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Cell contact and positional communication in hydra

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SUMMARY

Positional communication and the functional coupling of muscular reflexes were examined in grafted hydra. A head and distal gastric region inhibited head regeneration by a host sub-hypostome within 4–5 h of grafting. Functionally coupled pathways which indicated the presence of gap junctions also formed between graft and host during this time. It is suggested that gap junctions provide a channel for positional communication.

INTRODUCTION

Hydra restores its simple pattern of head, body and foot by regenerating lost parts in their correct polarity and by regulating the proportions of the axis despite variations in the overall size of the animal. Normal regeneration of a new head is inhibited however when an intact head is grafted to the body. Experiments like this have given rise to a model of pattern formation which proposes that differentiation arises from a cell's positional information (Wolpert, 1971). This property is determined by the interaction of two gradients: one is cell bound and registers the positional value of each cell while the other is a long range inhibitor of head development which moves rapidly from a source in the intact head (Wolpert, Hornbruch & Clarke, 1974). The molecular basis of this mechanism is unknown but it has been shown on theoretical grounds that communication within a positional field could involve the diffusion of a low molecular weight morphogen (Crick, 1970; Wolpert, Clarke & Hornbruch, 1972). If this is true it is unlikely that the morphogen passes freely between the cells since escape from the epithelium into the gut would disturb the gradient. Consequently diffusion between the cells may be restricted to small, highly permeable channels which are laterally insulated against leakage. These requirements are satisfied by gap junctions which have been shown in many cells to allow the movement of nucleotides and hydrated ions (Gilula, Reeves & Steinbach, 1972; Bennett, 1973). As gap junctions are also found in hydra (Hand & Gobel, 1972) their role in positional communication is examined here in detail. The course of formation of gap junctions is compared with re-

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generation at a graft–host border for if these contacts channel positional communication they must form during the time in which a regenerating head is inhibited.

A suitable combination for demonstrating head regeneration and inhibition can be obtained by grafting a head and distal gastric region to a decapitated body. This is described as a H12/12...F combination using the H1234B56F notation in which H, B and F mark the head, bud and foot and numbers refer to equally spaced portions of the body (Wolpert *et al.* 1974). A head may regenerate at the graft–host border but if cultured at a lower temperature the H12/12...F combination will regulate to produce a single axis. These diverging pathways result from the strong temperature dependence of determination of the head boundary value by the host sub-hypostomal region (Hicklin, Hornbruch, Wolpert & Clarke, 1973). At the higher temperature determination occurs before communication can be established with the grafted head but at the lower temperature healing leads to the passage of inhibition across the graft–host border (Wolpert, Hicklin & Hornbruch, 1971). The onset of communication during healing can then be plotted by incubating grafted animals for increasingly longer periods at a lower temperature before shifting them to a higher temperature. When an animal fails to respond to the higher temperature by regeneration at the graft–host border the positional communication channel may be considered to be re-established.

Gap junctions can be recognized in thin sections of normal tissue by the characteristic reduction in membrane separation (Hand & Gobel, 1972) but it is difficult to detect the formation of new junctions between healing cells or to assess them in a quantitative manner. An assay can however be based on functional rather than structural grounds. The myoepithelial cells that are responsible for movements of the body are electronically coupled for both contraction and electrical activity are unaffected by denervation (Campbell, Josephson, Schwab & Rushforth, 1976). Hydra thus resembles mammalian tissue such as smooth muscle and cardiac muscle (Dewey & Barr, 1962; Barr, Dewey & Berger, 1965) and it is likely that the low resistance pathways, known to be present in the Hydrozoa (Spencer, 1971) are mediated by gap junctions. Pairs of dissociated heart cells will synchronize their beating when they are electrically coupled (DeHaan & Hirakow, 1972) and this experiment suggests that the co-ordination of reflex movements in hydra could also be used as an indicator of junctional communication.

