Regeneration and growth control in Nereis

III. Separation of wound healing and segment regeneration by experimental endocrine manipulation

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SUMMARY

Loss of posterior segments from immature Nereis is followed by the associated processes of wound healing and segment regeneration. In the absence of a hormone secreted by the brain, wound healing alone ensues. However, proliferation of segments can be initiated by intracoelomic implantation of a living brain (removed from a donor) during the course of wound healing or subsequent to its completion. In this way, wound healing and regenerative growth can be separated experimentally. Wound hormones may have a role in wound healing, but are probably not involved in the initiation of segment regeneration in Nereis. However, developments at the site of the wound which follow loss of posterior segments in the absence of regeneration progressively reduce the regenerative activity which follows subsequent brain implantation. The nature of the changes is unknown. Maintenance in a decerebrate condition also reduces regenerative capacity. This effect is independent of changes at the wound and may be due to accelerated maturation.

INTRODUCTION

Loss of caudal segments from immature Nereis is followed by wound healing, reformation of the postsegmental pygidium bearing the paired anal cirri, and after an initial time-lag, rapid segment proliferation. However, these three processes do not invariably accompany each other. Mature animals exhibit wound healing and pygidial formation after loss of segments but fail to engage in regenerative segment proliferation (Golding, 1967e; Porchet & Dürchon, 1968; Baskin & Golding, 1970), and this indicates the existence of a significant difference in the physiological control of these processes.

The basis of the distinction between mechanisms controlling these processes has been established by experimental investigations into endocrine phenomena in nereids. Posterior segment regeneration is dependent on a hormone secreted by the supraoesophageal ganglion or 'brain' (Hofmann, 1966; Golding, 1967a; for earlier references, see review by Dürchon, 1967). In the absence of the brain, one or two parapodial rudiments at the most are produced, but differentiated

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segments are not developed. In contrast, wound healing and pygidial formation are not blocked by decerebration. In *Nereis* which possess a brain (either *in situ* or implanted in the coelomic cavity) the first segments to be regenerated appear a little more than one week after segment loss. Proliferation continues in the presence of the brain but slows down and stops if the brain is removed (Golding, 1967b). This indicates that the cerebral principle exerts a prolonged and continuous influence on segment proliferation during regeneration. There appears to be no critical stage in regeneration in connexion with which the action of the hormone can be described as a 'triggering' effect.

Segment regeneration is also influenced by other factors. The rate of segment production is directly correlated with the number of segments lost. Furthermore, the rate is initially high, but declines rapidly as regeneration proceeds (Golding, 1967c). Such contrasting rates of proliferation are not due to fluctuating hormone concentrations, but to differences which constitute a gradient in inherent potentiality, which declines posteriorly. Thus, although the brain hormone provides some indispensable prerequisite for regenerative growth, it cannot be said to ‘control’ this process (Golding, 1967d).

Wound hormones – substances liberated by damaged cells – have been thought to constitute an important influence on regenerating tissues (review by Needham, 1964). Such factors have received little attention with respect to annelids.

Wound healing is a complex process involving, among other aspects, the dedifferentiation, proliferation, migration and phagocytosis of various cell types (Clark & Clark, 1962; Herlant-Meewis & Nokin, 1962; Hofmann, 1966; Boilly, 1969). This process and the events leading up to segment proliferation normally take place at the same time and in the same area, and this situation hinders the elucidation of the nature of the structures and processes which are subject to the influence of the brain hormone. This study was initiated in the hope of finding a procedure by which regenerative segment proliferation could be studied in isolation from the process of wound healing by which it is usually accompanied, and to investigate the possibility that wound hormones are involved in triggering such proliferation.

**MATERIALS AND METHODS**

Specimens of *Nereis diversicolor* were collected from the river Wansbeck, Northumberland. They were maintained in the laboratory in full strength sea water to which 140 i.u. of benzyl penicillin and 0·0014 g of streptomycin (as streptomycin sulphate) per ml of sea water had been added.

After collection, the *Nereis* were initially put into large bowls of sea water at 12–20 °C for about 24 h to allow the contents of the gut to be evacuated. They were transferred to a cold room (2–4 °C) where they were kept until required. If the water was replaced periodically (twice each week) animals
kept in this way remained healthy for prolonged periods of time, and this was an essential condition for the experiments described below.

