A QUANTITATIVE ANALYSIS OF BEHAVIORS DURING HABITUATION TRAINING OF THE CILIATED PROTOZOAN, SPIROSTOMUM AMBIGUUM

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This is to certify that the

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ABSTRACT

A QUANTITATIVE ANALYSIS OF BEHAVIORS DURING HABITUATION TRAINING OF THE CILIATED PROTOZOAN, *SPIROSTOMUM AMBIGUUM*

By

Thomas Charles Hamilton

Previous studies have shown that a large group of *Spirostomum ambiguum* will show a gradually decreasing probability of contraction (habituation) to a repetitive mechanical stimulus. This study characterizes other behaviorally identifiable modifications which occur to repetitive mechanical stimulation, and evaluates the possible influences of secondary effects (such as migration within the stimulation chamber) upon the observed behavioral modifications. Such a study should provide the behavioral groundwork upon which molecular investigations of these learning related phenomena can be based.

Forty *Spirostomum* were given ten minutes of repetitive mechanical stimulation (0.1 Hz). Retention stimuli were given at two, five, and ten minutes after the end of the stimulation period. Each of the forty animals was tested individually and movements of the animals during the stimulation period and during the retention trials were recorded on video tape. The tapes were then analyzed to determine modifications in the responses elicited to the mechanical stimuli and changes in the ongoing swimming activities of the animals.

These analyses have shown that several behavioral modifications are induced by repetitive mechanical stimulation. 1. There is a change over time in the probability with which contractions are elicited such that animals with a high initial probability of contraction habituate and those with a low initial probability of contraction appear to sensitize (increase in contraction probability). Furthermore, the "high" responders remain more responsive than the "low" responders throughout a ten minute stimulation period. 2. The magnitudes of contraction are decreased by about 10% after an animal has contracted to several consecutive stimuli. 3. The magnitude of avoidance reactions decreases over time. These behavioral modifications were found to be independent of considerations of the animal's location in the stimulation chamber, swimming activity at the time of stimulus delivery, attitude (orientation with respect to gravity), and body shape.

Latencies of contraction to mechanical stimulation were found to be independent of stimulus intensity and were not changed by repetitive stimulation. Previous electrical stimulation, which lowers contraction probability to mechanical stimulation and which causes increased latencies of contraction to electrical stimuli, does not change latencies of contraction to mechanical stimulation.

The results suggest that changes in calcium concentrations are important in controlling the observed behavioral modifications. Preliminary electron microprobe analyses further suggest that differences in initial contractile responsiveness may be due to differences in the calcium to magnesium ratio.

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By

Thomas Charles Hamilton

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INTRODUCTION

Interpreting results from experiments designed to investigate learning and memory is often difficult due to the great complexity of neural systems. Even the increasingly popular "simple system" preparations must evaluate the influences of intercellular connectivities upon individual neurons chosen for study. Such concerns make investigations on learning related phenomena in protozoans of interest.[1-17,24,29-32,35,36,44,46,52-54] For all behaviors and behavioral modifications are confined to one cell. But one cell does not equal one behavior. These single celled organisms possess a complex repertoire of behaviors,[32] and thus may provide extremely useful systems in which to investigate the cellular and molecular bases of behavioral modifications.

One protozoan often used for such studies is *Spirostomum ambiguum* (see Figure 1). This cigar-shaped organism, which can grow up to 3 mm in length, has a large repertoire of behaviors. It possesses both a ciliary system, capable of propagating the animal in the direction of its long axis in the typical forward, spiraling mode of ciliate swimming, and a contractile system capable of shortening the animal to less than 50% of its resting length in about 5 msec. The animal also responds to several modalities of stimulation (e.g., electrical, tactile [mechanical], chemical, light).[19] The animal may contract when a sufficiently strong stimulus is given. In conjunction, it may also reverse the direction of its ciliary beat and swim backwards (avoidance reaction), or it may simply alter its ciliary activity such that it either stops or throws its

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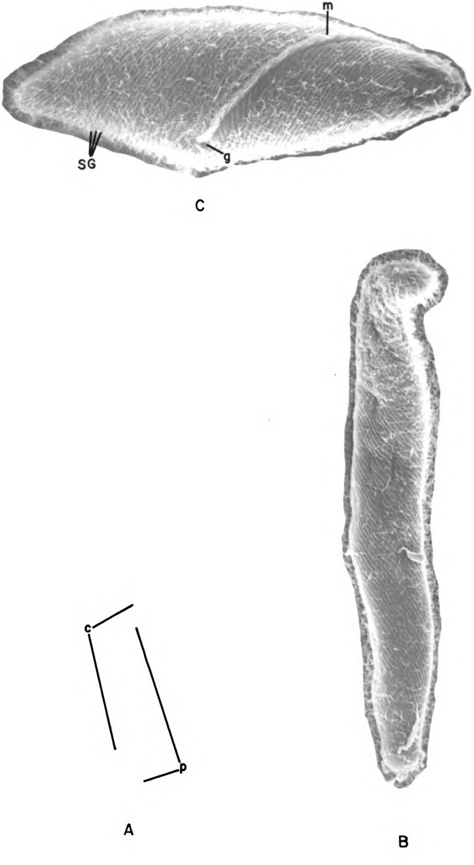

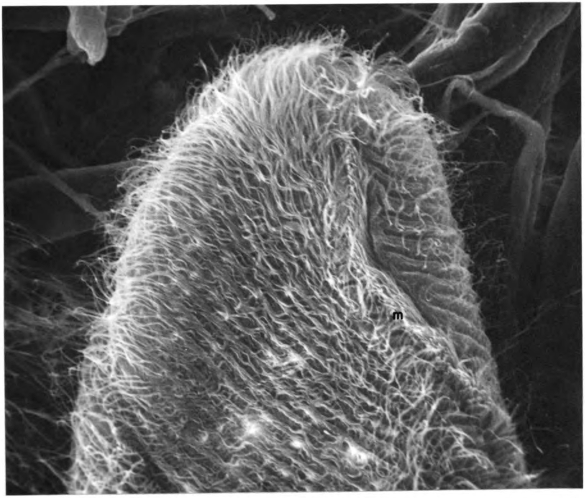

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Figure 1. Light and scanning electron micrographs of *Spirostomum*. Figure 1A shows light micrographs (220x) of a *Spirostomum* during normal swimming behaviors and of a contracted animal. Anterior is toward the top. Note the contractile vacuoles (c), anal pores (p), and the dark cytoplasmic inclusions. Figure 1B shows a scanning electron micrograph (200x) of a freeze-dried, partially elongated *Spirostomum*. Anterior is towards the top. Note the somatic grooves and the cortical cilia. Figure 1C shows a scanning electron micrograph (400x) of a contracted *Spirostomum*. Anterior is toward the right. The somatic grooves (SG), cilia, membranelle (m), and opening to the oral groove (g) are visible. Note the change in the pitch of the somatic grooves between Figures 1B and 1C. Figure 1D (1000x) is a scanning electron micrograph of the anterior tip of a *Spirostomum*. Note the thick coat of cilia, somatic grooves, and the membranelle (m). The cilia emanate from within the somatic grooves.

anterior end slightly sideways (startle response). In addition to its normal forward and backward swimming behaviors, an animal can also rotate on its posterior end, sometimes in conjunction with bending its body, and "probe" its immediate environment, thereby randomizing its orientation before resuming normal swimming behaviors. Thus, *Spirostomum* would appear to offer a rich behavioral repertoire for examining learning and memory related phenomena.

It has been shown that a decrease occurs over time in the number of contractions elicited by a large group of *Spirostomum* to repetitive mechanical stimulation.[35,36] This phenomenon of gradually decreasing responsiveness is common throughout the animal kingdom and is known as habituation. Investigations were undertaken to determine those biochemical alterations which occurred in conjunction with habituation in *Spirostomum*.[4,6-10,29] But these studies did not investigate or even consider the possibilities that while the group as a whole showed habituation (1) some of the animals being stimulated might actually be sensitizing (increasing in response probability), (2) some may not have changed in response probability, and (3) some other behavioral modifications might be occurring in the animals investigated. The occurrence of these other possibilities might cause additional molecular alterations which would tend to complicate the interpretation of biochemical results designed to explain contractile habituation. Furthermore, previous studies on *Spirostomum* have not even demonstrated that the contractile habituation is indeed a modification of the contractile system itself, rather than a secondary effect resulting from some other alteration. For instance, the animals might simply migrate to portions of the stimulation container where the stimulus intensity is weaker; or the stimulation may cause increased or decreased amounts of ciliary activity which may indirectly affect the stimulus sensitivity of an animal's contractile system. This study evaluates the possible contributions of such factors to the contractile habituation phenomenon observed in *Spirostomum ambiguum* and characterizes those behaviorally identifiable modifications which occur in the contractile and ciliary systems of *Spirostomum* over a period of repetitive mechanical stimulation. Based on this behavioral framework it will become meaningful to examine in *Spirostomum* the molecular correlates of the various behaviors and to causally relate specific behavioral and molecular changes.

Studies using the electron microprobe analyzer in conjunction with electron microscopy were also undertaken to investigate some of the possible physical-chemical correlates of observed behavioral differences among animals.

METHODS

Culturing Procedures

Spirostomum ambiguum were obtained from Connecticut Valley Biological Supply Company, Southampton, Massachusetts, and were thereafter cultured and grown at room temperature (23.5 ± 1 C) in our laboratory. The animals used in behavioral series A were grown in unbuffered Carter's medium[20] which had a few preheated wheat seeds added to about 100 ml of culture medium in a four inch diameter glass culture bowl. The wheat seeds supported a fairly dense population of bacteria upon which the *Spirostomum* fed. The light cycle varied with room use, usually being turned on about 7:30 a.m. and turned off at about 11:00 p.m. during the week. Weekends were more variable. Animals used for behavioral series B were all derived from one original animal and were kept in the same culture bowl. They were grown in Carter's medium buffered to pH 7.2 (see Appendix 1) and kept under constant room illumination. The culture was occasionally thinned to maintain an exponential growth phase and the culture medium (100 ml) was changed as completely as possible once each week. (Later studies by Thompson[*50] have indicated that culture bowls should also be changed biweekly.) Extremely heavy growths of *Aeromonas hydrophila* and lighter growths of *Enterobacter (Aerobacter)* *sp.* and *Mima polymorpha var. oxidcons* were determined to comprise the

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[*] Jeffrey Thompson has assisted in the research investigations of our laboratory for the past 2-1/2 years. His efforts, as yet unpublished, will be cited often in this text.

bacterial population. Each animal was used in only one experiment and data from any unhealthy looking animals were discarded.

Behavioral Studies

In all behavioral experiments large, free swimming animals were selected for individual study (see Appendix 2). They were first placed in a round slide well (about 1 mm deep and 6 mm diameter) which was covered by a moist evaporation cover (Figure 2). The cover prevented cooling of the medium in the slide well due to evaporation and also prevented the concentration of the medium's ionic milieu over the test period. The slide was then placed on the stage of a Nikon M inverted microscope and allowed to equilibrate under experimental conditions for either 15 min (behavioral series A) or for 10 min (behavioral series B) before stimulation was begun. Control experiments showed that the temperature of the slide well equilibrated at room temperature within one minute after the slide was placed on the microscope stage. (Medium temperatures in the culture bowls were usually found to be about 0.5 to 1.0 C below room temperature.) The swimming behaviors of the animals also stabilized after about 10 min in the slide well. (Earlier results[46] have shown the initial contractile responsiveness of the animal to stabilize after a five to ten minute equilibration period.) After the equilibration the animals were stimulated for 15 min (behavioral series A) or for 10 min (behavioral series B) with a repetitive (0.1 Hz) mechanical stimulus elicited by spring driving an 11.5 g weight from a height of 9.5 mm onto the slide on which the *Spirostomum* was swimming. (The stimulus intensity was found to be stable for the duration of an entire experiment.) All stimuli within any given experiment were of a constant intensity, but the stimulus intensity was varied slightly

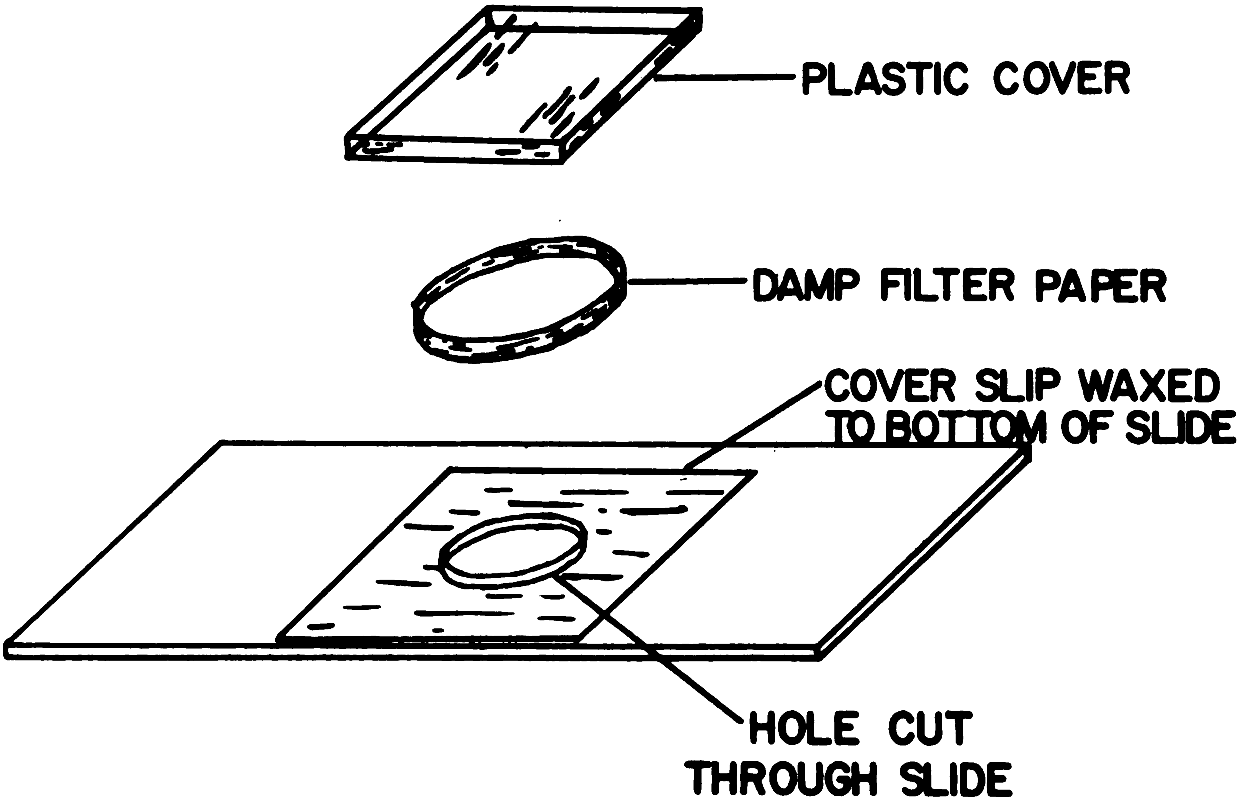

Figure 2. Mechanical stimulation slide. Figure 2 is an illustration of the 76 x 26 x 1 mm glass slide used for behavioral studies using mechanical stimulation. The hole cut through the slide was 6 mm in diameter. A damp ring of filter paper, placed on the slide, enclosed the well. This was sealed by placing the plastic cover on the filter paper and fastening it to the slide with masking tape.

between experiments in behavioral series A in order to artificially produce animals of differing initial contractile responsiveness.

Several additional stimuli were presented to the animal at two minutes, five minutes, and ten minutes after the termination of the initial stimulation period to measure the rate at which the modified behaviors returned to their baseline values. Three stimuli were given at both the two and five minute marks and six stimuli were given at the ten minute check. Thompson[50] has shown that giving retention stimuli at each of these three times does not affect the rate of recovery relative to animals stimulated at only one, rather than all, of the three periods.

In all cases, movements of the animals during the ten minutes of stimulation and during the two, five, and ten minute retention trials were recorded on video tape. The tapes were then replayed at a later time to make the desired behavioral measurements. The following behavioral measurements were made for behavioral series B:

1. The response exhibited by the animal in response to the stimulus. The following response classifications were used: (a) contraction - a rapid shortening to about one-half resting length, (b) avoidance reaction (ciliary reversal) - a definite, stimulus induced movement in the posterior direction, (c) no response - no visually detectable movement in response to the stimulus, (d) startle response - any visually observable response to the stimulus which did not involve either a contraction or a ciliary reversal (e.g., throwing the anterior end sideways or stopping). Startle responses were similar to avoidance reactions in that both involved a ciliary response.

2. The location of the animal in the slide well when the stimulus was delivered. The animals were classified simply as being in the center, intermediate, or edge zone of the wall. The center was defined to be that portion of the well closer to the exact center than 1/3 the radius of the well. The edge was that portion within 1/3 the radius of the well from the edge.

3. The attitude of the animal when the stimulus was given. The animal was simply classified as being either horizontal or vertical.

4. The body shape of the animal at the time the stimulus was presented. The animal was classified as being either bent or straight.

5. The behavior in which the animal was engaged at the time the stimulus was delivered. The following behavioral categorizations were used: (a) forward swimming - normal ciliary mediated movement in the anterior direction, (b) backward swimming* - movement in the posterior direction following a ciliary reversal, (c) probing - a variable behavior by which the animal seems to explore his immediate environment by rotating his anterior end while remaining in place or by pivoting on his posterior end, (d) stopped - the absence of any detectable movement.

6. The amount of time occupied by each of the behaviors described in point 5 above during the 10 sec prior to each stimulus during the first, third, and tenth minutes of stimulation and during the retention trials. This provided a measure of changes in on-going activity over the stimulation period.

7. The timecourse (change in body length over time) of those contractions which did occur. The measurements were made by manually advancing the video tape one half frame at a time. Each half frame was 1/60 sec.

8. The distance backed up and the time spent backing up on those ciliary reversals which did occur.

The more time consuming measurements (points 6-8) were not made on all animals. (Only behavioral measures from points 1, 7 and 8 were made for series A.)

The following experimental conditions were also recorded for series B:

1. The room temperature at the microscope at the beginning and end of each stimulation period.

2. The temperature of the culture medium when an animal was removed from the culture bowl and placed in the stimulation slide.

3. The date and time of day that stimulation was begun on each animal tested. This information was used to look for the presence of biological rhythms.

4. The person taping the stimulation session and the person analyzing the video tape were also recorded.

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*Backward swimming is the result of avoidance reactions. However, in this text the term avoidance reaction will be used to describe only those responses elicited to the mechanical or electrical stimuli used during experimentation. Backward swimming will refer to backward movement following both stimulus induced and spontaneous ciliary reversals.