METHODS

(*a*) *Grafting*

Large budding *Hydra attenuata* and *Hydra littoralis* fed the day previously on brine shrimps were axially grafted (Hicklin *et al.* 1973). Animals were left to relax and extend in culture medium in a Plasticene-floored dish and were

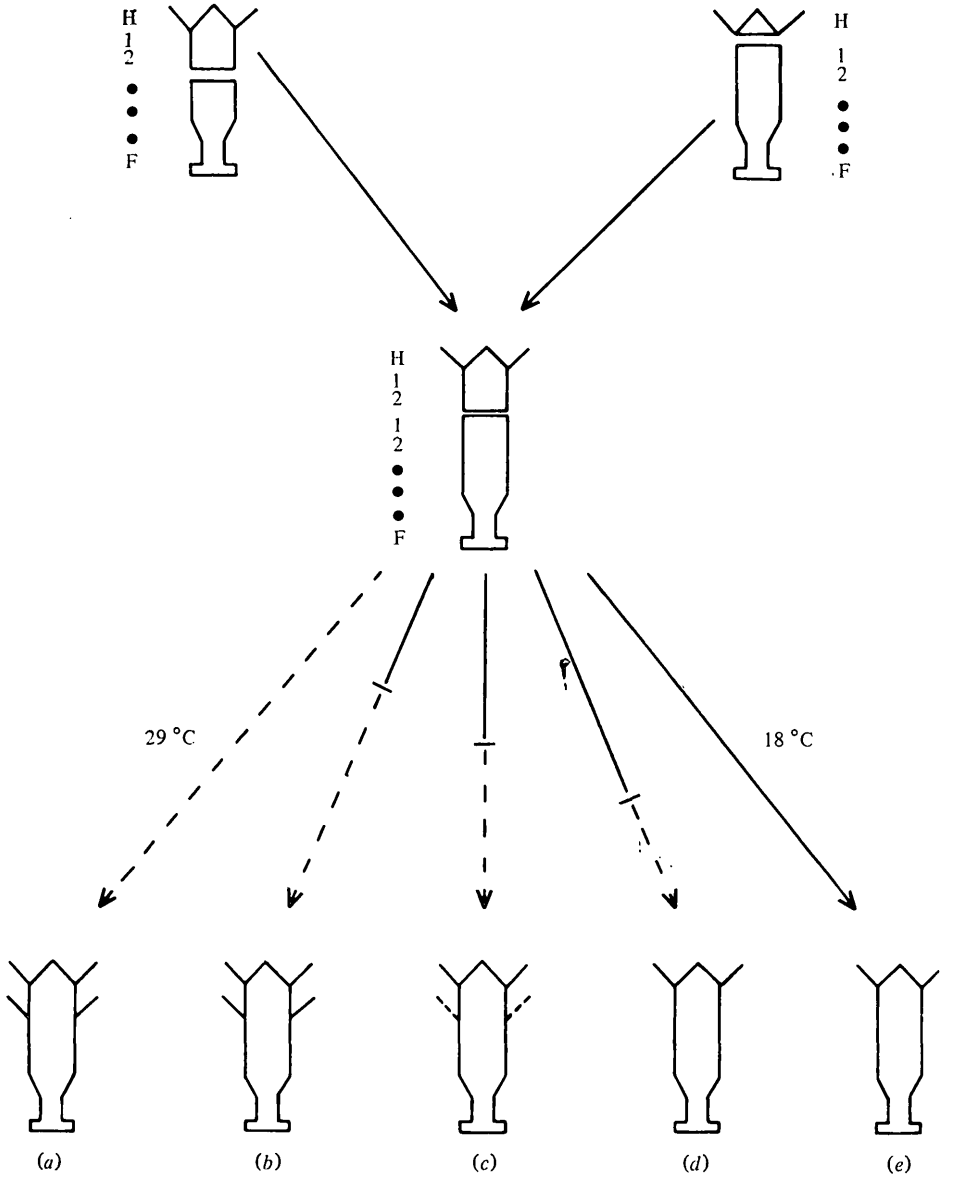


Fig. 1. Inhibition of host head regeneration: (*a*) 29 °C control combination regenerating at the graft–host border, (*b*), (*c*) and (*d*) show progressive stages of inhibition of host regeneration after a temperature shift from 18 to 29 °C at increasing intervals, (*e*) 18 °C control showing regulation at the graft–host border.