Animals were anaesthetized using a solution of 0.5% MS 222 (Sandoz) in sea water or in 5% ethyl alcohol in sea water (the latter method is preferred). Brains were always removed intact, together with the overlying epidermis, and were implanted into the coelom through a puncture in the body wall. Experimental animals were kept together in plastic boxes (approximately 12 × 6 in.) the bottoms of which were covered with glass tubes of an appropriate diameter. The water was aerated and the temperature maintained at 20 ± 1 °C.

RESULTS

The effect of decerebration and wound healing on subsequent segment regeneration

This experiment was designed to determine if segment regeneration can be initiated by brain implantation after the commencement, progress or completion of wound healing. Eighty immature animals (i.e. with oocytes less than 120 μm in diameter) were randomly divided into eight groups of equal size. The members of one group were subjected to decerebration, amputation of all but 30 anterior segments and distinctive parapodial clipping (see Golding, 1967a); and were maintained together at 20 °C. The other groups were transferred to the cold room. Approximately 3½ days later a second group was removed from the cold room and similarly subjected to decerebration, amputation and parapodial clipping, and introduced into the box containing the first group. This procedure was repeated at regular time intervals (7, 10½, 14 and 17½ days) after the start of the experiment and on day 21 was applied to the remaining pair of groups. On day 21 the brains of 70 intact immature Nereis were removed. Each member of the groups described above (with the exception of one of the last pair) received one immature brain as an intra-coelomic implant. This meant that the different groups had been allowed to engage in wound healing (in the absence of segment regeneration) for different time intervals before subjectation to the influence of the hormone. The animals which received no brain implants constituted a control group.

The animals were kept together and the number of segments regenerated by the survivors was determined after 21 days. The results, and a regression line obtained from them by statistical analysis, are shown in Fig. 1. The correlation coefficient of −0.93 has a probability $P$ lying between 0.01 and 0.001. The results demonstrate that segment regeneration can be initiated by the presence of the brain hormone in animals maintained in a decerebrate condition, and in which wound healing has proceeded for 10 days or more. They also show that the regenerative capacity of decerebrate animals in which wound healing is proceeding is subject to a progressive decline. After about 3 weeks little or no endocrine-stimulated proliferation is possible.
The experiment does not, however, establish the basis of the decline in regenerative ability. Decerebrate Nereis cannot feed, and consequently their condition constitutes a state of physiological decline. Such a deterioration or other changes involving the physiological condition of the animal as a whole may be the basis of the reduction in regenerative ability. Alternatively, changes associated with the progress and completion of wound healing may be responsible. The following three experiments were designed to elucidate this problem.

**The effect of decerebration on subsequent segment regeneration**

Seventy immature Nereis were randomly divided into seven groups of ten. Members of one group were subjected to decerebration and distinctive parapodial clipping, but otherwise left intact. They were maintained at 20 °C. The other groups were transferred to the cold room. Approximately 3½ days later, another group was brought to room temperature and subjected to the same treatment as that employed with the first group. The other groups were treated similarly at intervals of 3½ days. On day 21 the operations were performed on the last group; all but 30 segments were then amputated from all the animals and brains from 70 immature donors were implanted. In this way, all groups of animals were identical with respect to wound healing, since the brain hormone was made available at the same time as loss of segments occurred. The groups differed with respect to the length of time during which they had been maintained without a brain prior to amputation of segments and brain implantation. They were subsequently kept together for 21 days, after which the number of segments regenerated was determined.
Regeneration in Nereis

The results, and a regression line obtained from them, are shown in Fig. 2. The correlation coefficient is \(-0.88\) \((P = 0.01-0.001)\). These results show that maintenance in a decerebrate condition progressively reduces the hormone-dependent regenerative capacity. However, after 3 weeks, brain implantation induces segment proliferation beyond that exhibited by decerebrate Nereis (for examples, see Figs. 1 and 3; also Golding 1967a, b, e).

The effect of wound healing on subsequent segment regeneration

Seventy immature Nereis were randomly divided into seven groups of equal size. All were subjected to brain ablation and distinctive parapodial clipping. All groups were left otherwise intact, with the exception of one group, from which all but 30 segments were removed. All groups were maintained at 20 °C. Approximately 3\(\frac{1}{2}\) days later, animals of a second group were subjected to amputation of segments, and so on. On day 21, segments were amputated from the seventh group and 70 immature donors were used to provide brain implants for all specimens.