All animals used in series B were tested (and recorded on video tape) on either July 8, 1971, or on August 26-28, 1971. Thus, results were obtained from two, relatively uniform groups of animals, and possible contributions due to long term culture variations could be evaluated.

A short series of experiments was also performed using electrical shock. The stimulation schedule was the same as in series B, but two different variations of stimulation were used. Both stimulus variations produced an initial contraction probability of about 70%, but one was a 2 msec, 50 V, biphasic shock and the other was a 0.2 msec, 95 V, biphasic shock. Only points 1, 7 and 8 of behavioral series B were made in these studies.

All video tapes from each behavioral series were completely analyzed before the data were examined for any trends. Some of the tapes were analyzed independently by a second investigator as a control for possible experimenter bias.

Latency of Contraction Studies

Determinations were made of the latency of the onset of contraction to both mechanical and electrical stimuli. These measurements were made to determine whether or not there was any relationship between latency and stimulus intensity. In addition, changes in latency were recorded over a period of repetitive stimulation of a constant intensity. It was also determined whether or not repetitive mechanical or electrical stimulation would modify the latency of contractions elicited by the other stimulus.

For studies using electrical stimulation an animal was placed in the square well of a slide having platinum electrodes painted on opposite sides (Figure 3). The well was lighted with darkfield illumination

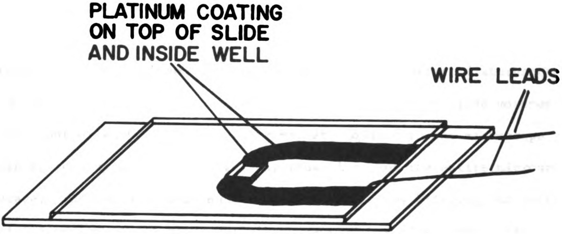

Figure 3. Electrical shock slide. Figure 3 is an illustration of the slide used for behavioral studies using electrical stimulation. A 5 mm square hole was cut through a 60 x 26 x 1 mm glass slide and platinum electrodes were painted on the top of the slide and opposite sides of the square hole. This slide was then waxed to a 76 x 26 x 1 mm glass slide and lead wires were attached to the electrodes. The square well produced a uniform electric field throughout the well.

(Figure 4). Light from the well was split two ways: 50% went to a video camera and was displayed on a TV screen and 50% was directed into a phototube mounted on a Nikon M inverted microscope. Measurements were made by displaying the phototube output on an oscilloscope (CRO). The CRO trace was triggered by the onset of the biphasic stimulus and the contraction onset appeared as a sharp deflection due to the decreased light reaching the phototube.

Measurements with mechanical stimulation required modification of both the stimulating apparatus and the slide well containing the animal. These modifications reduced interference with phototube recordings by reducing gross slide movements and by eliminating surface disturbances caused by using a dropping weight as a stimulus. Gross slide movements were controlled by using an electromagnet, mounted to the microscope stage, to snap an 11.5 g weight up, thus vibrating the entire microscope stage. The surface disturbances were controlled by bringing the surface of the medium in the slide well in contact with a glass cover slip. This was accomplished by supporting a cover slip approximately 1 mm above a slide (Figure 5) and suspending a drop of medium containing an animal between them. Electrical tape was placed on the bottom of the slide to mask stray light scattered by the meniscus.

Experiments which measured the effect of mechanical stimulation on latencies to electrical stimuli were conducted by mechanically stimulating animals in the square-welled, electrical shock slide for 2-7 min at 0.2 Hz, prior to measuring latencies to electrical stimulation. Two platinum wires, which served as electrodes for electrical stimulation, were inserted under the cover slip of the slide designed to measure latencies to mechanical stimulation (Figure 5). The slide was then used

Figure 4. Setup for latency measurements. Figure 4 is a schematic of the apparatus used for measuring latency of contraction. The stimulator both triggered the oscilloscope trace to coincide with the stimulus onset and delivered either an electrical shock or a mechanical stimulus to the *Spirostomum* in the slide well. Darkfield illumination was used. This caused the *Spirostomum* to appear bright against a dark background. When the animal contracted it became smaller and caused less light to reach the phototube. This resulted in a sharp deflection in phototube output to be viewed on the oscilloscope screen.

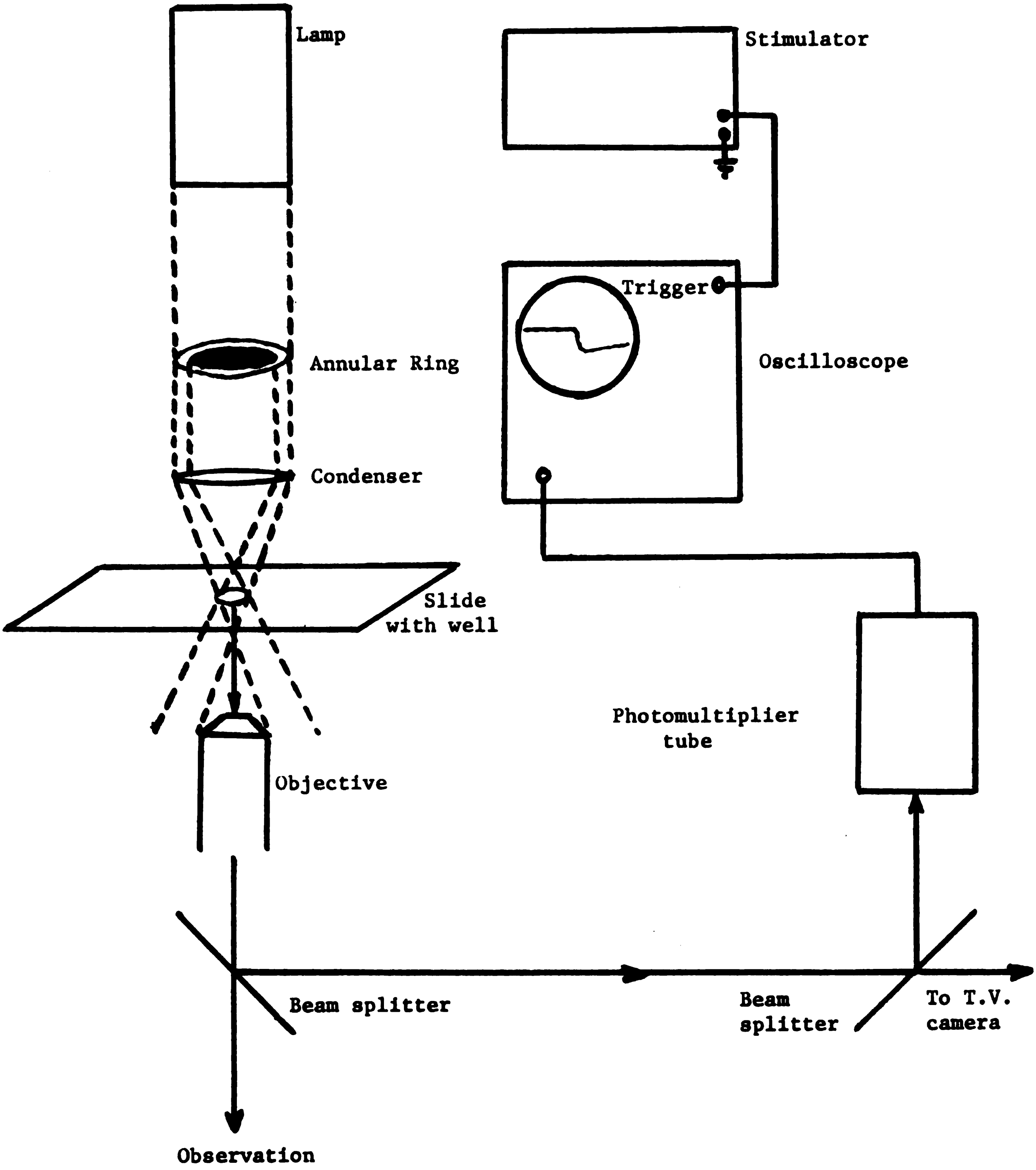
Lamp
Stimulator
Trigger
Annular Ring
Oscilloscope
Condenser
Slide with well
Photomultiplier tube
Objective
Beam splitter
Beam splitter
To T.V. camera
Observation

Figure 4

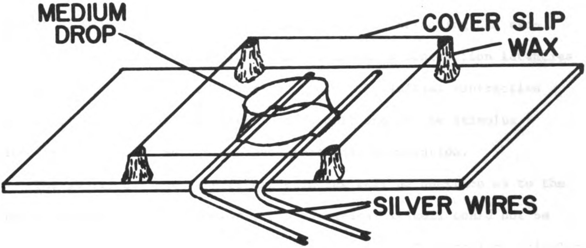

Figure 5. Mechanical latency slide. Figure 5 is an illustration of the slide used to measure latencies of contraction to mechanical stimulation. A glass cover slip was supported with wax about 1 mm above a 76 x 26 x 1 mm glass slide. A drop of medium containing a *Spirostomum* was placed between the slide and cover slip. A piece of electrical tape with a 6 mm diameter hole was placed on the bottom of the slide to block light scattered by the edges of the medium. For experiments measuring the effect of electrical stimulation on latencies of contraction to mechanical stimulation, two silver wires were inserted into the medium to serve as electrodes.

to measure the latency of contractions to mechanical stimuli following two to seven minutes of 0.15 Hz electrical stimulation.

All latency measurements were made on individual *Spirostomum* by maintaining a constant stimulus intensity and stimulating repetitively at a rate of from 0.1 to 0.2 Hz. Difficulties were encountered in accurately synchronizing the CRO sweep delay with the mechanical stimulus onset, for the onset varied with stimulus intensity. The intensity of electrical stimuli could be easily varied without affecting CRO synchronization: however, a rapid change occurred in contraction latencies to repetitive electrical stimulation. Thus, the initial contraction latency of each animal was determined at only one of the stimulus intensities for both mechanical and electrical stimulation.

The short, 0.2 msec electrical stimulus left no question as to the time of stimulus onset. However, the mechanical stimuli could not be so neatly evaluated. Figure 6A illustrates a typical mechanical stimulus as monitored by a barium titonate strip. It was arbitrarily decided to use the beginning of the mechanical disturbance as the time of stimulus onset. The delayed sweep on the CRO was set to begin, as illustrated in Figure 6B, at this point in time.

The construction of the mechanical stimulation slide used for latency studies prevented the use of an evaporation cover. Also, the darkfield illumination used resulted in a much greater intensity of light reaching the slide well for both mechanical and electrical stimulation than reached the slide during the behavioral studies. This extra light often caused noxious reactions in the animals. Therefore, very little time, perhaps one minute, was allowed for the *Spirostomum* to equilibrate in the slide before stimulation was begun.

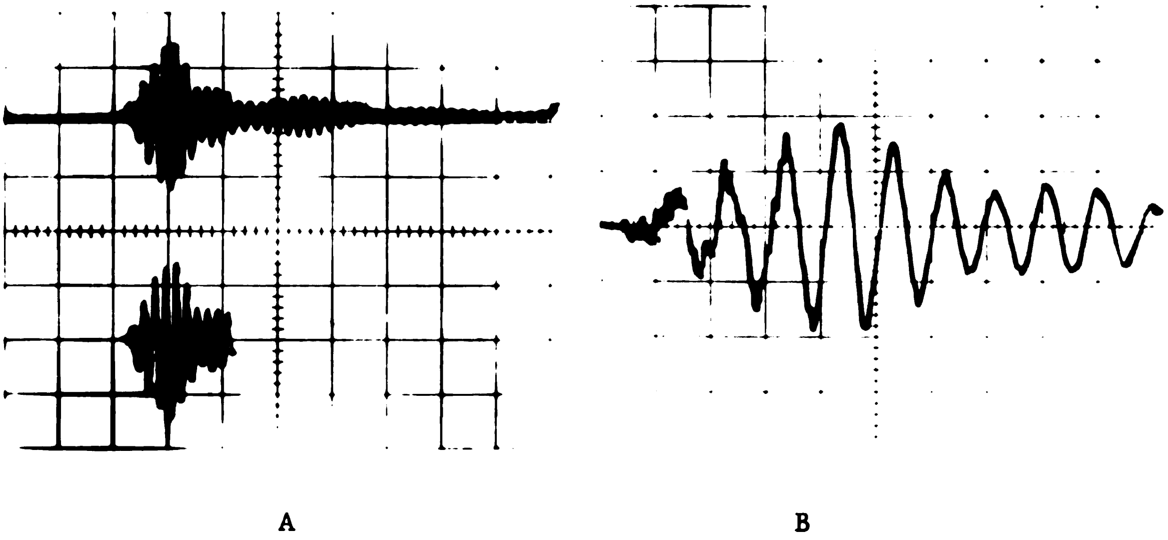

Figure 6. Shape of the mechanical stimulus. Figure 6A, upper trace, shows the shape of the mechanical stimulus as displayed on an oscilloscope and recorded by a barium titonate strip mounted to the microscope stage. The lower trace shows the 20 msec portion of the stimulus during which the phototube output (not shown here) was displayed on the oscilloscope to record contraction latencies. Time scale is 10 msec/division. Figure 6B shows an expansion, 2 msec/division, of the lower trace of Figure 6A. Notice that time zero on the grid corresponds to the beginning of the stimulus envelope.

Response Group Correlations

In order to determine whether or not there was any correlation between the initial contractile responsiveness to mechanical stimulation and that to electrical stimulation, animals were tested individually to determine responsiveness to each modality. Each animal was given five mechanical stimuli and its response level (zero to five contractions) was determined. Five electrical stimuli were then presented and this response level was determined. (Osborn[46] has shown that repetitive mechanical stimulation does not alter contractile responsiveness to electrical stimulation.) The stimulus intensities of both modalities of stimulation were selected to produce about a 50% probability of contraction for the population average.

Response Stability Studies

Studies by Kung[38-40] have shown that certain behavioral traits are genetically controlled in *Paramecium aurelia*. It therefore became of interest to determine if the initial contractile responsiveness of individual *Spirostomum* was propagated from parent to daughter cells.

Individual *Spirostomum* were selected from a culture of buffered Carter's medium and were placed in buffered Carter's medium in separate, 24 hr aged, two inch, plastic culture dishes (home cultures). The initial responsiveness was measured for each animal 24 hr after subculturing[*] and for successive daughter cells at random intervals during the succeeding four days (approximately three generations) to determine the consistency of response group classifications among animals in each home culture. After five days, each daughter cell was tested individually and subcultured to a separate 24 hr aged, two inch, plastic petri dish and allowed to propagate for about three generations. The response group consistency among all animals propagated from such subcultures of each home culture was then determined. Thus, the effects of local culture environment on responsiveness could be assessed. Additionally, animals from selected home cultures yielding predominantly high responders were interchanged with animals from "low responder cultures." The effects of growing the high responders in "low responder culture medium" (and vice versa) was then evaluated by determining the responsiveness of each transferred animal after 24 hrs of incubation in the new environment.

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[*] The first division after subculturing does not occur for 2-3 days; thereafter divisions occur about once per day.

Electron Optics Studies

Osborn[45] has studied the spatial distribution of calcium (Ca) and phosphorous (P) in 2 μm thick sections of *Spirostomum*. The possible interaction of magnesium (Mg) and Ca in the contractile process has been suggested.[6,9,18,25,46,47] If this is so, then one might expect Ca and Mg to be co-distributed near the contractile filaments. However, the levels of Mg present in *Spirostomum* were below the resolving power of a standard electron microprobe analyzer. The use of a multichannel analyzer* increased the Mg signal-to-noise ratio sufficiently to permit its spatial resolution. Five *Spirostomum* of differing initial contractile responsiveness to mechanical stimulation were prepared for electron microscopy by fixing in 3% glutaraldehyde, post-fixing in 1% OsO_4, dehydrating in a graded series of acetone and embedding in Epon. Three of these animals were fixed concurrently and identically using cacodylate buffer (pH 7.2) to prevent the precipitation of phosphate salts of Ca. The other two animals were fixed concurrently and identically using 125% Carter's medium (pH 7.2). Both thick (1.5 μm) and thin (150 nm) sections were obtained. The thick sections were examined with the microprobe to determine (a) the distributions of Ca, P, and Mg, (b) the Ca to Mg ratio, and (c) the approximate concentrations of Ca, Mg, and P in the fixed sections. The thin sections were examined by transmission electron microscopy and by the electron microprobe analyzer.

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*Packard Instrument Co., Chicago, Illinois.

RESULTS

Behavioral Studies with Mechanical Stimulation

Response Groups

The contractile responsiveness of 100 animals was determined during the behavioral studies. Forty animals each from series A and B were video taped and studied in detail. Because the stimulus intensity was altered to artificially fill all of the initial responsiveness subgroups (i.e., the number of contractions to the first six stimuli), little can be said about the population response group distribution of behavioral series A. However, since the stimulus intensity was maintained constant throughout behavioral series B, this information was obtained. No more than nine animals were actually video taped and analyzed in any one response subgroup, but the subgroups were noted for all animals that were not taped. Figure 7 graphically shows the response group distribution of all animals from behavioral series B. Those portions of the bars that are all black represent animals tested in July, the cross hatched bars represent animals tested in August. The subgroups are categorized on the basis of the first six rather than the first five stimuli as used in the remainder of the study.