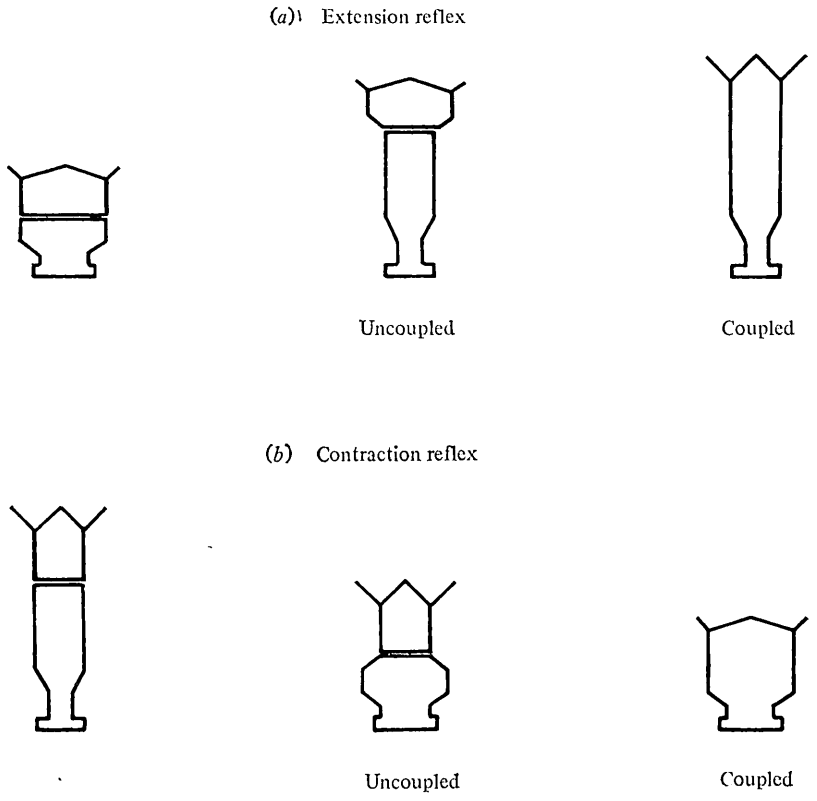


Fig. 2. Patterns of behaviour of grafted animals showing the recoupling of extension and contraction reflexes.

cut with a razor blade scalpel to give a straight clean edge. H12/ and /12...F segments were threaded on a 1 cm length of hair and were floated in a dish so that the surface tension compressed the wound. After 30 min the hairs were withdrawn and the grafted animals submerged. The operation had about a 90 % success rate and any grafts which did not immediately make and maintain contact were discarded. Animals were grafted and kept in a temperature-controlled room at $18 \pm \frac{1}{2}$ °C until transferred at intervals to a water-jacketed incubator at $29 \pm \frac{1}{2}$ °C where they remained for 2 days until morphogenesis was complete. The structures formed at the graft–host border were then scored and later checked for regression. Only complete heads and tentacles over 1 mm long were considered to be positive responses. These operations are shown diagrammatically in Fig. 1.

(b) Reflex coupling

Animals were grafted as described above and the time was recorded as each was placed in a separate square of a compartmented Petri dish. After 30 min the supporting hair was withdrawn and the animal was immersed in the culture

medium and left until its foot stuck to the floor of the dish. The extension reflex was then tested at 10 min intervals with a fine tungsten needle by touching both head and foot to induce complete contraction and observing the animal's behaviour in the following minute. Initially the extension of the distal graft was both delayed and slower than that of the proximal host attached to the dish and gave a 'lollipop' shape. In time the behaviour of the animal changed and it extended in one smooth telescopic movement (Fig. 2*a*). Coupling of the contraction reflex was tested by touching the foot of a fully extended animal. At first contraction occurred only in the proximal host and the graft remained extended giving an inverted 'lollipop' but eventually this pattern changed and the body shortened in a single unambiguous movement to a quarter of its extended length (Fig. 2*b*).