By means of this procedure, the different groups resembled each other with respect to the length of time during which they were deprived of the presence of a brain, and therefore with respect to the extent to which they had suffered physiological decline. They differed with respect to the length of time which intervened between loss of segments and provision of a brain – that is, with respect to wound healing. The numbers of segments regenerated by the various groups during the 21 days following brain implantation did not show significant differences. This is understandable in the light of the results of the previous
Fig. 3. Segment regeneration induced by brain implantation carried out at different time intervals subsequent to loss of segments. The interval between decerebration and brain implantation was identical with respect to the different groups. Standard errors represented by vertical lines.

experiment, which show that regenerative ability is greatly reduced in *Nereis* deprived of the presence of the brain for 3 weeks, even in the event of simultaneous brain implantation and segment loss. Such a prolonged period of hormone deprivation was also employed in this experiment, and in this situation there is little room for a further significant reduction in regenerative activity on account of wound healing.

A further experiment was set up which resembled the abortive one above, but involved a smaller range in the periods of time which intervened between amputation and brain implantation. Seventy-five immature *Nereis* were divided into five groups of equal size. The procedure was identical to that above, except that the groups were subjected to amputation 0, 3½, 7, 10½ and 14 days after decerebration, respectively. On day 14, 75 immature donors were used to provide brain implants. The experimental animals, together with a decerebrate control group of 15 individuals, were kept together for 21 days, after which the number of segments regenerated was determined.

The results, and a regression line obtained from them, are shown in Fig. 3; the correlation coefficient was found to be $-0.92 (P = 0.02-0.05)$. This indicates that delay in the provision of the brain hormone after segment loss progressively reduces the endocrine-dependent regenerative ability.

*The effect of prior wound healing on the time course of segment regeneration*

Frequent anaesthetizing and observation of experimental animals were avoided in the experiments described above, since they are probably inimical to health and survival. However, as shown above, segment proliferation can be initiated when wound healing is well advanced or complete, and it seemed
possible that the first segments would be proliferated more rapidly upon brain implantation in such circumstances.

Twenty immature *Nereis* were randomly divided into two groups of equal size. Both groups were subjected to decerebration and parapodial clipping. All but 30 segments were removed from members of one group. Both groups were kept together at 20 °C. Seven days later, segments were amputated from the individuals with intact tails, and brains removed from 20 immature *Nereis* were implanted into all animals. They were examined under anaesthetic on day 7 and at various intervals thereafter. The results are summarized in Fig. 4. No segmental rudiments were visible 7 days after brain implantation; on day 10, almost identical numbers were present and thereafter, proliferation was more rapid in animals which were subjected to simultaneous amputation and brain implantation than in specimens which had undergone prior wound healing. Since the first segments are subject to most rapid proliferation (Golding, 1967c) they were probably differentiated 8–9 days after provision of the brain hormone in each case. The experiment suggests that the initiation of segment proliferation by the brain hormone is subject to a delay of rather more than 1 week, irrespective of the progress of wound healing at the time of brain implantation.
The effect of circulating hormone on segment rudiment development in decerebrate Nereis

Exirpation of the brain at the time of segment loss blocks regeneration; however, one or two segmental rudiments do appear under these circumstances. I have suggested elsewhere (Golding, 1967c) that 'normal' and regenerative growth in Nereis are dependent on the same hormone. This hypothesis is supported by a number of observations. They are essentially similar processes; brains removed from intact animals are as competent to induce regeneration in decerebrate hosts as brains of regenerating donors (a finding supported by the results of Olive, 1974); and furthermore, a decerebrate fragment grafted into a host regenerates segments even if the host is intact (i.e. non-regenerating) (Durchon & Marcel, 1962; Golding, 1967d). In the light of these observations it seemed possible that segment rudiment development in decerebrate Nereis is a consequence of the presence of hormone, secreted by the brain prior to its removal. Elimination of this stimulus, if present, would effect a more complete separation of the processes of wound healing and segment proliferation, and hence facilitate their study.

However, it was found that maintenance in a decerebrate condition for 7 days at 20 °C, to allow for the reduction or elimination of hormone from the body prior to amputation of segments, does not prevent the development of up to two segmental rudiments. This suggests that the production of the rudiments is not hormone-dependent.

Finally, ten immature Nereis were subjected to brain removal and segment amputation. They were observed at frequent intervals to determine the time course of the process of wound healing. Judging by observations of the living material under the dissecting microscope, wound healing appears to be complete by the end of the first week, by which time the anus has formed. Development of the pygidium and anal cirri occurs during the second week.