All animals video taped for analysis in behavioral series B were categorized into subgroups on the basis of the number of times they contracted to the first five stimuli. Animals contracting either four or five times were classified as high responders, those contracting zero

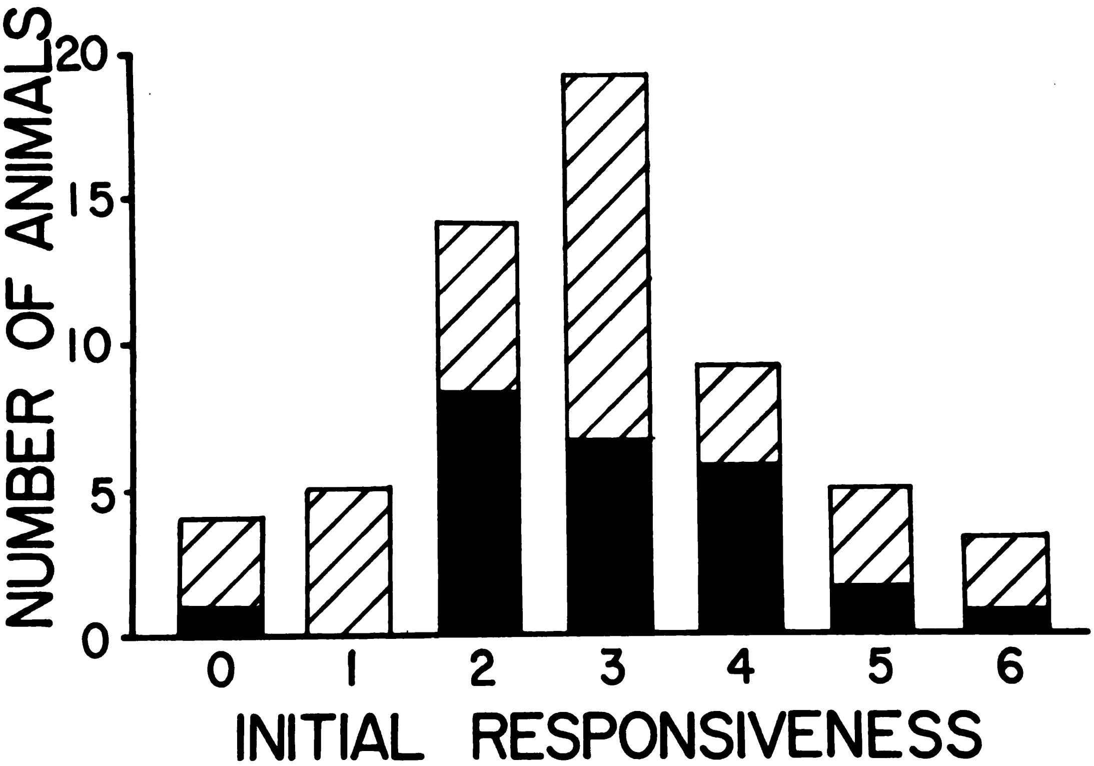

Figure 7. Initial responsiveness distribution. The *Spirostomum* tested in behavioral series B showed a normal distribution of initial contractile responsiveness (i.e., the number of contractions elicited to the first six stimuli). Regions of the bars which are all black represent animals tested during July. The cross-hatched regions represent August animals. (N = 59)

or one time were low responders, and those contracting two or three times were medium responders.* Ten high, 16 medium, and 14 low responders were taped and used for analysis in behavioral series B.

There was no relation between initial responsiveness of the animals tested and the time of day at which they were stimulated. The one or two degree (C) variations between experiments in room and culture temperatures also produced no noticeable effects. These considerations were true for both the July and the August animals of behavioral series B. Subsequent studies have shown, however, that responsiveness is affected for several days by subculturing or changing culture medium.[50]

Contraction Probability

Both behavioral series A and B showed that animals, differing in their initial contractile responsiveness, would also differ in modifications in contractile responsiveness determined over a period of repetitive mechanical stimulation. Figure 8 shows the results of behavioral series B. The high responders habituate ($p \leq 0.05$, Daniel's test for trend, one tailed). The low responders, on the other hand, do not habituate, but show a tendency to sensitize. The response probability of the medium response group decreased slightly. The separation among groups was highly significant ($p \leq 0.002$, Kruskal and Wallis' multi-sample test, two tailed). A significant increase in contraction probability is present between the first and second 5 trial blocks for the low responder subgroup ($p < .005$, Wilcoxon matched pairs test, one tailed). However, the percent contractions of second and twelfth 5 trial blocks are not significantly different. This suggests that low responders either (1) maintain a

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*Henceforth these high, medium, and low responder groups will be referred to as the response subgroups.

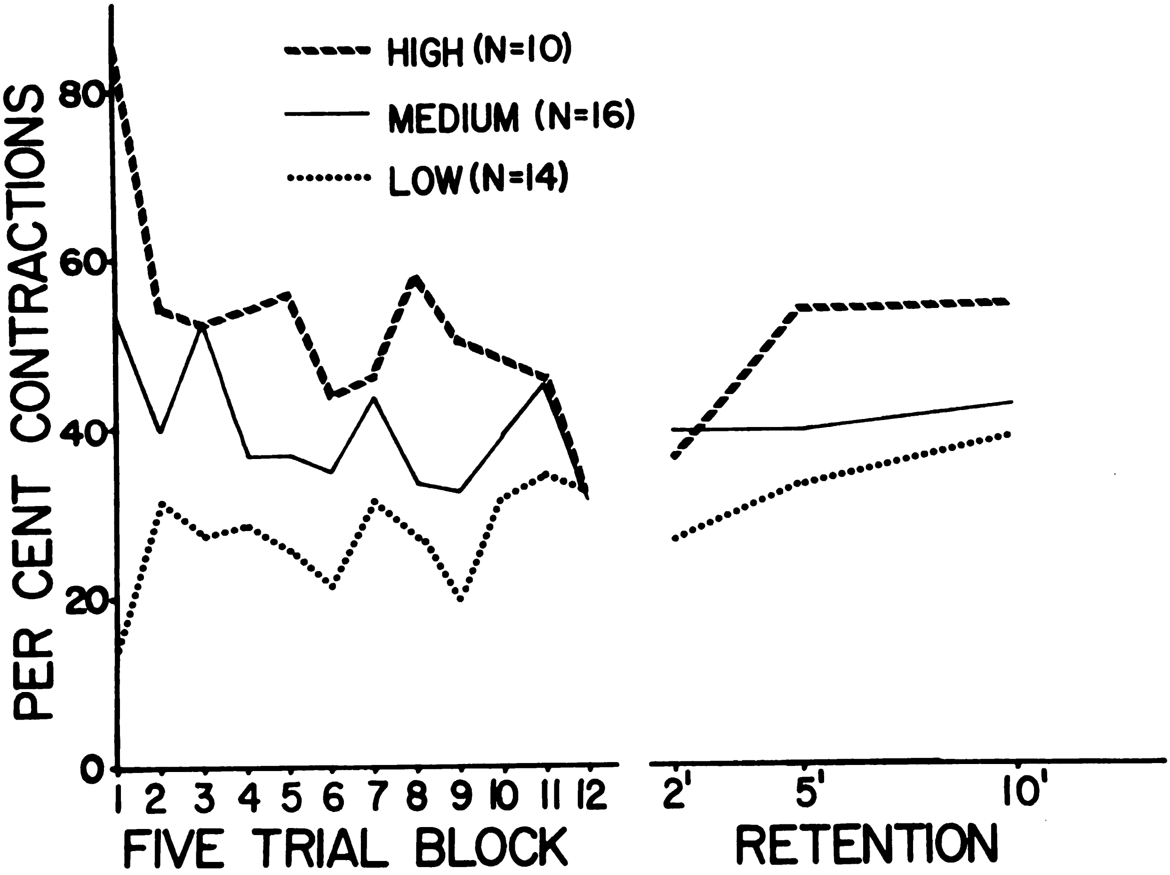


Figure 8. Percent contractions by subgroups to repetitive (0.1 Hz) mechanical stimulation. Each point represents the percent contractions elicited by all of the animals of a subgroup to the corresponding five trial block (abscissa), or to the 2, 5, or 10 min retention trials. The separation among groups is significant ($p \leq 0.002$, Kruskul and Wallis' multi-sample test, two tailed). The high responders subgroup habituates ($p \leq 0.05$, Daniel's test for trend, one tailed).

relatively constant, and rather low, probability of contraction over the stimulation period (the low percentage of contractions for the first 5 trial block being simply a by-product of animal classification), or (2) sensitize during the first minute of stimulation. Results from the response stability studies show that initial responsiveness remains quite stable in unstimulated animals for relatively long periods of time. Therefore, the rapid initial rise in contractile responsiveness observed in low responders may indeed result from sensitization processes. The results from behavioral series A were essentially identical.

Contraction probability was also analyzed on a trial by trial basis for the first two minutes of stimulation. As shown in Figure 9 the separation among the groups is still evident. Notice that the animals of two of the subgroups show an increased responsiveness on the second trial (the high responders could not increase) with the first marked decrement occurring on the third trial. This phenomenon has been observed in habituation studies on other animals as well.[51]

Ciliary Response Probabilities

Neither behavioral series, A or B, demonstrated any changes over time in the probability of an animal exhibiting an avoidance reaction in response to the stimulation (Figure 10). The animals are classified into subgroups on the basis of their initial contractile probabilities. Reclassifying the animals on the basis of their initial probability of giving an avoidance reaction did not introduce any noticeable trend in the data.

The probability with which startle responses occurred was extremely low. No change occurred over time in the rate at which they were elicited.

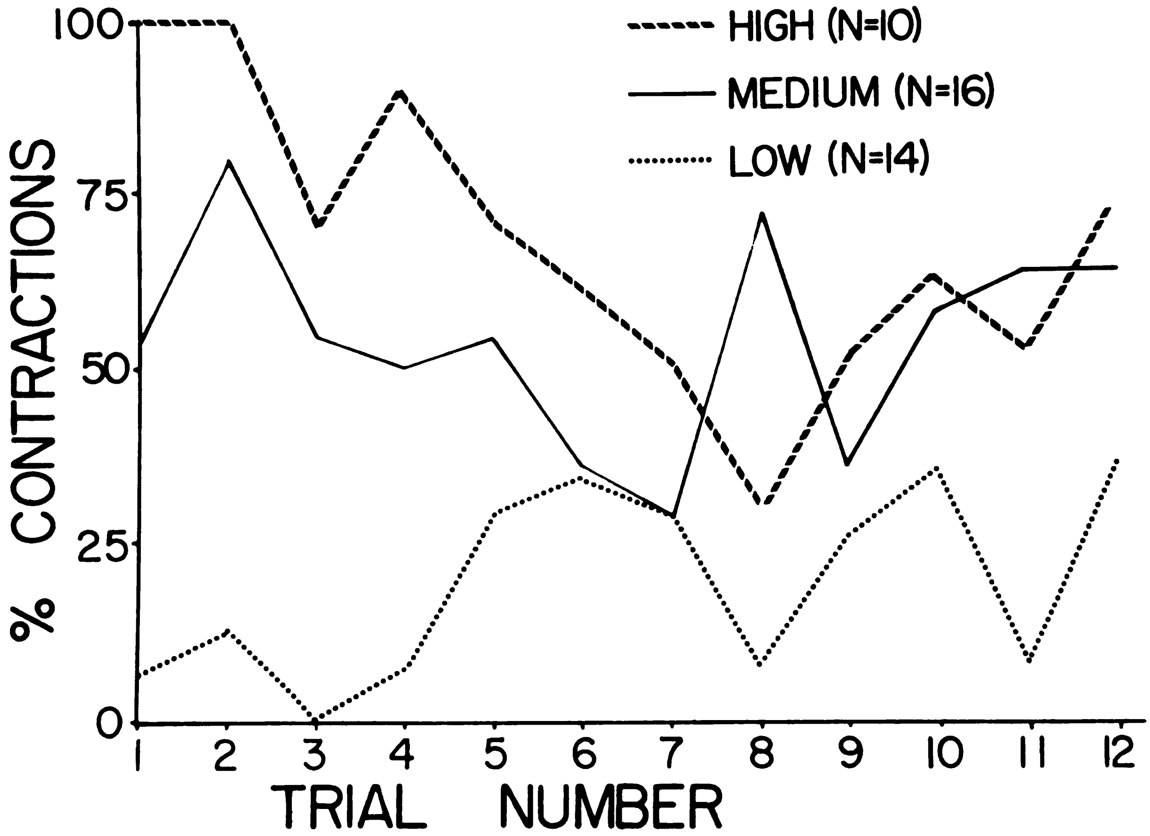


Figure 9. Percent contractions per trial. The percent contractions of all animals in each response subgroup is shown on a per trial basis for the first twelve stimuli. Note the tendency for increased responsiveness on the second trial for two of the three subgroups and sharply decreased responsiveness on the third trial for all groups. Habituation is evident for the high responders, but the low responders seem to sensitize.

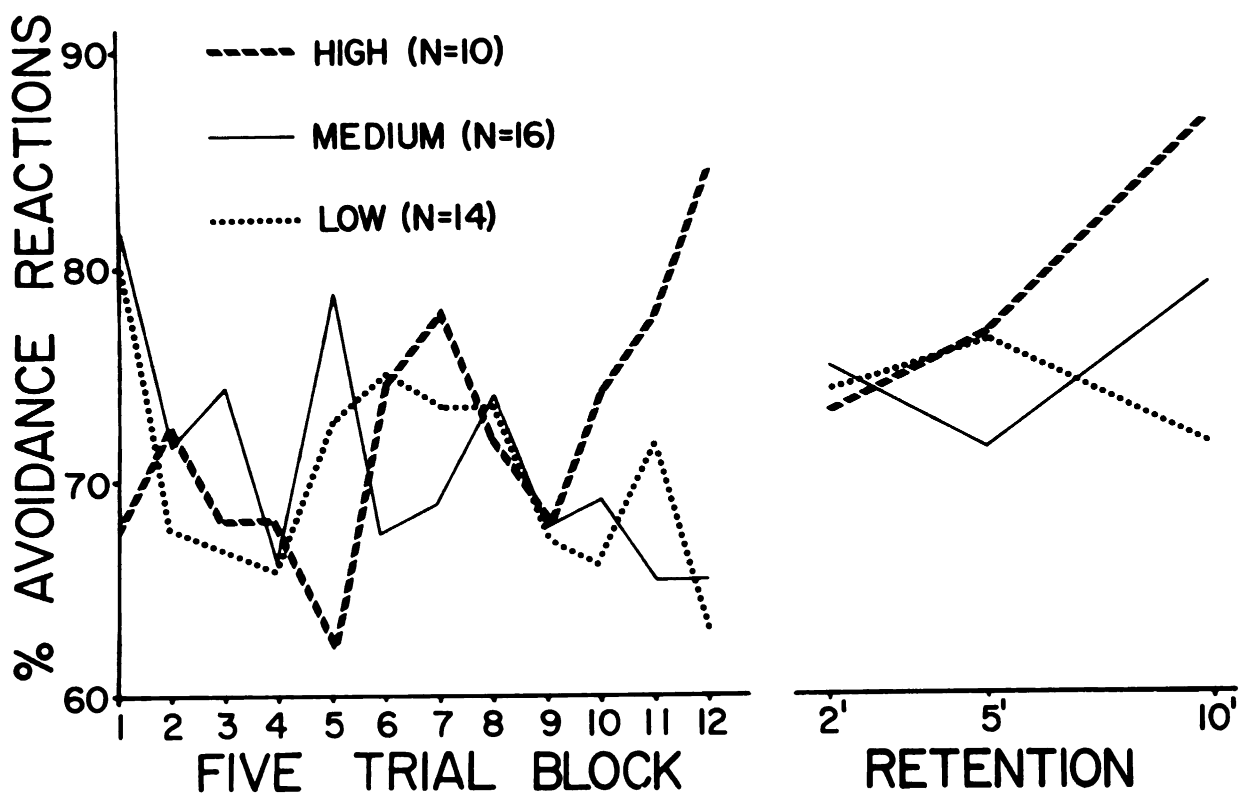


Figure 10. Percent avoidance reactions by subgroup. The percentage with which animals of each contractile responsiveness subgroup elicited avoidance reactions is shown for consecutive 5 trial blocks and for the 2, 5, and 10 min retention trials. No change was observed during the stimulation period in the average frequency with which animals elicited avoidance reactions. Also, no separation among groups is present (behavioral series B).

Interactions Between Responses to Stimuli and Conditions at Time of Stimulus Delivery

Location. Figure 11 summarizes the results of measurements made on the location of the animals within the slide well at the times stimuli were delivered during the ten minutes of stimulation. Members of the low response group (Figure 11A) were located in the center of the well less often than animals of the high or medium response groups. Figure 11C shows data on the location at the edge of the well. The differences between the groups are not significant; but the total population of 40 animals shows a trend over time toward increased location at the edge of the well significant at the 0.01 level (Daniel's test for trend, two tailed).

Also interesting to note from Figure 11 is the average frequency at which animals were stimulated in the various designated zones of the well. The animals were located at the edge approximately 25-50% of the time, in the intermediate zone about 50% of the time, and in the center about 20-25% of the time. The relative areas of the three zones are πR^2, $\pi(2R)^2 - \pi R^2 = 3\pi R^2$, and $\pi(3R)^2 - 3\pi R^2 = 6\pi R^2$ for the center, intermediate, and edge zones, respectively. Thus, one would expect to find the animals in the edge zone about 60% of the time, in the intermediate zone about 30% of the time, and in the center only 10% of the time. The observed values were 29%, 49%, and 22%, respectively.

Table 1 lists the percentages with which the animals of behavioral series B elicited the various behavioral responses during minutes 1-3, 4-7, and 8-10 of the stimulation period while located in either the center (C), intermediate (I), or edge (E) zones of the slide well. High, medium and low responders are shown separately. Numbers in parentheses indicate the number of times in each time period that

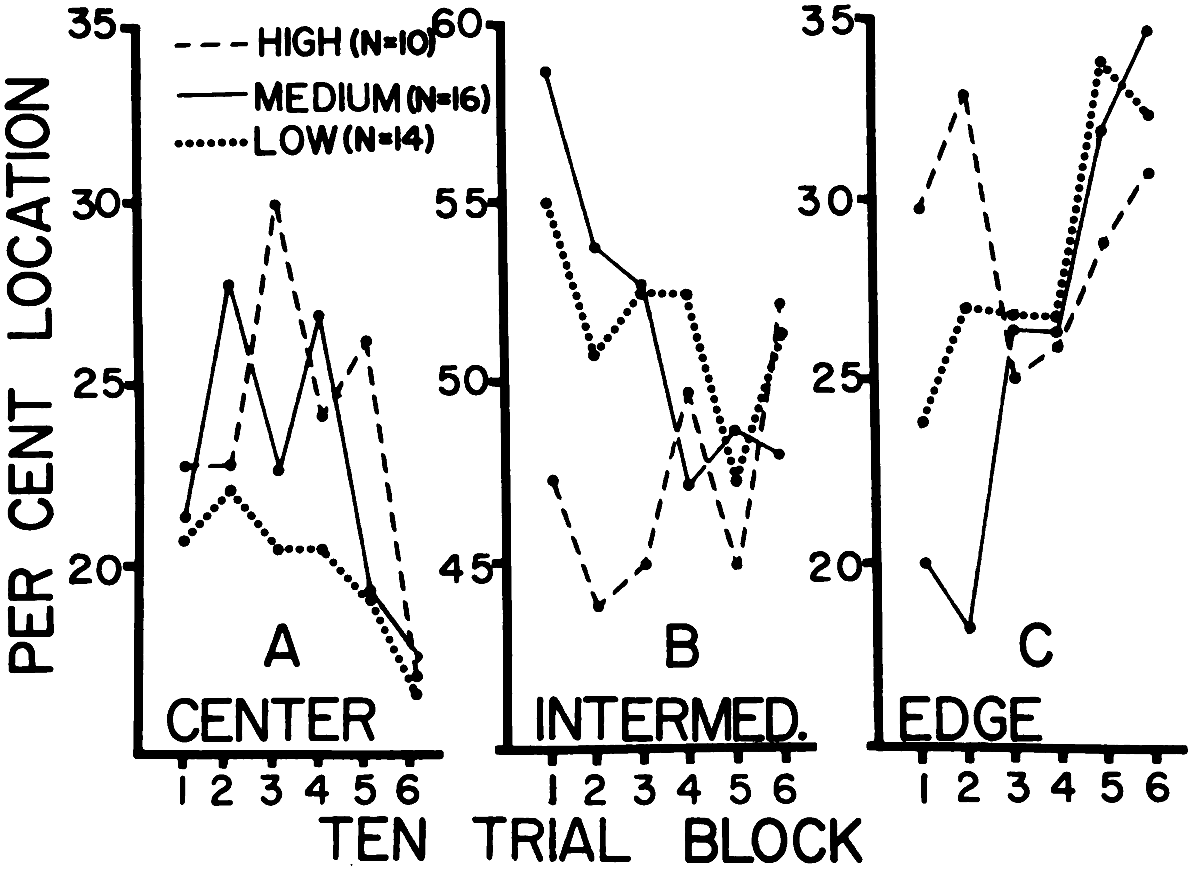


Figure 11. Location in slide well at time of stimulus delivery. Figures A-C, respectively, show the percentages per ten trials that the animals of each response subgroup were located in the center, intermediate, or edge zones of the slide well at the time each stimulus was delivered. The average values for each zone do not correlate well with those values expected by chance when the relative areas of the three zones are considered. (Note the differences in ordinate scales.) There is a significant migration toward the edge of the well over the stimulation period ($p \leq 0.01$, Daniel's test for trend, two tailed).