(*c*) *Cytology*

Grafted animals were incubated at 18 °C in culture medium for varying periods and checked for reflex coupling. They were then drained and fixed for 1 h in a mixture of equal volumes of quarter strength Karnovsky fixative (Karnovsky, 1965) and 2% osmium tetroxide in distilled water. Specimens were washed in 0·2 M cacodylate buffer, dehydrated in alcohol, infiltrated with propox–Araldite and embedded in Araldite. After remounting on Araldite blocks, sections were cut on a Cambridge ultra-microtome, stained with uranyl acetate and lead citrate and photographed on a Philips EM300 electron microscope.

RESULTS

(*a*) *Grafting*

The effect of temperature in stimulating regeneration is shown by the response of the continuously incubated control H12/12...F grafted animals. At a constant temperature of 18 °C 100% regulated (total number = 96) but at 29 °C 42% had distal structures at the graft–host border (total number = 132). The course of distal inhibition is shown by the decline in regeneration at the graft–host border at 29 °C after increasing periods of incubation at 18 °C (Fig. 3). The time for the response to fall to half the initial value is used as a measure of a significant degree of inhibition (Wolpert *et al.* 1972). In this case the response fell from 42% at 0 h to 21% after 4–5 h. It should be noted though that 100% regulation was only achieved after 24 h incubation at 18 °C. Morphogenesis at the graft–host border was similar to that described by Webster & Wolpert (1966) and Hicklin *et al.* (1973). Distal structures grew as complete heads with hypostomes inside coronets of four to six tentacles or with one to four individual tentacles scattered around the circumference of the body. There was no change in the frequencies of these different types of structure with increasing periods of incubation at 18 °C.

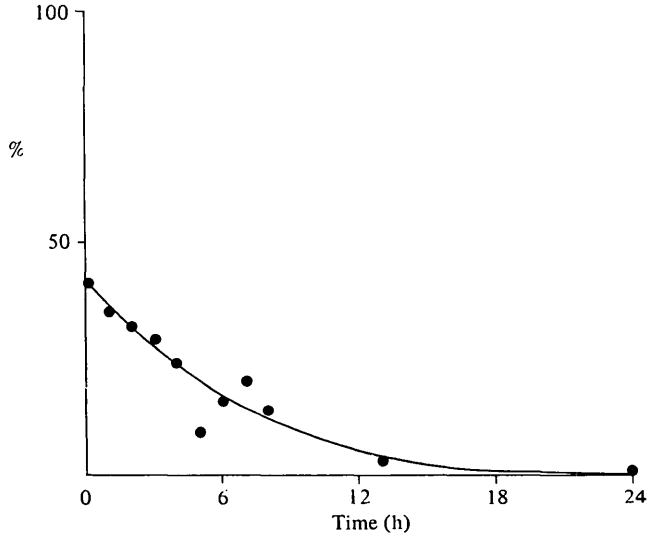


Fig. 3. Inhibition of host head regeneration. The % regeneration of groups of animals after shifting from 18 to 29 °C is shown on the vertical axis. Each group contained 30–40 grafted animals. The time of incubation at 18 °C before transfer to 29 °C is shown on the horizontal axis.

(*b*) *Reflex coupling*

The point at which coupling occurred in an individual grafted animal was estimated to lie halfway between the last negative and first positive responses for both extension and contraction reflexes. Coupling in a H12/12...F combination is presented as a pair of curves which show the percentage of the total number of grafted animals coupled at 10 min intervals for each reflex (Fig. 4). The times at which coupling occurred in 50 % of a group of grafted animals are given for several other graft–host combinations (Table 1). In every case extension coupling occurred in the first hour after grafting and contraction coupling followed in the second hour. Neither the temperature nor the type of graft–host combination had any significant effect on the time or rate of coupling. Continuous observation of individual animals showed that coupling was a rapid process. This could most easily be seen by following the contraction reflex when stimulated at half minute intervals. Only 2–3 min elapsed between the first tentative movements in the tentacles and a complete and rapid withdrawal by the entire graft synchronized with the contraction of the host.

(*c*) *Cytology*

Both epidermis and gastrodermis made contact within the first hour of healing. The gastrodermis, which had sloughed off and expelled its damaged cells through the mouth, quickly returned to normal with intact membranes, vacuoles and few lysosomes. Epidermal cells on the other hand were retained at the cut surface

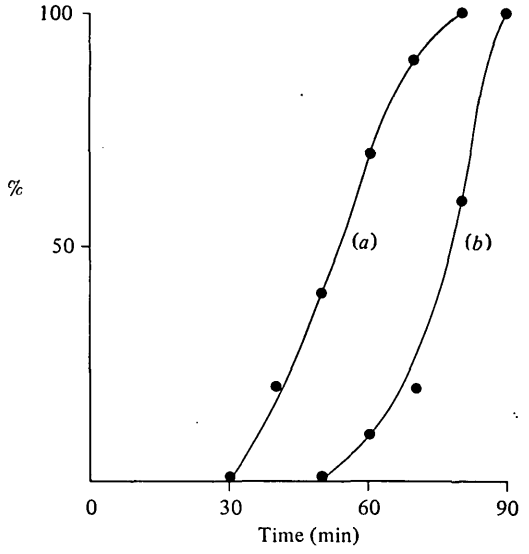


Fig. 4. Reflex coupling of a H12/12...F combination at 18 °C. Total number of animals = 10. (*a*) Extension coupling. (*b*) Contraction coupling.

and showed many broken membranes (Fig. 5). After 3 h these cells had also replaced their vacuoles, secretory vesicles and mitochondria along with many ribosomes, small areas of Golgi apparatus and endoplasmic reticulum (Fig. 6). The mesogloea was absent from the wound margins leaving the two epithelial layers in close contact. Initial contacts across the wound in both layers were not made by interstitial cells but by epithelial cells which were recognized by their size, vacuoles and secretory vesicles. Interstitial cells such as those shown in Fig. 5 were lost at the wound margin. No nerve cells or synapses were seen near the wound or crossing it at any stage.

Short lengths of close membrane contact, up to 0·2 μm long, were found around the host–graft border after 1 h of healing in the gastrodermis and after 3 h in the epidermis. However the sampling of gap junctions in well defined regions of host–graft membrane apposition was hindered by traumatic changes. The border could only be located precisely at the outer edge of the epidermis where it was marked initially by broken membranes in the opposing cells (Fig. 5). After repair of these cells an indentation remained in the epidermis over the site of direct contact between the epidermis and gastrodermis (Fig. 6). Beneath the apical region of the epidermis and throughout the line of gastrodermal contact, cell contortions and interdigitation of the lateral membranes obscured the course of the border. Unmistakable gap junctions are common away from the wounded area but the close membrane contacts at the border were indistinct and failed to show a quadrilaminar structure or periodicity. Nevertheless the specimens taken for cytology were coupled for extension after 1 h of healing and for both extension and contraction after 3 h. Mature gap

Table 1. *Reflex coupling*

Graft	Host	No.	Temp. (°C)	Extension T_{50} (min)	Contraction T_{50} (min)
	(*a*) *H. attenuata–H. attenuata*				
H12	34...F	11	29	47	67
H12	34...F	8	18	51	81
H12	56F	10	18	36	87
H...5	5...H	13	18	–*	93
	(*b*) *H. attenuata–H. littoralis*				
H12	12...F	15	22	39	177

* Extension reflex not obtained in reverse polarity grafts lacking feet.

junctions between host and graft can be demonstrated in an interspecific combination of *H. attenuata* and *H. littoralis* which heals leaving a deep and long lasting indentation at the border (Fig. 7). After 48 h of healing the gap junctions between the apposed lateral membranes have come to resemble those found in normal tissue for they are up to 0·4 μm long, have a distinct quadrilaminar structure and a 9 nm repeat within the gap. This interspecific combination is coupled both for positional communication (at 18 °C 100 % of H12/12... F grafted animals regulated: total number = 14) and for reflex movements (Table 1). Septate desmosomes were found at the borders of *H. attenuata–H. attenuata* combinations after 3 h of healing and are indicated by the dense apical lateral membranes in Fig. 6. At the same time small areas of dense membrane were found attached to myoneme filaments at the cell bases as the fascia adherens desmosomes reformed.