DISCUSSION

This investigation was undertaken partly to find a method by which the normally associated processes of wound healing and segment regeneration could be separated. Its results indicate that such a separation is possible. In decerebrate Nereis, loss of posterior segments is followed by wound healing in the virtual absence of segment regeneration. Subsequent implantation of a living brain will initiate prolific segment production in these circumstances, provided that implantation is not delayed for more than about 14 days. I have remarked previously (Golding, 1967c) on the utility of annelids in the investigation of growth phenomena on account of their segmented structure. The ability to initiate regenerative growth after completion of wound healing could enhance their value in this respect; it should be particularly useful in the elucidation of
the structures and processes that are affected by the brain hormone. Indeed, this procedure has already been exploited in studies undertaken in this laboratory (Olive, 1974).

The results of this study also show that prolonged delay between decerebration and amputation on the one hand, and brain implantation on the other, reduces the ensuing regenerative activity. It was thought that this reduction might be due either to the deleterious effects of maintenance in a decerebrate state (i.e. to changes involving the physiological condition of the animal as a whole) or to changes taking place at the site of wound healing and regeneration (i.e. to local developments). The experiments described above indicate that changes of both types are contributory factors.

There are at least two ways in which maintenance of Nereis in a decerebrate condition may result in a decline in regenerative ability. Firstly, decerebrate Nereis are in a state of physiological decline since they cannot feed. However, it is doubtful if nutritional deficiency is solely responsible for the decline under consideration, since decerebrate Nereis which have brains implanted in the coelom exhibit little reduction in regenerative ability over more prolonged periods of time (Golding, 1967a). Secondly, maintenance in a decerebrate state may reduce regenerative capacity by inducing precocious maturation, since the brain hormone not only promotes regenerative growth but also exerts an inhibitory influence on sexual maturation in Nereis (review by Durchon, 1967). Naturally occurring maturation is accompanied by loss of regenerative activity (Golding, 1967e; Porchet & Durchon, 1968), and it is probable that Nereis whose maturation has been experimentally accelerated by decerebration are similarly unable to proliferate segments, although this question has not been investigated. Loss of regenerative ability may be due to the inability of the mature body to respond to the brain hormone and/or to a feedback effect whereby the mature body inhibits the secretory activity of the brain removed from an immature donor and implanted into the coelom (Golding, 1967e; Porchet, 1967). However, it must be pointed out that the effect of decerebration on the development of the gametes was not determined in these experiments. The animals involved were at a very early stage of maturation, and decerebration usually results in abortive development of the gametes in such cases (e.g. see Porchet, 1970; Schroeder, 1971).

The finding that prolonged delay between loss of posterior segments and subsequent brain implantation reduces the amount of ensuing regenerative activity (irrespective of the effect of maintenance in a decerebrate condition) is more difficult to interpret. It bears a superficial similarity to that of Herlant-Meewis & Deligne (1964), who investigated the trophic influence of the ventral nerve cord on regeneration in the oligochaete Eisenia foetida. They concluded that the nerve cord induces regeneration at the cut surface but that, in the event of its absence at the wound, a trophic influence can be exerted by nerve fibres growing into the healing zone if they reach it whilst the epidermis is still in a
state of dedifferentiation. Otherwise, regeneration is blocked. However, the results given in Figs. 1 and 3 show that there is little or no reduction after 3½ days or 7–8 days, yet it is during this time that wound healing takes place (see Olive (1974) for a more detailed histological study of this process).

Consequently, it is unlikely that prior wound healing or the disappearance of wound hormones is responsible for the decline in regenerative ability. Similarly, Wilkerson (1963) found that injections of growth hormone leads to the regeneration of well-formed limbs in hypophysectomised newts, even if the hormone is administered 15 days after amputation (i.e. subsequent to the formation of a fibrous pad over the wound). Wound healing, but no segment regeneration, is exhibited by amphipodid lumbricid oligochaetes subjected to removal of posterior segments during an active phase of their life-cycle. Regenerative growth ensues during the subsequent period of diapause, and thus a time interval of several months may intervene between wound healing and regeneration (review by Golding, 1974). In crabs, loss of appendages is followed by wound healing and ‘basal growth’, after which no further regenerative activity occurs until the onset of the next moult. Regenerative growth then proceeds under the influence of the moulting hormone (Bliss, 1960; Passano & Jyssum, 1963). Other work on crustaceans (Needham, 1947) and investigations into limb regeneration in insects (Bodenstein, 1955) also lead to the conclusion that wound hormones have no role in the initiation of regenerative growth. They may affect wound healing by stimulating epidermal mitosis (described for *Rhodnius* by Wigglesworth, 1937). They may also initiate the activation and migration of neoblasts in planarians and annelids (Dubois, 1950; Stephan-Dubois, 1955, 1956, 1958).

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REFERENCES


Regeneration in Nereis


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