Table 1. Location-response correlation[*]

		Contractions			Avoidance			Startle		
			minutes		minutes			minutes		
		1-3	4-7	8-10	1-3	4-7	8-10	1-3	4-7	8-10
High	C**	63 (40)	53 (68)	50 (34)	70	68	68	3	3	9
	I	57 (90)	55 (111)	54 (96)	60	73	77	2	3	1
	E	52 (50)	51 (61)	42 (50)	84	67	56	0	3	0
Medium	C	48 (69)	43 (90)	35 (54)	75	72	74	1	1	4
	I	45 (165)	37 (190)	41 (136)	68	74	65	5	4	4
	E	50 (54)	27 (104)	34 (98)	80	64	62	0	7	6
Low	C	34 (56)	43 (68)	45 (44)	73	80	71	2	4	5
	I	22 (134)	22 (176)	29 (135)	72	78	64	2	3	4
	E	16 (64)	25 (91)	23 (73)	69	59	60	8	4	4

[*] Table 1 lists the percentages with which the animals of each response subgroup of behavioral series B exhibited the indicated behavioral responses during minutes 1-3, 4-7, and 8-10 of the stimulation period. The percentages are further categorized by the region of the well in which the responses were elicited. Numbers in parentheses indicate the number of trials on which the percentages were calculated.

[**] C = center zone; I = intermediate zone; E = edge zone.

animals were stimulated in a given region of the well, and thus the number of trials on which the corresponding percentages were calculated.

Animals in the center seem most likely and animals in the edge zone least likely to contract.

The decreased contractile responsiveness near the edge combined with the migration toward the edge over time could help explain the contractile habituation observed for high responders. However, the magnitude of these trends (an approximately 10% migration toward the edge, and about a 10% center-edge contraction probability difference) suggests that their contribution to habituation is negligible, especially since high responders showed no migration toward the edge zone. Also, high responders can be seen (Table 1) to habituate within each zone of the well, and low responders to sensitize. Therefore, changes in location in the slide well at the time of stimulus delivery cannot account for the habituation and sensitization observed.

Attitude. The average number of times per ten trials that the animals were found to be oriented vertically in the well is shown in Figure 12. The low responders were vertically oriented about 5-10% less often than either the high or medium response group animals (this difference was not statistically significant). This fact may have contributed to the relatively higher contractile responsiveness of the high and medium responders; for as evidenced in Table 2 (organized as Table 1) animals oriented vertically (V) contracted about 30% more frequently than animals in a horizontal (H) attitude. However, the approximately 30% difference in contractile responsiveness between vertical and horizontal attitudes can account for at most a 2-3% separation between high and low response groups, since high responders were vertical only about 5-10% more often than were low responders (30% x 10% = 3%). This 2-3% is not sufficient to explain the observed 20%

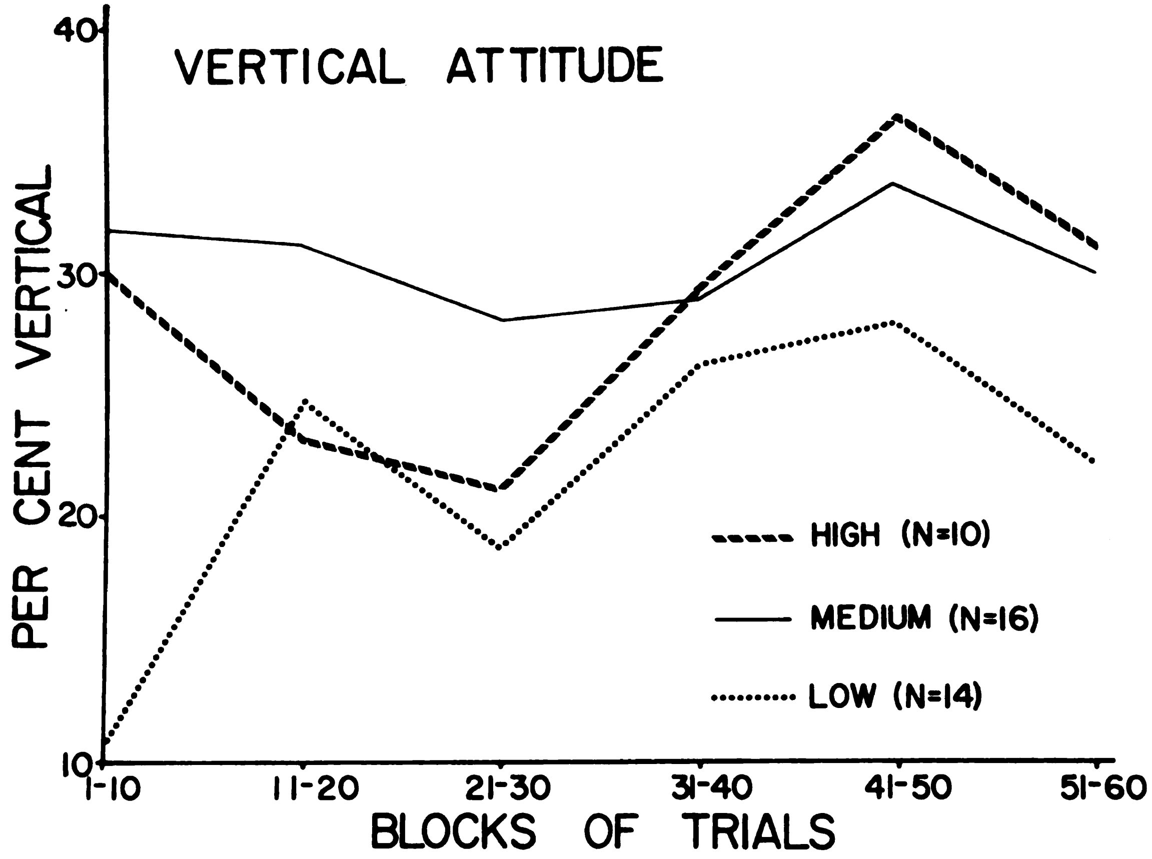


Figure 12. Attitude at time of stimulus delivery. The ordinate shows the percentage of times per ten trials that the animals of behavioral series B were oriented vertically when a stimulus was presented. The abscissa indicates the ten trial block to which each point corresponds.

Table 2. Attitude-response correlation*

		Contractions			Avoidance			Startle		
			minutes			minutes			minutes	
		1-3	4-7	8-10	1-3	4-7	8-10	1-3	4-7	8-10
High	H**	56 (131)	40 (174)	36 (123)	75	78	83	0	2	1
	V	75 (47)	76 (66)	63 (57)	55	56	63	4	5	5
Medium	H	34 (196)	22 (275)	24 (194)	73	73	67	4	5	5
	V	71 (94)	70 (109)	63 (94)	69	69	60	1	1	3
Low	H	15 (210)	20 (262)	19 (187)	69	76	67	4	2	5
	V	57 (46)	57 (74)	66 (65)	65	65	58	0	8	3

*Table 2 shows the percentages with which the animals of each response subgroup of behavioral series B exhibited the various behavioral responses during minutes 1-3, 4-7, and 8-10 at the 10 minute stimulation period. The percentages are further categorized as to whether the animals were horizontal or vertical when the stimuli were delivered. The numbers in parentheses indicate the number of trials upon which the corresponding percentages were calculated.

**H = horizontal; V = vertical.

separation between high and low response groups. Also, the fact that no changes in attitude were observed over time indicates that this factor is not responsible for contractile habituation or sensitization.

Vertically oriented animals, which were more likely to contract, were about 10% less likely to elicit an avoidance reaction than were horizontal animals. No trends were evident for startle responses.

Body shape. Figure 13 shows the percentage of times per ten trials that the *Spirostomum* tested were observed to be bent when the stimuli were delivered. The low and medium response groups were bent about 10% more often than the high responders (not statistically different). Since bent animals (B) were about 20% more likely to contract than were animals which were relatively straight (N) (Table 3), body shape did not contribute to the contractile responsiveness differences between subgroups. Similarly, since no changes were observed over time in the body shape of the animals tested, contractile habituation and sensitization are also seen to be independent of considerations of body shape.

Bent animals, which were more likely to contract, were observed to be about 15% less likely to elicit an avoidance reaction than were relatively straight animals.

Behavior. No differences among groups or any trends over time were observed in the rate at which the animals were engaged in each of the various behaviors (i.e., forward swimming, backward swimming, and probing) at the time the stimuli were delivered.

Table 4 lists the percentage of times the animals elicited each of the categorized responses during minutes 1-3, 4-7, and 8-10 of the ten minute stimulation period while swimming forward (F), probing (P) and swimming backward (A). Animals were about 10% more likely to contract while probing than while either swimming backward or swimming forward. However, animals swimming forward were most likely to give an avoidance reaction. Again, the contractile responsiveness differences between subgroups and any response modifications over time are not due to considerations of swimming activities at the time of stimulus delivery.

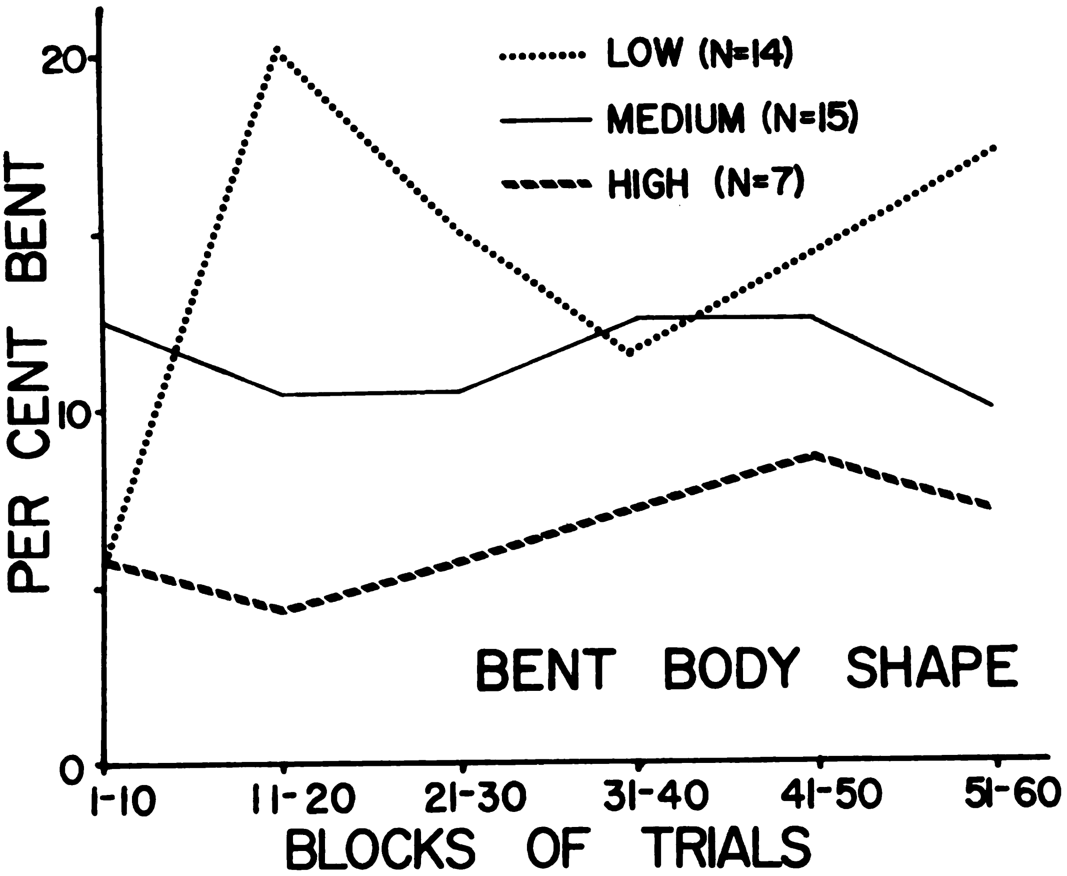


Figure 13. Body shape at time of stimulus delivery. The ordinate showsthe percentage of times per ten trials that the animals tested in behavioral series B were bent at the time a stimulus was delivered. The abscissa indicates the ten trial group block to which each point belongs.

Table 3. Body shape-response correlation*

		Contractions			Avoidance			Startle		
		minutes			minutes			minutes		
		1-3	4-7	8-10	1-3	4-7	8-10	1-3	4-7	8-10
High	N**	62 (119)	48 (157)	44 (117)	75	76	85	1	1	1
	B	71 (7)	73 (11)	67 (9)	57	82	56	0	0	0
Medium	N	43 (240)	30 (316)	35 (240)	75	74	68	3	5	5
	B	70 (30)	68 (44)	60 (30)	57	64	63	3	0	0
Low	N	21 (222)	25 (286)	28 (215)	75	76	66	3	4	4
	B	43 (30)	44 (50)	43 (37)	50	60	59	7	4	3

*Table 3 shows the percentages with which the animals of each subgroup of behavioral series B exhibited the various behavioral responses during minutes 1-3, 4-7, and 8-10 of the 10 minute stimulation period. The percentages are further categorized as to whether the animals were bent or straight at the time of stimulus delivery. The numbers in parentheses indicate the number of trials upon which the corresponding percentages were calculated.

**N = straight; B = bent.

In summary, interactions capable of explaining any of the observed response modifications over time or any differences among response subgroups are not found from considerations of (1) the location of animals within the slide well, (2) the body shape of the animals, (3) the attitude of the animals, and (4) the swimming behaviors of the animals at the time of stimulus delivery. However, it was noticed that those conditions

Table 4. Swimming behavior-response correlation[*]

		Contractions			Avoidance			Startle		
			minutes			minutes			minutes	
		1-3	4-7	8-10	1-3	4-7	8-10	1-3	4-7	8-10
High	F**	58 (71)	50 (101)	33 (63)	73	76	86	1	2	0
	P	63 (104)	55 (134)	50 (116)	66	68	73	2	4	3
	A	20 (5)	50 (4)	50 (2)	60	75	0	0	0	0
Medium	F	28 (109)	25 (143)	25 (121)	76	82	74	5	3	4
	P	58 (166)	43 (224)	47 (159)	70	67	62	2	5	4
	A	46 (13)	31 (13)	43 (7)	62	54	14	0	0	14
Low	F	15 (87)	24 (106)	23 (92)	80	88	70	6	2	5
	P	28 (155)	33 (203)	35 (150)	71	70	62	3	4	4
	A	20 (10)	6 (17)	33 (9)	20	53	67	0	0	0

[*] Table 4 shows the percentages with which the animals of each response subgroup of behavioral series B exhibited the various behavioral responses during minutes 1-3, 4-7, and 8-10 of the 10 minute stimulation period. The percentages are further categorized as to whether the animals were swimming forward, swimming backward, or probing at the time of stimulus delivery. Numbers in parentheses indicate the number of trials on which the corresponding percentages were calculated.

[**] F = forward swimming; P = probing; A = backward swimming.

(i.e., body shape, attitude, and behavior) which increased the probability of contraction, decreased the probability of an avoidance reaction being elicited.

Modifications in Swimming Activity

Figure 14 shows the average amount of time that the animals of each response subgroup of behavioral series B spent engaged in each of the three behavioral categories (forward swimming, backward swimming, probing) during the ten second periods prior to each trial in the first, third, and tenth minutes of stimulation, as well as prior to each retention trial. (Since the animals remained essentially continually active, the "stopped" classification was not required.) Figure 14A shows the data for backward swimming. The separation among groups for the first ten seconds is not significant, nor is there any separation among groups over the stimulation period. There is, however, a significant trend ($p \leq 0.002$, Daniel's test for trend, two tailed) for the total group of animals to exhibit decreased amounts of backward swimming over the ten minute stimulation period (through stimulus number 60). The observed trend of increased backward swimming during the ten seconds prior to the second trial relative to the ten seconds prior to the first trial is significant at the 0.04 level (Wilcoxon matched pairs test, two tailed).

No differences among groups was observed over the stimulation period for either forward swimming (Figure 14B) or for probing (Figure 14C). There is, however, a significant increase in probing for the total group of 40 animals over the first 2.5 min of stimulation ($p \leq 0.02$, Daniel's test for trend, two tailed).

Figure 14. Percent time per behavior. Figures 14A,B,C, respectively, show the percentage of time, on a per trial basis, that the animals of behavioral series B spent swimming backward, swimming forward, and probing during the 10 sec prior to each trial during the first (stimuli 1-6), third (stimuli 13-18) and tenth (stimuli 55-60) minutes of stimulation and during retention trials (3 stimuli, a-c, after two minutes and after 5 minutes, and 6 stimuli, a-f, after 10 minutes). The ordinate indicates the percent time per behavior; and the abscissa, the corresponding trial number. Note differences in ordinate scales. There is (14A) a significant decrease in backward swimming over the stimulation period ($p \leq 0.002$, Daniel's test for trend, two tailed). The increase in backward swimming between the 10 sec prior to the first and the 10 sec immediately after the first (prior to the second) trial is also significant ($p \leq 0.04$, Wilcoxon matched pairs test, two tailed). There is also (14C) a significant increase over the first 2.5 min of stimulation in the amount of time spent probing ($p \leq 0.02$, Daniel's test for trend, two tailed).

