Time-Action Relationships of the Specific Inhibitor of Caudal Regeneration in *Clymenella torquata.** (29166)

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The author recently reported(2) the presence of a very specific regeneration-inhibiting substance in the anal collar of *Clymenella torquata*. Ordinarily, if a posterior segment is removed from this 22-segment marine annelid of the northeastern Atlantic coast, a regenerative process is initiated. This process ultimately results in replacement of the number of segments lost and of the typical funnel-shaped anal collar. Regeneration may be totally blocked by application of sea water extracts of the anal collars of these worms, or by extracts of regions immediately posterior to the point of section. However, extracts of the prostomium or of a region immediately anterior to the point of section have no effect on the regeneration.

The present studies were undertaken to examine two aspects of the inhibition in more detail. First, experiments were designed to determine the length of treatment required to produce the effect, and second, to determine how long one can delay treatment and still produce the inhibition.

Materials and methods. Length of treatment tests. For this series of experiments, extracts of the anal collars of *Clymenella torquata* were prepared by isolating and grinding the collars in glass homogenizers in ice cold culture solution. This culture solution contained 10 mg % chloramphenicol (Chloromycetin, Parke, Davis & Co.) and 50 units per ml mycostatin (Nystatin, E. R. Squibb) in filtered pasteurized sea water (ca. pH 8.1). The resultant homogenates were diluted to a concentration of one anal collar per ml, and were then centrifuged at 5,000 $\times$ g for 10 minutes to remove large cellular debris.

One hundred seventy-three single posterior segments (15 through 17) were then isolated and divided into 6 groups. Twenty-eight were placed in individual containers (the depressions of an Hutzler ice ball tray) filled with 5 ml of culture solution. Twenty-eight more were placed in a Stender dish containing 28 ml of the anal collar extract. After one hour in this dish at 23°C, the segments were removed and placed in individual containers as above. A third group of 28 segments was treated with anal collar extract in the same way for 2 hours. The fourth and fifth 28-segment groups were exposed to anal collar extract for 3 and 4 hours respectively. After treatment, the trays containing the test and control segments were kept at 15°C. A sixth group of 33 segments received continuous treatment. The isolated segments were placed immediately into individual containers filled with 4 ml of culture solution. One ml aliquots of the extract were added, and the trays were then incubated at 15°C like the rest. After 8 days in the incubator, the trays were removed, and frequency of regeneration within each group was recorded.

Delayed treatment tests. For these experiments, 454 posterior segments were isolated. They were immediately placed in individual containers filled with 4 ml of culture water and transferred to the incubator at 15°C. To 48 of the worm-bearing containers, one ml aliquots of anal collar extract prepared as above were added without delay. Forty-eight others received one ml aliquots of plain culture water at the same time. These two groups served as the zero-delay series and its control. Six hours later, one ml aliquots of extract were added to 40 more containers, and 40 others received culture water. After 8½ hours 48 containers received extract and 48 received culture water. At 12 hours, extract was added to 48 containers, culture water to 38. Finally, after 18 hours delay, 48 containers received extract and 48 received plain culture solution. Thus, a time-graded series was obtained. The segments were allowed to regenerate for 8 days at

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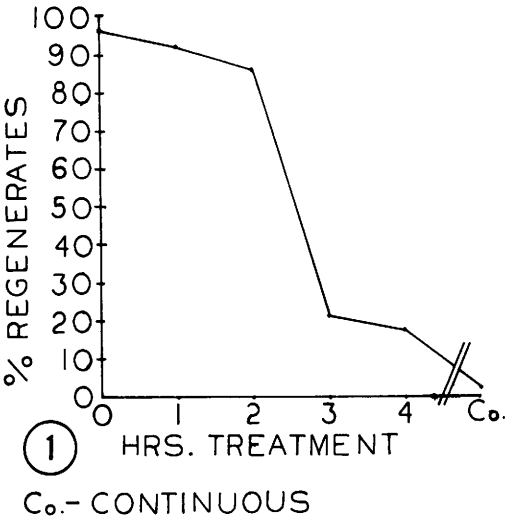
15°C and were then examined.

Results. Length of treatment tests. The results are shown in Fig. 1. Twenty-seven of the 28 posterior segments placed immediately into plain culture solution (zero-hour treatment) regenerated perfect new anal collars. Regeneration of those treated for one hour with extract was not significantly different, 26 out of 28 regenerating. Those treated for 2 hours regenerated nearly as well, 24 or 28 showing perfect new tails. However, this frequency represents a 10% drop from the control level. The remarkable difference in frequency is that which existed between the 2- and 3-hour treatment groups. Only 6 out of the 28 segments treated for 3 hours regenerated. This represents a highly significant 64% drop in frequency from the 2-hour level, and a 75% drop from control level. The segments treated for 4 hours responded as did the 3-hour group, 5 out of 28 regenerating. The segments treated continuously with extract showed a very low frequency, only one out of the original 33 test segments growing a new anal collar.