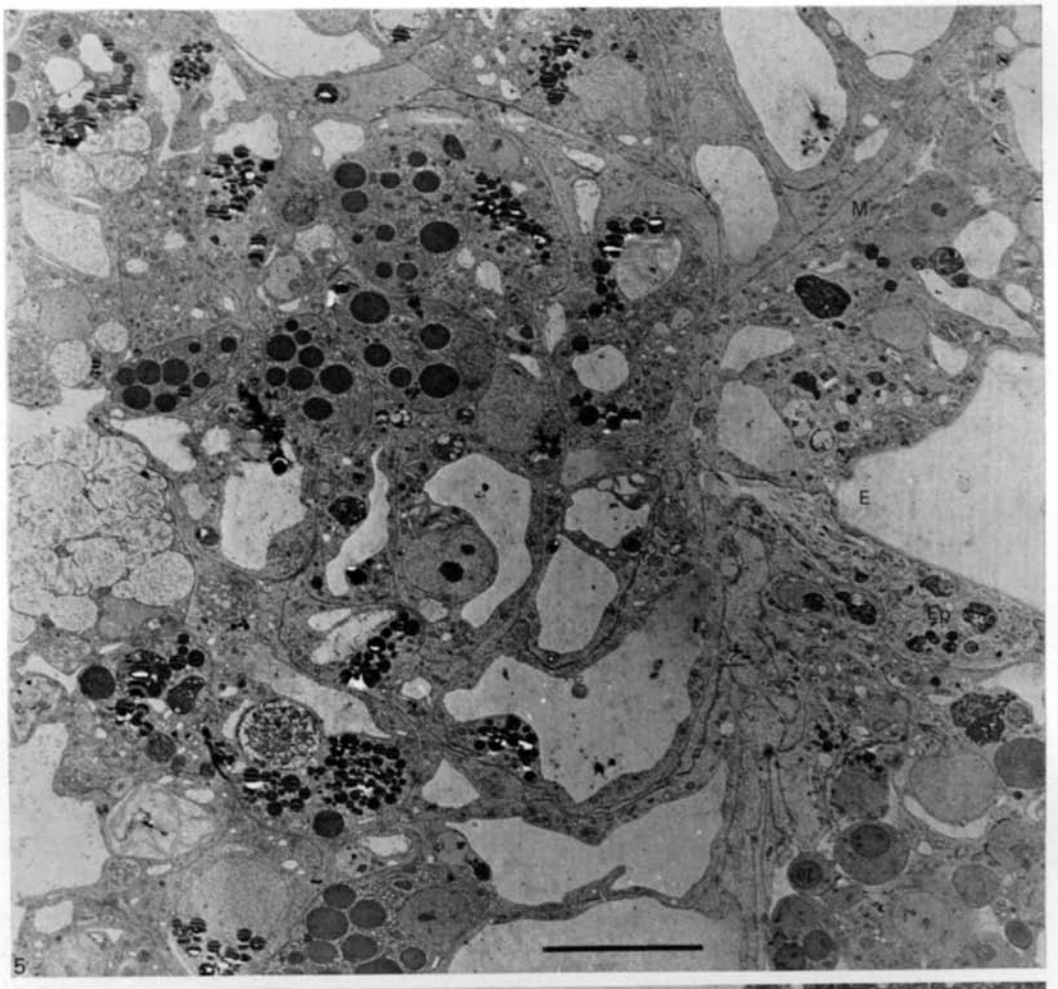
DISCUSSION

The course of healing in *H. attenuata* closely resembles the events described by Bibb & