplysia (Calabri, 1985).

In addition to these similarities, there are also some differences between conditioning of the Aplysia siphon withdrawal reflex and vertebrate conditioning. For example, many vertebrate behaviors show a tight interstimulus interval function (e.g., Smith et al., 1949) or a strong effect of contingency (Rescorla, 1968), but not both. Furthermore, it is often more than five trials to establish an effect of contingency in vertebrates (e.g. Rescorla, 1968). Finally, vertebrate conditioning sometimes involves the appearance of a new response, rather than the strengthening of a preexisting response (e.g., Germaine, 1972). We do not feel that this difference is fundamental, however, since in several cases, neurophysiological experiments in vertebrates have shown that conditioning simply strengthens a preexisting neural response until it is above a threshold for producing an observable behavioral response (e.g., Cagan et al., 1970). On balance, therefore, we feel that the similarities between conditioning in molluscs and in vertebrates are more important than the differences, which suggests that similar neurochemical mechanisms could underlie conditioning in these different phyla.

In addition to providing a test of generality, our behavioral results also place constraints on possible cellular mechanisms of the conditioning. The neuronal mechanisms we believe underlie the change in the neuronal correlates of a reflex activity-dependent amplification of preyspecific facilitation. In brief, the US's in our behavioral experiments produces prey-specific facilitation of transmitter release from sensory neurons in the CS pathway. This facilitation involves a sequence of steps that begin with the change in cyclic AMP and elevate the cAMP levels in the sensory neurons (see Kandel and Schwartz, 1982). We have found that the facilitation is amplified if there is spike activity in the CS sensory neurons just before the US is delivered, as occurs during behavioral conditioning, and we believe that a change in the conditioning (Harding et al., 1983; see also Walters and Byrne, 1983). Preliminary experiments suggest that this amplification of facilitation may be due to "priming" of the adenyl cyclase by Ca2+ which enters the sensory neuron during the spike activity, so that the cyclase produces more cAMP in response to the US (Abrams et al., 1983; Kandel et al., 1983; Ovear et al., 1983). If this amplification of facilitation is, in fact, a mechanism of the conditioning, we would predict that it should have the same ISI function as the behavior. Preliminary data indicate that forward pairing of spike activity and the US is more effective than backward-pairing (Clark, 1984). We do not yet understand why spike activity in the sensory neurons has to occur shortly before the US is delivered to amplify its effectiveness, but we hope that a more detailed understanding of the molecular mechanisms of the conditioning will provide an explanation for this temporal requirement.

We also do not yet have any experimental evidence concerning the neuronal mechanisms of the effect of contingency. That effect actually seems contrary to the neuronal mechanisms of conditioning proposed above, which might be expected to predict that presentation of extra-US's should simply produce more facilitation. Rescorla and Wagner (1972) have suggested that the effect of contingency is due to conditioning of background stimuli, which blocks conditioning of the experimental CS. Recent behavioral evidence from Aplysia tends to support this idea (Calabri, 1981). Hawkins and Kandel (1984) have attempted to translate Rescorla and Wagner's hypothesis into a neuronal model based on known Aplysia circuitry. They have also proposed a somewhat similar hypothesis, which is that repeated presentations of the US may lead to habituation of its effectiveness, perhaps due to synaptic depression in the US pathway. A quantitative simulation based on this idea has produced results similar to the results shown in Figure 3 (Hawkins and Kandel, 1984).

This hypothesis is probably somewhat simplistic, however, and we do not feel that it will provide a complete explanation for the effect of contingency. We mention it primarily to illustrate a more general idea, which is that the neuronal mechanisms of higher-order features of learning, such as the effect of contingency, may be built by putting together combinations of the mechanisms of simpler forms of learning, such as synaptic depression and activity-dependent facilitation. At the moment, this proposal is speculative, but we hope that it will provide a useful framework for further investigations into the neuronal mechanisms of learning.

References