Delayed treatment tests. Fig. 2 graphically illustrates the results of this series of tests. In all groups, the controls regenerated very well, varying from ca. 90-96% perfect new anal collars. The zero-delay group, as might be expected from the above series of experiments, regenerated very poorly. Only one out of 48 grew a new tail. Only 4 out of the 40 segments treated after a 6-hour delay were able to regenerate. Of those treated after an 8½-hour delay, 8 out of the 48 regenerated—a slight but significant increase in frequency over the zero-delay group. Of the 48 test segments exposed to the extract after a 12-hour delay, 13 were able to regenerate. In contrast to the above results, after an 18-hour delay the frequency of regeneration of the test segments was essentially the same as that of the controls. No evidence of inhibition was apparent. It might be noted here that when inhibition was present, it was nearly always total. Only a very few of the inhibited segments showed any signs of beginning regeneration, in marked contrast to the perfect new anal collars of the non-inhibited segments.

Discussion. Two things are immediately apparent from the length of treatment tests: (1), that the inhibitor penetrates to its target site rather rapidly, and (2), that its effect is of very long duration. The latter observation leads to the conclusion that the inhibitor, whatever it may be, is not readily metabolized and that it probably blocks an initial step necessary for regeneration. The site, mode of action, and the chemistry of the inhibitor are unknown. Further studies are in progress.



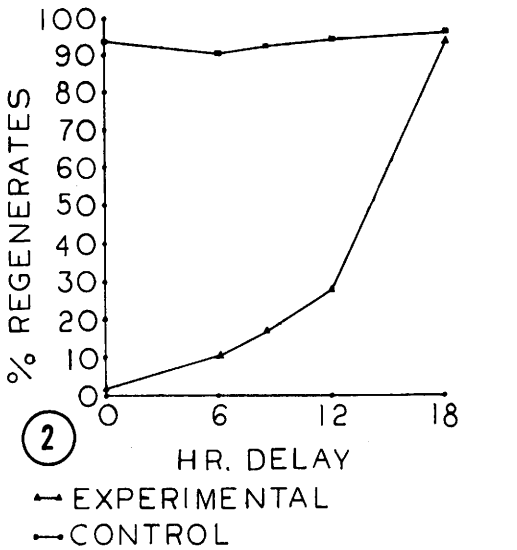


FIG. 1. Results of length of treatment tests.
FIG. 2. Results of delayed treatment tests.

The delayed treatment experiments were designed to test the hypothesis that the inhibitor must enter the test segment via an open wound surface. Since Sayles(1) has shown that new tissue appears at the wounded surface in *Clymenella* about 24 to 48 hours after cutting, it seems reasonable to assume that by 18 hours after cutting, wound closure would be essentially complete. The sudden increase in regeneration frequency between the 12- and 18-hour delay groups may thus be explained on the basis of a closing wound which becomes impermeable to the inhibitor.

Summary. These data suggest that one may extract from the anal collars of *Clymenella torquata* an inhibitor which acts within 3 hours, and whose effect lasts at least 8 days. The data furthermore suggest that the inhibitor is not easily metabolized, and that it blocks an initial step in regeneration. The data from the delayed treatment tests suggest that the inhibitor must enter an open wound surface, and that a closed wound is impermeable to inhibitor.

1. Sayles, Leonard P., *J. Exp. Zool.*, 1932, v62, 237.
2. Smith, Stephen D., *Biol. Bull.*, 1963, v125, in press.

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Effect of Intravenous Estrogen on an Inhibitor of Hyaluronidase and on Clotting Factors in Blood.* (29167)

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Intravenous estrogen has been used extensively in attempts to control bleeding(1-5). Well controlled, double-blind studies and laboratory data have, however, often been lacking. The present study deals with the effect of i.v. estrogen† on the clotting system and the ground substance. We have not attempted here to assess the clinical efficacy of the hormone.

Materials and methods. Subjects were healthy medical students, nurses and patients with no known bleeding disorders on the wards of a large general hospital. The double-blind method was used to determine bleeding and clotting times, blotter weights, one-stage prothrombin times, factors V, VII-X complex, antithrombin, and spread of intracutaneous hyaluronidase. Ampoules containing 40 mg of estrogen or placebo were coded and paired. If the subject received both preparations, a 48- to 72-hour interval elapsed between injections. If a single ampoule was used, its contents were unknown to both subject and investigator. Results are related to a single dose of either preparation. No evidence of toxicity or side effects was noted. Double-blind techniques were omitted for determination of prothrombin by the synthetic substrate (TAMe) method, and for serum hyaluronidase assay. Unless otherwise stated, all studies were performed 1½ hours after injection of either hormone or placebo, since Johnson(6) has shown this to be the period of optimum activity. Bleeding time was measured by the method of Ivy(7). Clotting time was determined by the three tube Lee-White method(8). Prothrombin time was measured by the method of Quick(9). Prothrombin content, employing the synthetic substrate TAMe, was assayed by the method of Glueck(10). Factor V was measured by the method of Stefanini and Dameshek(11). Factor VII-X complex was determined as follows: substrate plasma with a prothrombin time greater than 25 seconds was obtained

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† Premarin®.