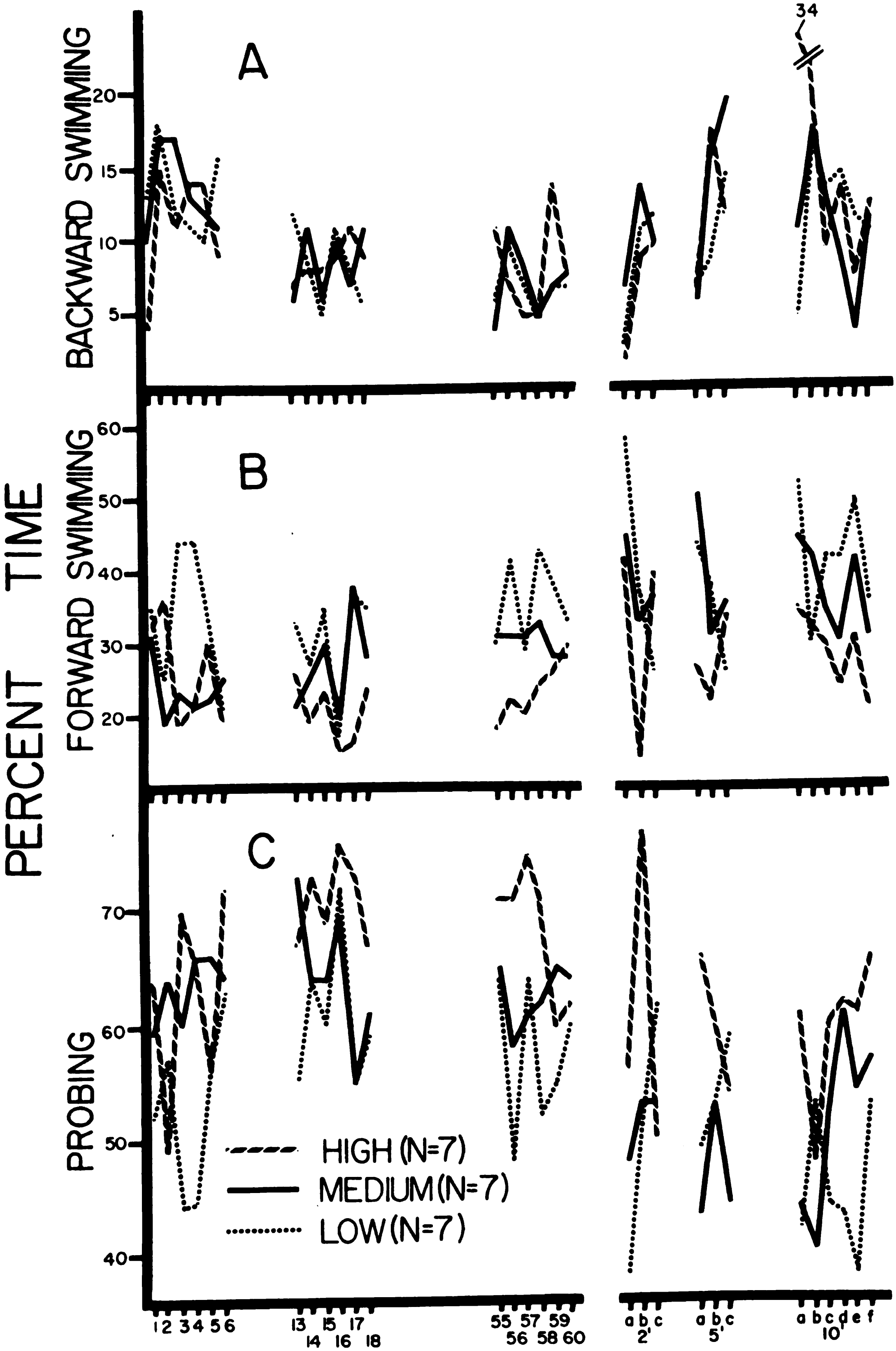

PERCENT TIME
A
BACKWARD SWIMMING
5
10
15
20
34
B
FORWARD SWIMMING
20
30
40
50
60
C
PROBING
40
50
60
70
HIGH (N=7)
MEDIUM (N=7)
LOW (N=7)
1 2 3 4 5 6
13 14 15 16 17 18
55 56 57 58 59 60
a b c 2'
a b c 5'
a b c d e f 10'
TRIALS
RETENTION

Figure 14

There was fairly good correlation between the percentage of time that the animals spent engaged in the various behaviors and the percentage of times that the animals were stimulated while pursuing those behaviors. The *Spirostomum* were stimulated while probing about 60% of the time, while swimming forward about 37% of the time, and while swimming backwards only about 3% of the time. Animals were stimulated while stopped on only two of 2880 trials (0.07%).

Behavioral Studies with Electrical Stimulation

The results obtained with 2 msec electrical stimulation are in agreement with those reported by Osborn,[46] i.e., animals stimulated with a 2 msec biphasic electric pulse at a rate of 0.1 Hz do not show any decrease in their probability of contraction over a ten minute period of stimulation and show a tendency to slow their rate of swimming over the stimulation period. A noticeable increase in the size of the contractile vacuole occurs. Furthermore, the animals were found to be more responsive when oriented parallel to the electric field than when oriented perpendicular to it.[37,46]

No changes were evident during the stimulation period in the probabilities with which avoidance reactions and startle responses occurred.

If a 0.2 msec electrical stimulus is used, however, the above findings were altered in one important respect. A sharp decrease was found in each animal's contractile responsiveness during the sixth to seventh minute of stimulation. But, as reported by Osborn[46] for more rapid (0.2 Hz) and more prolonged electrical stimulation, the shape of the "habituation" curve obtained differs from that seen with mechanical stimulation. As illustrated in Figure 15, habituation to mechanical stimulation (curve M) exhibits a negative exponential shaped curve, whereas the electrical stimulation (curve E) produced a sigmoidal shaped curve. Recovery of contractile responsiveness is seen after the termination of stimulation with either modality of stimulus.

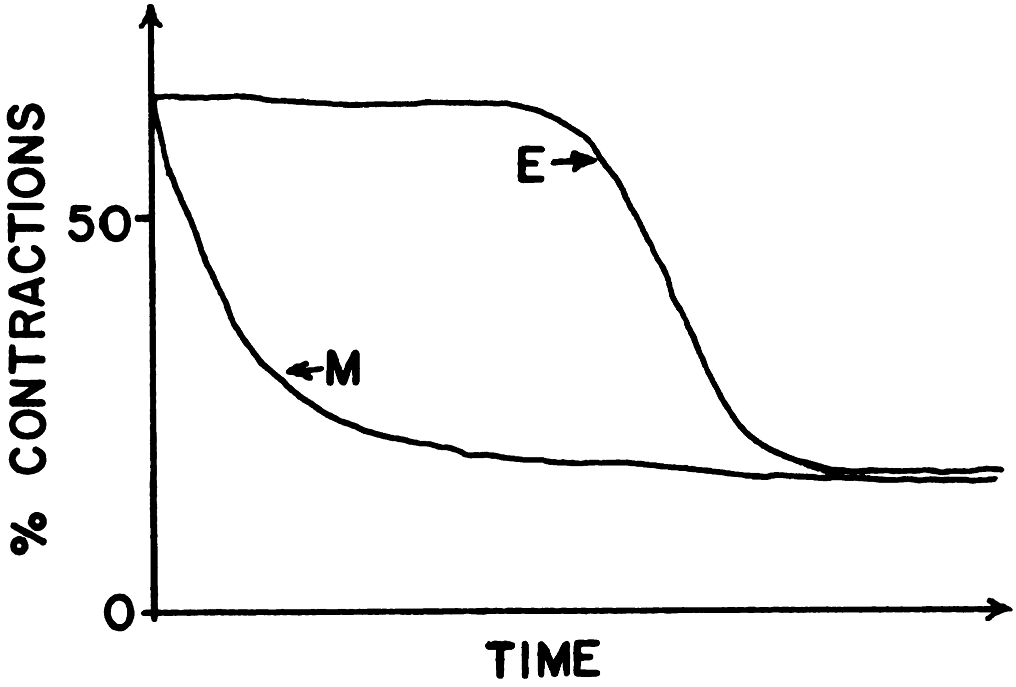


Figure 15. Comparison of habituation curves. Curves "M" and "E" show the characteristic ways in which contractile responsiveness decreases over time to repetitive mechanical (curve "M") and electrical (curve "E") stimulation.

Correlation of Mechanical and Electrical Response Groups

Twenty-five animals were tested to determine the response groups to which they should be assigned using both mechanical and electrical stimulation. No correlation was found to exist between the responsiveness of the animals to the two modalities of stimulation.

Timecourses of Individual Contractions

High speed films (up to 1200 frames per second) were taken of several animals contracting. Length measurements made from these films were used to determine an average contraction timecourse. The results from the high speed film measurements are shown in Figure 16. The animal contracts to less than one half its length very quickly, about 3-5 msec. Over the next 100-150 msec an additional, approximately 10% shortening occurs. These "fast" and "slow" components of the contraction are labeled "F" and "S", respectively, in the figure. Re-extension occurs over a period of several seconds.

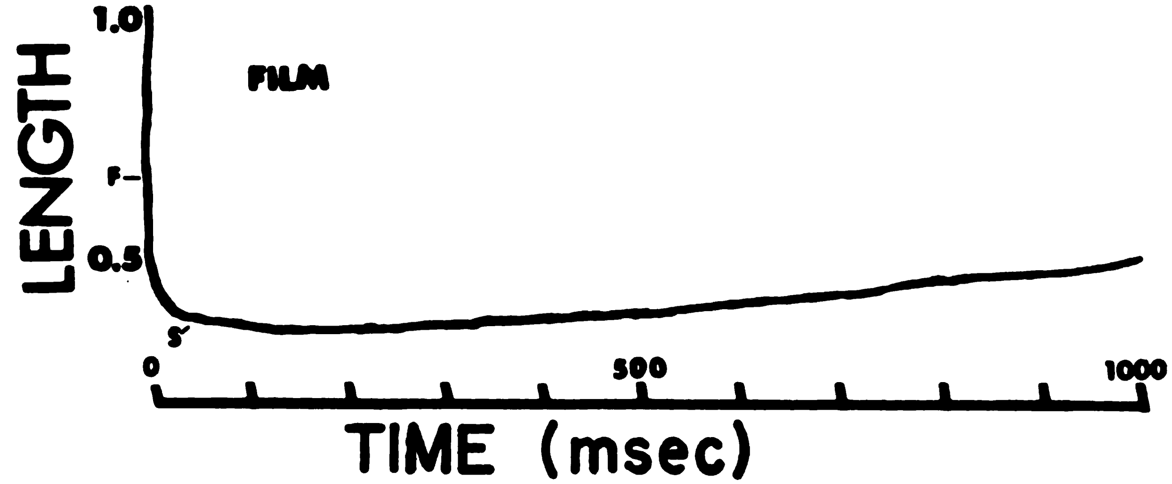


Figure 16. Contraction timecourse. The length of a contracting animal relative to its resting length (1.0) is shown as a function of time after the start of contraction. Measurements were taken from a high speed film taken at approximately 1200 fps. Note the fast ("F") and slow ("S") components of the shortening phase.

For the behavioral series, contraction timecourses were taken from the video tapes. Measurements were made just prior to the onset of contraction and at one half second intervals thereafter until the animal had relengthened to about 75% of its starting length. An additional measurement was also taken about 100-120 msec after the onset of contraction to determine the maximal extent of contraction.

Figure 17 shows some typical data from one animal of behavioral series A. This animal contracted to the first 13 stimuli. Note that the magnitude of contraction decreases with successive contractions; but that the starting (resting) length remains constant. The rate of re-extension also remains constant. The approximately 10% decrease in the magnitude of contraction is a function of the recent previous contractile activity of the animals. That is, if the animal remains responsive, the magnitude of contraction will remain depressed. However, if the animal becomes unresponsive for a few stimuli the timecourse of any succeeding contraction will again approximate that of the initial few contractions. Also, low response group animals which remained relatively unresponsive throughout the entire period of stimulation showed no modification in the magnitude (timecourse) of contractions which did occur.

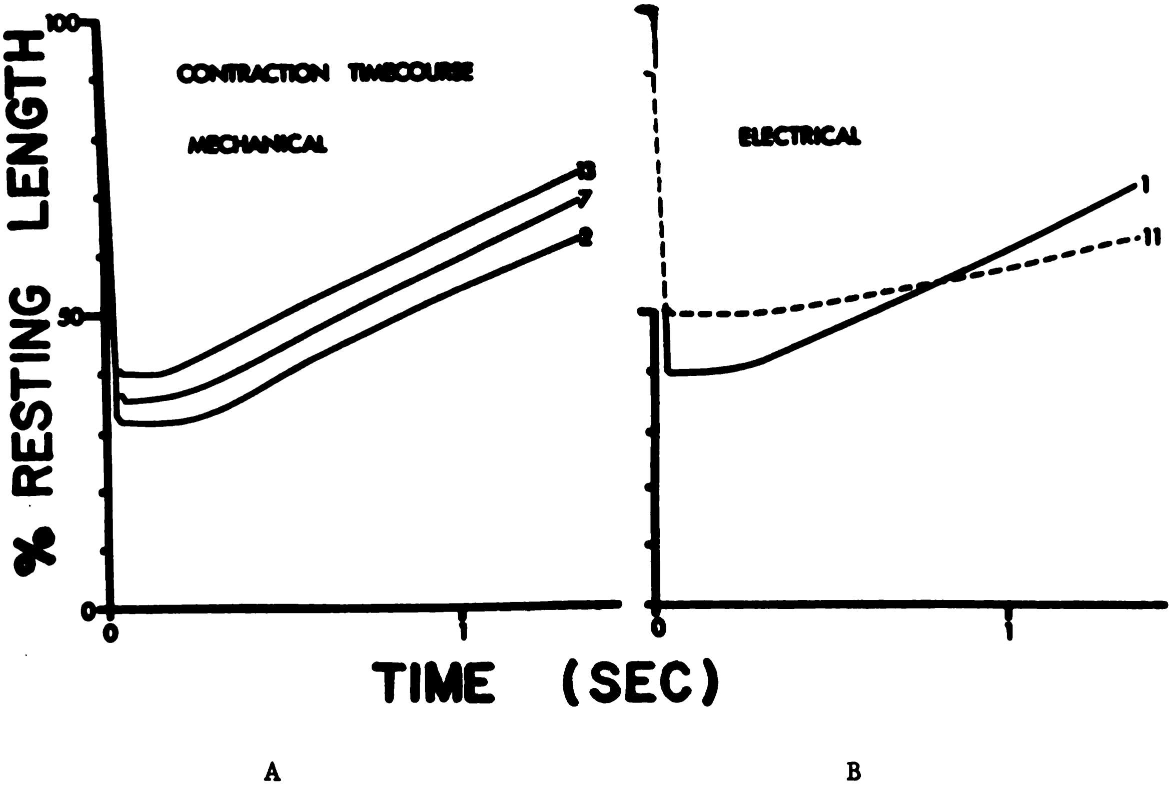


Figure 17. Representative contraction timecourse modifications. Figures 17A and 17B show the contraction timecourses from progressive trials of two individual animals, one stimulated with mechanical stimulation, and one given electrical stimulation. Note that only the extent of contraction is modified by repetitive mechanical stimulation. The trial number of each curve is indicated. All measurements were made from video tapes.

Figure 17B shows typical data from an animal stimulated with a 2 msec biphasic electrical stimulus. The initial contraction timecourse is indistinguishable from timecourses obtained using mechanical stimulation. Note that there is an approximately 10% increase in the maximum contracted length with successive contractions, just as there is for mechanical stimulation. Note also that the modifications in contraction timecourses caused by electrical stimulation differ from those caused by mechanical stimulation in two respects. (1) There is an approximately 10% decrease in resting length over time with electrical stimulation. (Thus, an up to 20% decrease in the magnitude of contraction is observed.) (2) The rate of re-extension decreases with successive contractions to electrical stimulation.

Avoidance Reaction Magnitudes

A dramatic decrease is seen in the magnitude (measured either in terms of the distance backed up or in terms of the time spent backing up) of those avoidance reactions which occur. Figure 18 shows some typical data from several animals, one from each response group, of behavioral series B. The distance backed up and the time spent backing up are shown for the first 5-6 trials of the stimulation periods. Note that the rate of response decrement is rather rapid (occurring over only a few trials) relative to the rate at which contraction timecourses are modified (10-20 trials). Note also that since the distance and time lines coincide, the rate of backward swimming remains constant. This behavioral modification will occasionally recover during the stimulation period and then decrease again, usually more rapidly than occurred earlier in the stimulation period. Identical modifications are observed to occur to both mechanical and electrical stimulation.

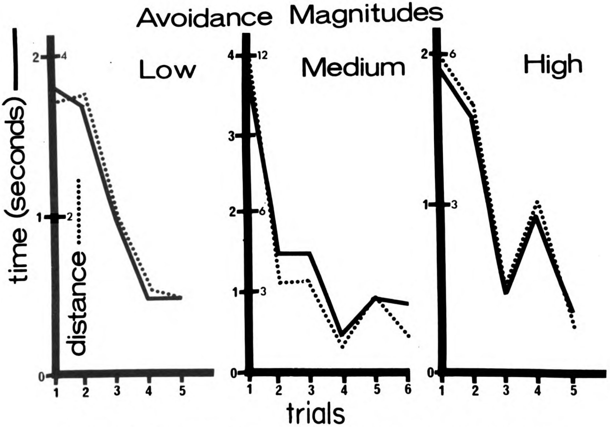
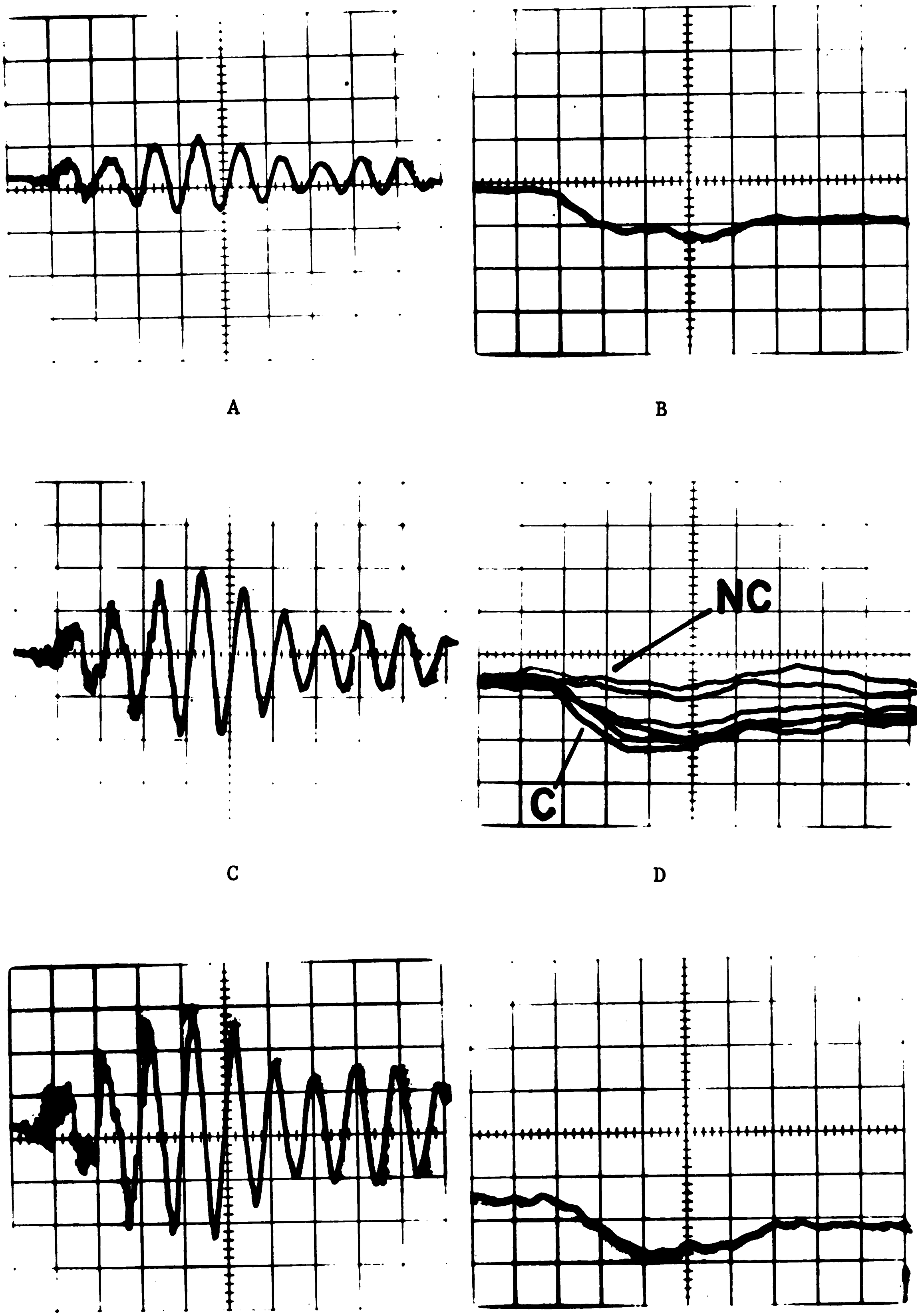


Figure 18. Representative avoidance reaction magnitude modifications. The time in sec (solid line) and the distance backed up in arbitrary units (dotted line) are shown for avoidance reactions elicited on the first five or six stimuli. Since the decrease in both time and distance are coincident, the rate at which the animals swim backwards can be seen to remain constant. The data were taken from three animals (a high, a medium, and a low responder) from behavioral series B. These trends are seen in essentially all animals. Note the differences in ordinate scales.

Latency of Contraction Studies

As illustrated in Figure 19, contraction latencies to mechanical stimulation are not a function of stimulus intensity. The contraction latencies for all animals tested (N ≃ 20) were about 3 msec for all intensities of stimulation used. Wood[53] has reported 2-3 msec contraction latencies for *Stentor*. Furthermore, no change in the contraction latencies was observed over time with repetitive mechanical stimulation, even though the animal's probability of contraction had decreased markedly. This is illustrated in Figure 20A,B. This contrasts with

Figure 19. Contraction latencies to mechanical stimuli of differing intensities. Figures 19A,C,E, respectively, show the relative intensities (as measured by the barium titonate strip) of mechanical stimuli produced by applying voltages of 17, 34, and 45 V to the electromagnetic coil. These stimulus intensities produced initial contraction probabilities of about 10%, 50%, and 95%, respectively. (Note that the stimulus onset begins about one millisecond late in Figures 19A,C. This must be considered when measuring latencies in Figures 19B,D.) Figures 19B,F show the phototube output to the first contraction to a mechanical stimulus of intensity shown in Figures 19A,E, respectively. Figure 19D shows a superposition of phototube output to the initial five mechanical stimuli (four contractions (C) and two noncontractions (NC)) of intensity illustrated in Figure 19C. Note that the sharp deflection in the phototube traces of Figures 19B,D,F all occur at about a 3msec latency (scale for all figures is 2 msec/division). This indicates that the latency of contractions which do occur remains fairly constant, regardless of the intensity of stimulation used.



A

B

C

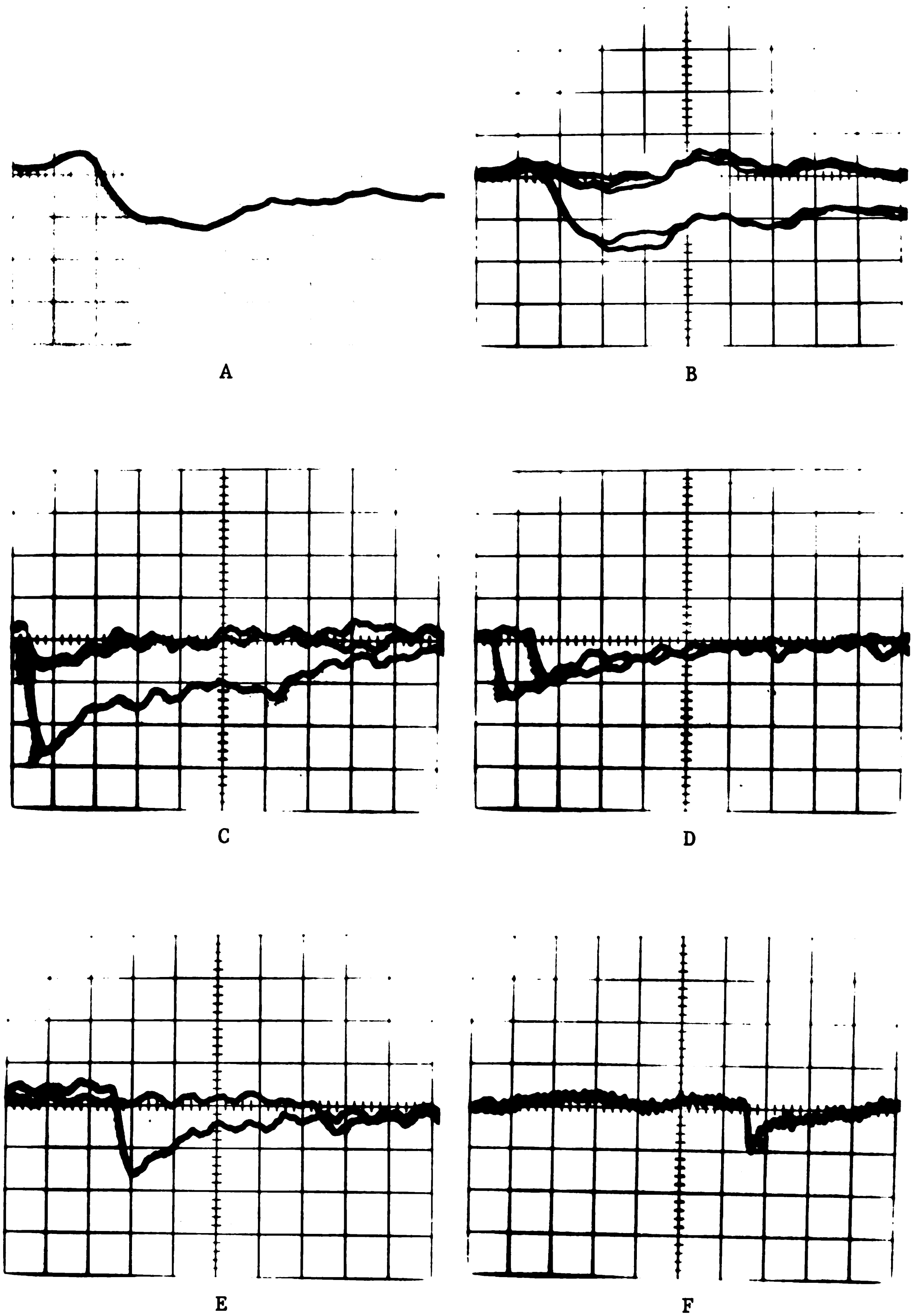
D

E

F

Figure 19

Figure 20. Effects of repetitive stimulation on latencies. Figure 16A shows the phototube output to the first contraction to repetitive *mechanical* stimulation (0.2 Hz). Figure 16B shows two contractions and three noncontractions after about five minutes of stimulation. The time scale for both figures is 2 msec/division. The latency in both figures can be seen to remain constant at about 3 msec. Figures 20C-E (time scale 10 msec/division) show the phototube output to contractions during various periods of repetitive *electrical* stimulation (0.1 Hz). The initial contraction (not shown here) had a latency of about 2 msec. Figure 20C shows contractions after about one minute of stimulation (stimuli 6-8). Note the latency has lengthened to about 3-4 msec. Figure 20D shows two contractions after three minutes of stimulation. The latencies are 5 and 15 msec. Two latencies of 25 msec and 72 msec were recorded after eight minutes of stimulation and are shown in Figure 20E. The latency of 325 msec shown in Figure 20F (time scale 50 msec/division) was recorded after about ten minutes of stimulation.



A

B

C

D

E

F

Figure 20

results reported by Wood for *Stentor*.[54] He found that electrophysiologically measured latencies of contraction in mechanically stimulated *Stentor* isolated in a methyl cellulose solution increased from about 3 to 6 msec over a period of repetitive mechanical stimulation.

Contraction latencies to electrical stimulation were dependent upon stimulus intensity in *Spirostomum*. For fixed stimulus durations, latencies increased as voltages were decreased. This effect was most pronounced at longer stimulus durations (20-200 msec) and was barely discernible for 0.2 msec stimuli. For a 70-80% probability of contraction when the *Spirostomum* were oriented parallel to the electric field, latencies were about 1.5 msec for a 0.2 msec biphasic stimulus whereas they were about 8 msec for a 20 msec biphasic stimulus.

Latencies were observed to be the shortest when the animals were parallel to, and were increased, when the animals were oriented perpendicular to the electric field. Increasing the stimulus duration and/or decreasing the voltage exaggerated this effect.

Dramatic increases in contraction latencies were observed over time in response to repetitive electrical stimulation. This is illustrated in Figure 20C-F. These changes in latency occurred prior to any changes in contraction probability. However, the shift to extremely long latencies (>50 msec) occurred about the same point in time that the animal's probability of contraction was observed to decrease markedly. For a given duration of stimulus the changes in latency occurred more rapidly to high intensity stimuli, but the magnitude of the change was greater at shorter stimulus durations. Several high intensity stimuli also markedly lengthened the expected latency to weaker intensity stimuli. Furthermore, the occurrence of stimuli seemed to lengthen the latencies from the expected initial value even if the animals did not contract to the first few stimuli presented.

If three to seven minutes of 0.2 Hz repetitive mechanical stimulation was given to an animal and then the latency of the first contraction elicited by an electrical stimulus was measured, no change in the expected first contraction latency to electrical stimulation was observed, even though the animal had contracted from 20-50 times to the mechanical stimulation. Similarly, if three to seven minutes of repetitive electrical stimulation was given prior to measuring the contraction latencies to mechanical stimulation, no change in latencies was observed to mechanical stimulation. This was true even though the electrically-caused contraction latencies had been noticeably lengthened. Thus, neither modality of stimulation affected the latency of contractions elicited by stimulation with the other modality.

Response Stability Studies

Results from these studies indicate that initial contractile responsiveness is not propagated from one generation of *Spirostomum* to succeeding generations. Rather, initial responsiveness seems to be largely a by-product of culture conditions.

The response distribution of all animals tested on the third through fifth day of incubation in one of the 12 home cultures is indicated in Figure 21. Animals chosen randomly from a population with such a distribution will meet the criterion of "not varying from the median response by more than one response group" about 64% of the time.

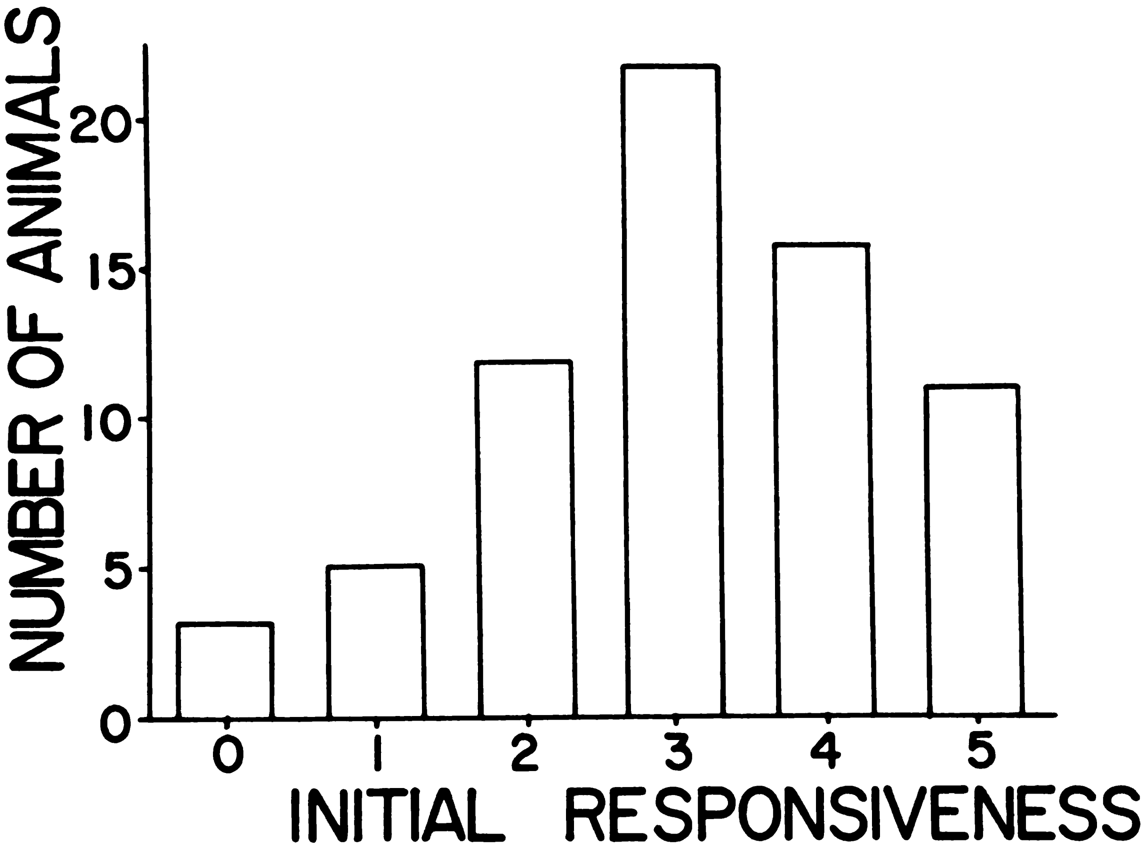


Figure 21. Population response distribution; inheritance studies. The ordinate indicates the number of animals tested which were determined to be members of each initial responsiveness group (number of contractions to the first five stimuli) shown on the abscissa. (N = 69)

With this in mind, compare the results of this study. Animals tested were within the median response group determined for their home culture on a given day 92.5% of the time (N = 53). If the response groupings of animals tested were compared to the median response group determined for their home culture over the entire five day incubation period, then only 85.5% meet the "not more than one response group deviation" criteria. Thus, response group uniformity was not completely consistent over time, even within the same culture dish. The response group consistency of those animals subcultured, when compared to the median response group of their parent home culture, was only 67% (N = 12). This is almost exactly the expected value for random selection.

The animals from two home cultures, one having predominantly high and the other predominantly low responders, were interchanged. Twenty-four hours later five of six low responders had become high responders. Two of the three high responders tested out to be low responders. Thus, 88% (N = 9) of the animals were changed from high to low (or vice versa) responders. Since the response changes occurred in both directions the effect cannot have been produced by the subculturing procedure.

Response group consistency within cultures was independent of whether the animals were tested individually or in groups of up to four at a time. This suggests that the contractile responsiveness of *Spirostomum* is not subject to intercellular influences at low population densities.

Electron Optics Studies

Figure 22A shows a cross section of an anterior portion of a *Spirostomum*. Note the somatic grooves from within which the cilia (c) extend and the increased density of mitochondria in the cortical region. Microfilamentous bundles (mf) of the contractile apparatus can also be seen immediately interior to the mitochondria. A cross section of the membranelle is evident at the left. Figure 22B shows an enlargement of a portion of the cortical region. The microtubular arrays (mt) are the kinetodesmal fibers emanating from the basal bodies (b).

Figure 23 shows two sections cut tangentially to the surface of the *Spirostomum* at the level of the somatic grooves. Note the basal bodies (b) just beneath the floor of the grooves and the kinetodesmal fibers (k) emanating from the basal bodies (Figure 23A). Figure 23B is a cut through the membranelle. Note how the cilia are arranged in 3 x 9 tufts. All of these findings are in agreement with previous work on *Spirostomum*[21,26-28,41,46] and follow the general pattern of ciliate ultrastructure.

No gross cytological differences were observed between high and low response group animals. However, studies using the electron microprobe analyzer suggested some interesting trends.

The general results of microprobe studies were consistent with results reported by Osborn.[46] Endoplasmic stores of Ca were found to be codistributed with P. This is illustrated in Figure 24, which shows Ca and P line scans taken through the center of a 2 μm thick section of a high responder fixed in cacodylate buffer. (All microprobe sections were oriented approximately the same as that section shown in Figure 22A.)

Figure 22.* Cross section through *Spirostomum*. Figure 22A shows a cross section through the anterior end of a *Spirostomum*. The subcortical microfilaments (mf) are believed to be the contractile apparatus. The microtubules (mm) emanating from the endoplasm approach the basal bodies of the membranelle. Note the increased density of mitochondria in the cortical region (3000x). Figure 22B shows an enlargement of the cortical and subcortical region. Cilia emanating from the somatic grooves (s), their basal bodies (b) and the lateral microtubules (mt) form the kinetosomal system. The ectoplasmic ridges (er), mitochondria (m) and microfilamentous bundles (mf) are evident (10,000x).

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*The micrographs in Figure 22 were reproduced[46] with the kind permission of D. Osborn.

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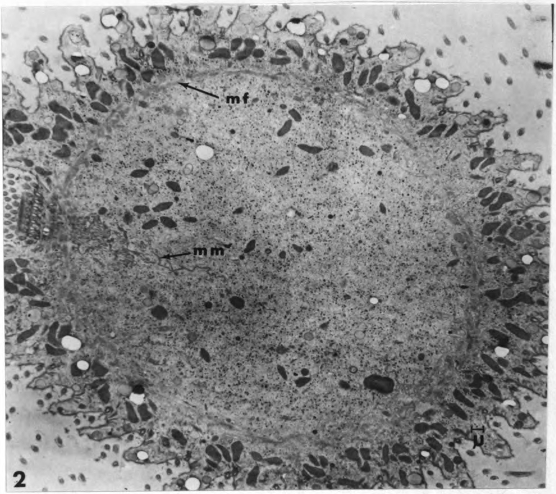

Figure 22A

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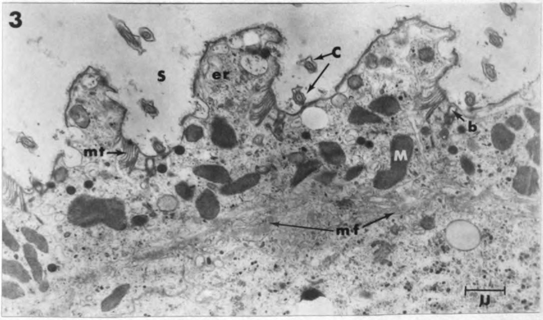

Figure 22B

Figure 23. Tangential sections through *Spirostomum* cortex. Figure 23A shows a section, almost parallel to the surface, through the somatic grooves (11,000x). Note how the cilia (c) originate from within the grooves with the basal bodies (b) lying just beneath the floor of the groove. Kinetodesmal fibers (k) can be seen to radiate from the basal bodies. Note also the dense population of mitochondria in the cortical region and what are believed to be the contractile filaments (mf) lying just interior to the mitochondrial zone. Figure 23B (8000x) shows a section oriented similarly to 23A, but which has been cut through the membranelle. Note how the cilia are arranged in tufts separated by membranes (mm) near the base of the cilia. The 9 + 2 structure is evident in individual cilia.

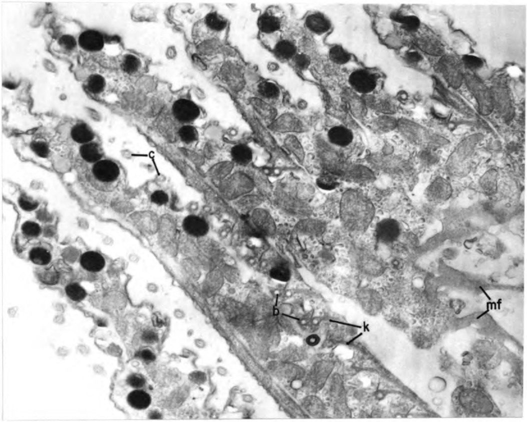

Figure 23A

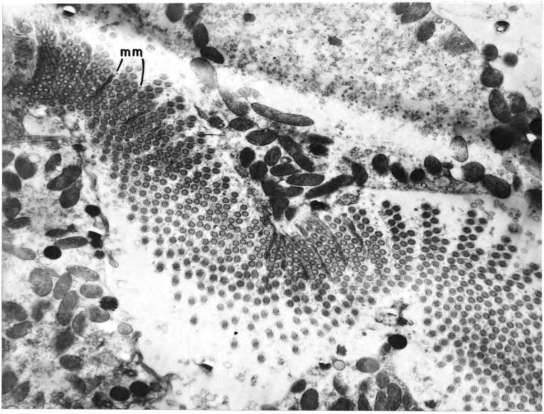

Figure 23B

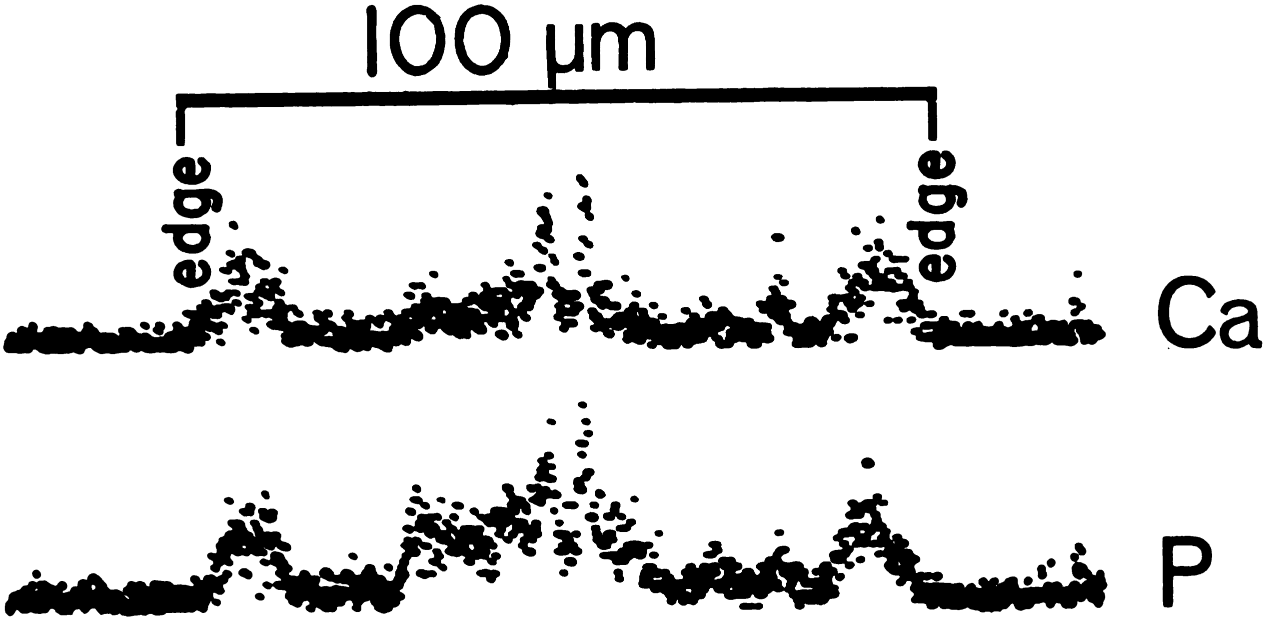

Figure 24. Ca and P distributions in *Spirostomum*. The Ca and P line scans were taken through a midsection from a *Spirostomum* fixed in cacodylate buffer. The section was oriented similarly to that shown in Figure 22A. The lines below the distance scale indicate the approximate edges of the animal. Note the coincident distributions of Ca and P in the endoplasm as well as their presence in the cortical regions. The analysis was done at 15 KeV with a sample current of 0.02 µa. The *Spirostomum* section was 2 µm thick and total scan time for each element was 500 sec.

Microprobe analyses of thin sections were unsuccessful. The 150 nm sections were stable under the electron beam, but estimates suggest that four to eight hours of beam time per line scan are necessary to resolve Mg above noise level. Such lengthy line scans were prohibitive at this time, but should be feasible in later studies.

Semiquantitative analyses also indicated that Ca and P are present in about equal concentrations, but that internal P is slightly more concentrated (Ca/P = 5/6). Internal Ca was calculated to be between 10 and 20 mM, which is consistent with an earlier study[33] measuring the uptake of 45-Ca. Magnesium was about 1 mM. Line scans of one hour or longer did not noticeably diminish or redistribute those stores of Ca, Mg, and P which were localized.

Figures 25A,B, respectively, show line scans for Ca, P, and Mg in one high and one low responder fixed in cacodylate buffer. Note how the P peaks are diminished at places where Mg peaks are present. (This is not always the case.) Note also that all Mg peaks are coincident with Ca peaks. (This seems to be a good generalization, but the contrapositive is not true.) Also, Mg peaks were usually found near the cortex (85%, N = 13), rather than in the endoplasm of the sections. This is consistent with the suggestion by Osborn[46] that Ca and Mg may be distributed at the microfilaments.

Area scans were done on ten of the sections from the five response group classified *Spirostomum* sectioned for this study. The ratio of the number of X-rays detected for Ca to the number detected for Mg are shown in Table 5. (The ratios do not reflect the absolute concentrations of these two elements.) A trend for high responders to have a higher Ca to Mg ratio than low responders is evident.

Figure 25. Ca, P, and Mg distributions in *Spirostomum*. Figure 25A shows 60 μm line scans for Ca, Mg, and P through a 2 μm section of a high responder fixed using cacodylate buffer. The edges of the animal are indicated. Note that Ca is coincident with Mg peaks, but that P is not. Note also that Mg is located near the cortical region, rather than in the endoplasm. Figure 25B shows 45 μm line scans for Ca, Mg, and P in a low responder fixed using cacodylate buffer. The scans start in the center of the animal and extend across only one edge. Note again how the Mg is distributed at the cortex and is coincident with Ca. All Ca and P analyses were based on 500 sec of scanning time. Mg was scanned for 1000 sec. Sample current was 0.02 μa and the beam tension was 15 KeV.

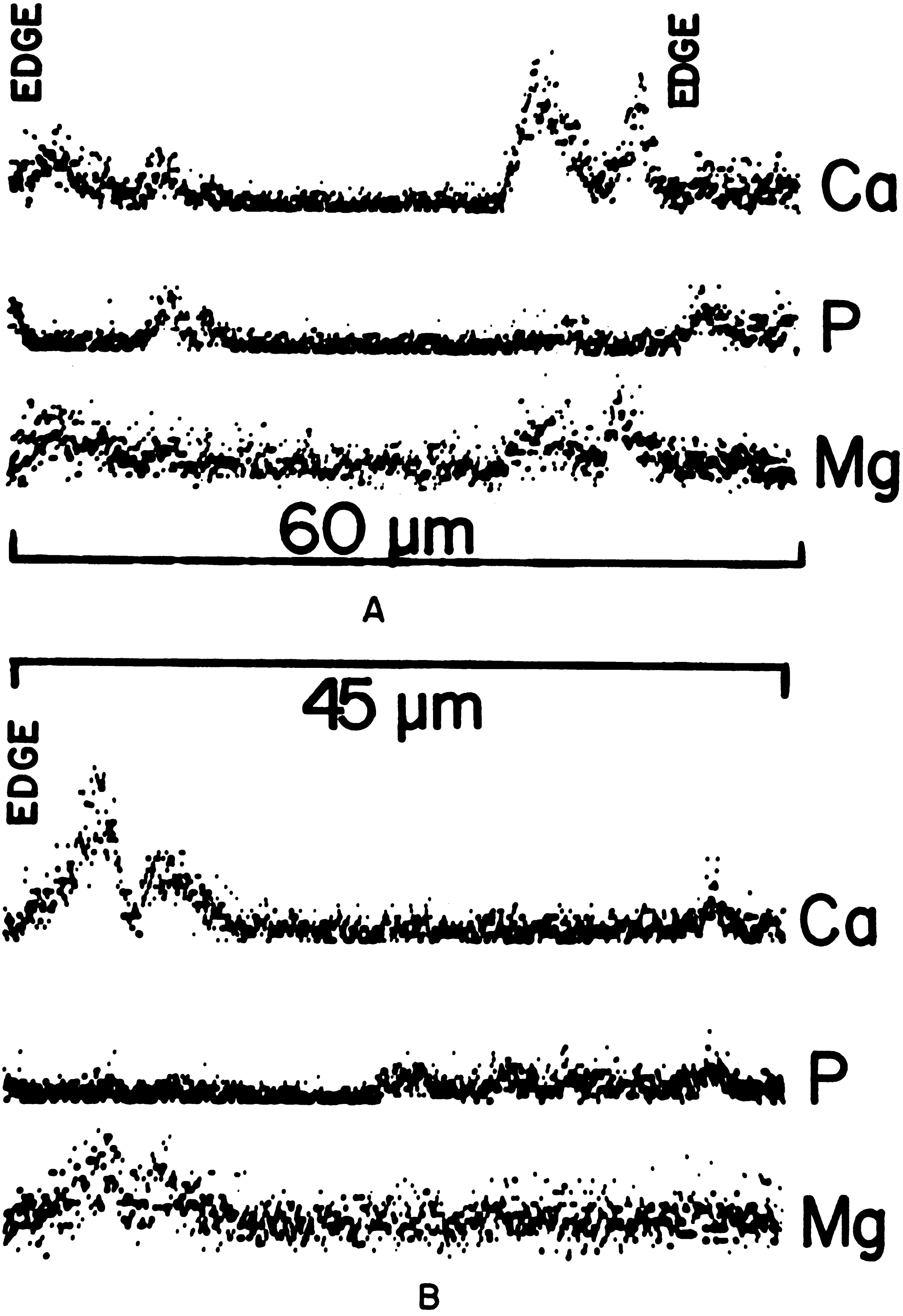

Figure 25

Table 5. Relative Ca/Mg ratios in high and low responders[*]

		Response Group	
		High	Low
Ca/Mg	Carter's	[1.52 M]	[0.93 M 0.44 P]
	Cacodylate	[0.81 M 1.19 M]	[0.72 M 0.73 A 1.45 M**]
		[1.14 A 1.30 M]	

A = anterior section
M = midsection
P = posterior section

[*] The ratios shown indicate the ratio of the number of X-rays detected for Ca to the number detected for Mg. Each ratio represents results from area scans of one entire 2 μm thick section of *Spirostomum*. Brackets indicate number of sections scanned from each animal. A total of 5 animals were examined.

[**] The Mg count in this animal was relatively high, but the Ca count was extremely high relative to the two other sections scanned from this animal.

DISCUSSION

Mechanical Stimulation Considerations

Three major modifications are observed over time in the responses elicited during repetitive (0.1 Hz) mechanical stimulation: (1) There is a change over time in the probability with which contractions are elicited such that animals with a high initial probability of contraction habituate. Those with a low initial probability of contraction appear to sensitize. Furthermore, the "high" responders remain more responsive than the "low" responders throughout a ten minute stimulation period. (2) There is an approximately 10% increase in the contracted length of an animal with successive contractions when the animal remains relatively responsive for a few trials. (3) There is a decrease over time in the magnitude of avoidance reactions (measured either in terms of distance backed up or the time spent backing up).

Measurements made at the time of stimulus delivery on the location within the well, body shape, attitude, and swimming activity of the animals of each subgroup indicated that changes in these factors were not responsible for the observed response modifications. Two other measures, which were not taken in this study, might also have induced the observed behavioral modifications. (1) The orientation (as opposed to location or attitude) of the animals relative to the nearest edge of the well may have altered over time. Since the effective stimulus intensity may vary with changes in this orientation (e.g., being perpendicular as opposed to parallel), any changes over time in the average orientation of the animals could account for the observed behavioral trends. However, the large percentage of time spent probing and therefore varying orientation suggests that this factor is not of significance. (2) The average number of times that the *Spirostomum* is physically touching the sides or bottom of the well when stimulated may vary over the stimulation period. Since physical contact with the slide (which cannot be determined for video tapes) could affect responsiveness by increasing the effective stimulus strength, this factor is of concern. Preliminary studies conducted in our laboratory[49] suggest that although the time spent touching does decrease somewhat over the stimulation period, it cannot account for the magnitude of the observed behavioral modifications. Thus, the reported modifications do not appear to be due to these additional factors.

The observed response modifications to mechanical stimulation (i.e., habituation, contraction timecourses, and avoidance reaction magnitudes) are not the result of stimulus injury, for repetitive electrical stimulation (1) yields identical changes over time in contraction magnitudes and in the magnitude of avoidance reactions and (2) causes a decrease in contractile responsiveness to mechanical stimulation even though no change is seen in the probability of contraction to electrical stimulation.[46]

The decreasing amounts of time spent swimming backward which occurred during the stimulation period are believed to be a direct result of the decrease in the magnitude of avoidance reactions. Since no change was seen during the stimulation period in the number of avoidance reactions elicited per unit time, the decreased magnitude of those responses which did occur would automatically result in a decrease over time in the amount of time spent swimming backwards.

The migration of the animals toward the edge of the slide well over the stimulation period (Figure 11C) is also believed to result from the observed decrease in avoidance reaction magnitudes. Normally the animals swim in all directions. When they touch the edge of the slide well, they generally initiate an avoidance reaction. This tends to drive them away from the edge of the well and explains why the *Spirostomum* are found in the edge zone of the well less often than would be expected by chance. However, as the magnitude of avoidance reactions decreases over the stimulation period (presumably this decrease also affects avoidance reactions initiated by touching the edge of the well) the animals become less likely to back completely out of the edge zone of the well. Thus, in agreement with observation, a net accumulation of *Spirostomum* in the edge zone would be expected to occur over the stimulation period.

Thus, the modifications in the three behavioral categories listed above (contraction probability, contraction timecourse, and avoidance reaction magnitude) are the only physiological behavioral changes which occur in individual *Spirostomum* over a period of repetitive mechanical stimulation. Furthermore, these changes are found to be independent of each other, since each modifies with a differing timecourse.

Because these three behavioral modifications are all occurring simultaneously to repetitive mechanical stimulation, interpretations of biochemical analyses are not as straightforward as Applewhite *et al.*[1-12,29,30] have assumed them to be. The situation is even further complicated by the fact that all animals do not undergo the same modifications. Whereas the contraction probability of high responders is seen to decrease, low responders show an increased probability of contraction over time. Similarly, low responders do not show as great, or as prolonged, a decrease in the magnitudes of contractions as do high responders.

The phenotypic inheritance studies showed response classifications to be a result of culture conditions. Thus, it seems likely that animals taken from different culture bowls may well yield differing chemical results. If large groups of *Spirostomum* are to be studied, one must ascertain not only that all experimental groups are handled equivalently during experimentation, but must also assume that the recent environmental history of all groups is identical.

The large size of *Spirostomum* suggest the possibility of examining the physical-chemical parameters of only one animal at a time. This would enable the physical-chemical results to be compared to and interpreted in conjunction with behavioral analyses performed on the same animal. The problems of variability among animals within a large group could thereby be avoided. (The microprobe studies reported here are an example of the feasibility of this approach.)

The rather arbitrary classification of animals into high, medium and low responder subgroups is not intended to imply that three such classes of *Spirostomum* exists. This study has, for a fixed stimulus intensity, suggested the existence of only two distinguishable groups: (1) animals which habituate to repetitive mechanical stimulation and (2) animals which do not habituate, but which may actually sensitize. It is interesting that these two classes of animals can be distinguished on the basis of their initial contractile responsiveness. Furthermore, since initial contractile responsiveness can be influenced by experimental parameters (stimulus intensity, chemicals, etc.), an animal's classification as a "high" or a "low" responder and hence the behavioral modifications exhibited over time are greatly affected by experimental conditions. If experimental variables (e.g., stimulus intensity, temperature, drugs) alter the initial contractile responsiveness of the animals[2,4,6-8,29,30] such that not all groups have the same initial level, it becomes unclear as to how comparable the groups are over time. Thus, in experiments of this nature perhaps two sets of animals should be tested: one set using a constant stimulus intensity and another set using an adjusted stimulus intensity to maintain a constant initial probability of contraction among all experimental groups.

The increased probability of contraction noticed on the second stimulus for animals of two of the response groups followed by a decreased probability on the third stimulus for all groups suggests that both habituation and sensitization occur in all animals but that habituation dominates in high responders, whereas sensitization dominates in low responders. "Miniexperiments", which would be designed to investigate the physiological changes in stimulated animals after the first, second and third stimuli relative to the physiological state of the animals prior to stimulation, might be useful in separating processes involved in these two phenomena.

The contraction initiating mechanism(s) to mechanical stimulation may well involve a prepotential. This can be deduced from the fact that contractions elicited to mechanical stimulation have a constant, approximately 3 msec, latency despite changes in stimulus intensity, contraction probability, and/or previous activity. For a prepotential which varied in amplitude with stimulus intensity, but which did not appreciably alter in timecourse, would affect probabilities of contraction without measurably affecting latency. However, such prepotentials have not yet been identified in *Spirostomum*,[26] although they have been found in *Stentor*.[53,54]

An interaction between the contractile and the ciliary systems does seem to exist. A total of 2400 stimuli were presented during the ten minute stimulation periods of behavioral series B. A total of 923 contractions and 1699 avoidance reactions were observed. Contractions thus occurred to 38.5% of the stimuli and avoidance reactions to 70.5%. However, contractions occurred on only 35.1% of the trials on which avoidance reactions were elicited, and avoidance reactions on only 64.7% of those trials exhibiting contractions. This difference between the obtained and expected values indicates that there is an interaction between contractions and avoidance reactions such that the occurrence of one suppresses the occurrence of the other* (97.5% one way confidence intervals). However, the ciliary and contractile motor systems can also operate independently. This is shown by the lack of habituation of the probability of exhibiting an avoidance reaction (Figure 10), even though the same animals habituate in contraction probability (high responders, Figure 8).

Eckert[23] found that Ca^{++} influx at the anterior end initiates ciliary reversals in *Paramecium*, but that influx at the posterior end causes forward acceleration. If the same situation exists in *Spirostomum*, then the occurrence of a contraction may cause changes in the Ca^{++} influx at the posterior end which occasionally override the anterior effects. This would explain the observed trend toward separation of contractions and avoidance reactions.

Transmembrane potentials, as well as Ca, have been demonstrated to control ciliary activity in *Paramecium*.[23,42-45] They control the rate

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*It is possible that the occurrence of a contraction could mask the observation of a concurrent avoidance reaction, but the use of video tape analyses helped to negate this concern.

of ciliary beating. The same situation may also exist in *Spirostomum*. If the transmembrane potential in *Spirostomum* is found to remain constant over a period of repetitive stimulation, then this would explain the constant rate of backward swimming observed for avoidance reactions of differing magnitudes. The decrease observed over time in the magnitudes of contractions and of avoidance reactions may well reflect the modifications in Ca release which are thought to control contraction probabilities.[9,10,25,46]

The operational distinction made between avoidance reactions and startle responses in this study may or may not have physiological significance; for, the magnitudes of avoidance reactions are continuously variable. Therefore, "startle responses" may in actuality be localized avoidance reactions which are not sufficient to reverse the direction of locomotion. This interpretation would be consistent with results reported for *Paramecium*.[23,42-45]

Site of Stimulus Action

The results from experiments using electrical stimulation suggest that electrical stimulation initiates contractions via a different mechanism than does mechanical stimulation.[26,46] This follows from the fact that the changes over time in contraction latencies to electrical stimulation are independent of previous contractions, but are dependent upon the number of previous stimuli delivered. This, plus the observed shortening of resting length over the stimulation period and the fact that contraction latencies are dependent upon stimulus strength are consistent with hypotheses invoking the importance of Ca^{++} in initiating contractions.[9,10,25,46] Since the animal is about 30 times more concentrated in Ca internally than externally, it is likely that large stores of Ca^{++} are being electrophoretically mobilized by the electrical stimulus.

If more detailed studies confirm that the Ca to Mg ratio is indeed higher in high responders than in low responders, then it is somewhat surprising that no correlation in response group classification was found between electrical and mechanical stimulation. For it would seem that the increased Ca to Mg ratio in high responders would make electrical stimuli more effective. Perhaps only a limited amount of the internal Ca is available for initiating contractions to mechanical stimulation. If changes in this limited Ca store reflect the major differences in the Ca to Mg ratio between high and low responders, then the importance of this ratio to mechanical stimulation may be overridden by the massive Ca release produced by electrical stimulation.

The fact that previous mechanical stimulation does not affect the latencies of contraction to electrical stimulation could also be explained if the Ca released by mechanical stimulation is tightly coupled to the contractile filaments, and if mechanical stimulation does not affect other Ca stores normally mobilized by the electrical stimulation. This would also be consistent with the facts (1) that previous electrical stimulation affects the probability of contraction to mechanical stimulation,[46] (2) that at most very small amounts of Ca are released during mechanical stimulation,[46] and (3) that large stores of Ca are released by electrical stimulation.[25,46]

The large amounts of Ca released during electrical stimulation also offer an explanation for the progressive shortening of resting length and the slowed rate of re-extension after contraction which occur over a period of repetitive electrical stimulation. For, the excess mobilized Ca may tend to flood the contractile filaments and thereby bias the level of tension toward increased tetanus.

The increased latencies over time with electrical stimulation suggest that the Ca stores activated by electrical stimulation are being depleted over time. This would be consistent with the sharp decrease in probability of contraction noted after prolonged stimulation, especially since a dramatic increase in latencies occurs concurrently.

Since the Ca concentrations in the microprobe sections were found to be about the same as previously reported for *in vivo* calculations, it seems reasonable to assume that Ca and other cations are not being leached out of the *Spirostomum* during the fixing procedure. However, it does not alleviate concerns about the possible redistribution of these elements. The specificity of coincident distributions of Ca and P and of Ca and Mg demonstrates, however, that a random redistribution is not taking place and suggests that the observed distributions resemble those occurring *in vivo*.

Comparisons of the contractile apparatus of *Spirostomum* and *Stentor* to those of other ciliates suggest that the membrane reticulum associated with *Spirostomum*'s contractile protein network is essential for powerful and repetitive contraction.[48] Perhaps this network binds Ca and Mg or makes them available for binding directly to the contractile proteins. Some rough calculations from the microprobe studies relate to this possibility. By referring to Figure 22, one can see that the cortical region of *Spirostomum* comprises about one-fifth the radius of the section. Thus, the cortical region comprises about $\frac{\pi(5r)^2 - \pi(4r)^2}{\pi(5r)^2} = \frac{25 - 16}{25} = 9/25 \cong 1/3$ of the cross sectional area of a *Spirostomum*. Studies on other ciliates have found that about 60% of the dry weight of a protozoan is protein and that dry weight $\cong$ 10% of the wet weight.[22] Assuming an even distribution of protein throughout the animal, a density of one, and an average protein molecular weight of 10^5, one finds about 3×10^{-12} moles of protein/*Spirostomum* cortex. By assuming that Mg is located predominantly in the cortex and that Ca is evenly distributed (as indicated by the microprobe scans), one also finds (by using the 15 mM and 1 mM microprobe determined concentrations of Ca and Mg, respectively) that there are 6×10^{-8} moles of Ca and 4×10^{-8} moles of Mg per *Spirostomum* cortex. But Osborn[46] has found that only 20% of the internal Ca is membrane bound. Thus, one calculates that there are about 3000 Ca molecules per molecule of protein. This amounts to about 30 Ca^{++}/amino acid residue! Similarly, assuming a range of from 20% to 100% of bound Mg, one finds that there are from 16 to 80 Mg^{++}/amino acid residue. Clearly, these rough calculations suggest that most of the cortical Ca and Mg is not bound directly to contractile proteins, but is rather membrane limited, perhaps in the membrane reticulum (or associated vesicles, as suggested by Lehman[41]) and is simply available for binding.

LIST OF REFERENCES

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1. Applewhite, P. 1968. Non-local nature of habituation in a rotifer and protozoan. *Nature 217*: 287-288.

2. ____________ 1968. Temperature and habituation in a protozoan. *Nature 219*: 91-92.

3. ____________ 1968. Retention of habituation in a protozoan improved by low temperature. *Nature 219*: 1265-1266.

4. ____________ 1970. Protein synthesis during protozoan habituation learning. *Commun. Behavioral Biol. 5*: 67-69.

5. ____________ 1971. Similarities in protozoan and flatworm habituation behavior. *Nature 230*: 284-285.

6. Applewhite, P. and S. Davis. 1969. Metallic ions and habituation in the protozoan *Spirostomum*. *Comp. Biochem. Physiol. 29*: 487-489.

7. Applewhite, P. and F. T. Gardner. 1968. RNA changes during protozoan habituation. *Nature 220*: 1136-1137.

8. ____________ 1970. Protein and RNA synthesis during protozoan habituation after loss of macronuclei and cytoplasm. *Physiol. Behavior 5*: 377-380.

9. ____________ 1971. A theory of protozoan habituation. *Nature 230*: 285-287.

10. Applewhite, P., F. Gardner, D. Foley and M. Clendenin. 1971. Failure to condition *Tetrahymena*. *Scand. J. Psychol. 12*: 65-67.

11. Applewhite, P., F. T. Gardner and E. Lapan. 1969. Physiology of habituation learning in a protozoan. *Trans. N.Y. Acad. Sci. 31*: 842-849.

12. Applewhite, P., E. Lapan, and F. T. Gardner. 1969. Protozoan habituation learning after loss of macronuclei and cytoplasm. *Nature 222*: 491-492.

13. Applewhite, P. and H. J. Morowitz. 1966. The micrometazoa as model systems for studying the physiology of memory. *Yale J. Biol. Med. 39*: 90-105.

14. Bergstrom, S. R. 1968. Induced avoidance behavior in the protozoa *Tetrahymena*. *Scand. J. Psychol. 9*: 215-219.

15. ____________ 1968. Acquisition of an avoidance reaction to light in the protozoa *Tetrahymena*. *Scand. J. Psychol. 9*: 220-224.

16. ____________ 1969. Amount of induced avoidance behavior to light in the protozoa *Tetrahymena* as a function of time after training and cell fission. *Scand. J. Psychol. 10*: 16-20.

17. ____________ 1969. Avoidance behavior to light in the protozoa *Tetrahymena*. *Scand. J. Psychol. 10*: 81-88.

18. Bien, S. M. and F. B. Preston. 1968. Calcification of *Spirostomum ambiguum*. *J. Protozool. 15*: 251-256.

19. Blättner, H. 1926. Beitraga zur Reizphysiologie von *Spirostomum ambiguum Ehrenberg*. *Archiv. für Protistenkunde. 53*: 253-311.

20. Carter, L. 1956. Ionic regulation in the ciliate *Spirostomum ambiguum*. *Exp. Biol. 34*: 71-84.

21. Daniel, W. A. and C. F. T. Mattern. 1965. Some observations of the structure of the peristomial membranelle of *Spirostomum ambiguum*. *J. Protozool. 12*: 14-27.

22. Dewey, V. C. 1967. Lipid composition, nutrition, and metabolism, p. 171, 205-214. In G. W. Kidder (ed.) *Chemical Zoology*, Vol I (Protozoa). Academic Press, New York.

23. Eckert, R. 1972. Bioelectric control of ciliary activity. *Science 176*: 473-481.

24. Eisenstein, E. M. 1967. The use of invertebrate systems for studies on the bases of learning and memory, p. 653-665. In G. C. Quarton, T. Malnechuk and F. O. Schmitt (ed.) *The Neurosciences*. Rockefeller University Press, New York.

25. Ettienne, E. 1970. Control of contractility in *Spirostomum* by dissociated calcium ions. *J. Gen. Physiol. 56*: 168-179.

26. ____________ 1972. Personal communication.

27. Finley, H. E. 1955. Electron microscopical observations on *Spirostomum ambiguum*. *Ann. N.Y. Acad. Sci. 62*: 229-246.

28. Finley, H. E., C. A. Brown and W. A. Daniel. 1964. Electron microscopy of the ectoplasm and infraciliature of *Spirostomum ambiguum*. *J. Protozool. 11*: 264-280.

29. Gardner, F. T. and P. B. Applewhite. 1970. Protein and RNA inhibitors and protozoan habituation. *Psychopharmacologia 16*: 430-433.

30. ____________ 1970. Temperature separation of acquisition and retention in protozoan habituation. *Physiol. Behavior 5*: 713-714.

31. Hamilton, T. C. and E. M. Eisenstein. 1969. A technique for continuous recording of the behavior of a single protozoan. *Amer. Zool. 9*: 548.

32. Jennings, H. S. 1901. Studies on reactions to stimuli in unicellular organisms. IX. On the behavior of fixed Infusoria (*Stentor* and *Vorticella*) with special reference to modifiability of protozoan reactions. *Amer. J. Physiol. 8*: 23-60.

33. Jones, A. R. 1966. Uptake of 45-calcium by *Spirostomum ambiguum*. *J. Protozool. 13*: 422-428.

34. ___________ 1967. Calcium and phosphorus accumulation in *Spirostomum ambiguum*. *J. Protozool. 14*: 220.

35. Kinastowski, W. 1963. Der Einfluss der mechanischen Reize auf die Kontraktilitat von *Spirostomum ambiguum*. *Acta Protozool. 1*: 201-222.

36. ___________ 1963. Das Problem "des Lernes" bei *Spirostomum ambiguum*. *Acta Protozool. 1*: 223-236.

37. Kinosita, H. 1938. Electric stimulation of *Spirostomum*. III. Orientation and time-intensity relation. *J. Fac. Sci. Imp. U. Tokyo 5*: 83-85.

38. Kung, C. 1971. Genic mutants with altered system of excitation in *Paramecium aurelia*. I. Phenotypes of the behavioral mutants. *Z. vergl. Physiol. 71*: 142-164.

39. ___________ 1971. Genic mutants with altered system of excitation in *Paramecium aurelia*. II. Mutagenesis, screening and genetic analysis of the mutants. *Genetics 69*: 29-45.

40. Kung, C. and R. Eckert. 1972. Genetic modification of electrical properties in an excitable membrane. *Proc. Nat. Acad. Sci. U.S. 69*: 93-97.

41. Lehman, W. J. 1969. The structural elements responsible for contraction in the ciliate *Spirostomum*. Ph.D. Thesis. Princeton University, Princeton, New Jersey.

42. Murakami, A. and R. Eckert. Cilia: activation coupled to mechanical stimulation by calcium influx. *Science 175*: 1375-1377.

43. Naitoh, Y. 1966. Reversal response elicited in non-beating cilia of *Paramecium* by membrane depolarization. *Science 154*: 660-662.

44. Naitoh, Y. and R. Eckert. 1969. Ionic mechanisms controlling behavioral responses of *Paramecium* to mechanical stimulation. *Science 164*: 963-965.

45. ___________ 1969. Ciliary orientation: controlled by cell membrane or by intracellular fibrils? *Science 166*: 1633-1635.

46. Osborn, D. 1971. Contractility in the protozoan *Spirostomum ambiguum*. Ph.D. Thesis, Mich. State Univ., East Lansing, Mich.

47. Pautard, F. G. E. 1959. Hydroxyapatite as a developmental feature of *Spirostomum ambiguum*. *Biochem. Biophys. Acta 35*: 33-46.

48. Pitelka, D. R. 1969. Fibrillar systems in protozoa, p. 364. In T. Chen (ed.) *Research in Protozoology*, Vol 3. Pergamon Press, New York.

49. St. Pierre, N. Unpublished.

50. Thompson, J. Unpublished.

51. Willows, D. 1972. Personal communication. Winter Conference on Brain Research, Vail, Colorado.

52. Wood, D. C. 1970. Parametric studies of the response decrement produced by mechanical stimuli in the protozoan, *Stentor coeruleus*. *J. Neurobiol. 1*: 345-360.

53. ____________ 1970. Electrophysiological studies of the protozoan, *Stentor coeruleus*. *J. Neurobiol. 1*: 363-377.

54. ____________ 1970. Electrophysiological correlates of the response decrement produced by mechanical stimuli in the protozoan, *Stentor coeruleus*. *J. Neurobiol. 2*: 1-11.

55. Yagiu, R. and Y. Shigenaka. 1963. Electron microscopy of the longitudinal fibrillar bundle and the contractile fibrillar system in *Spirostomum ambiguum*. *J. Protozool. 10*: 364-369.

APPENDICES

APPENDIX 1

Buffered Carter's Medium for *Spirostomum ambiguum*[50]

0.5 mM KCl

2.0 mM NaCl

0.2 mM $MgCl_2$

0.5 mM $CaCl_2$

1.0 mM KH_2PO_4

1.0 mM Na_2HPO_4

pH = 7.02

APPENDIX 2

Considerations in Handling *Spirostomum*

Our experience with *Spirostomum* has indicated a number of details which should be considered when using these animals for experimentation:

1. Transferring the animals in a pipette will partially habituate them to mechanical stimulation. Therefore, pipetting should be conducted as gently as possible, and an approximately ten minute equilibration period should be allowed before any additional stimulation is begun.

2. *Spirostomum* will tend to form tightly clumped groups. Animals from these groups are usually more flexible and more sensitive to transfer than are animals found swimming singly in the culture medium.

3. The first animal of the day tested in a given slide is often unhealthy looking. Several washes of the slide well with distilled water and culture medium can help to combat this problem.

4. Slide wells are subject to contamination which causes animals to become unhealthy shortly after placement in the well. To help prevent this, rinse the wells out thoroughly with distilled water immediately after each use and dry with a clean Kimwipe or other relatively lint-free towel. DO NOT PUT FINGERS OR OTHER OILY OBJECTS INTO A SLIDE WELL. Store slides in a relatively clean and dust-free environment. If the slide well should become contaminated, an overnight soaking in distilled water or in culture medium will sometimes help.

5. The level of the medium in the slide well can affect the intensity of mechanical stimulation which the animal receives. Use a constant level for all experiments.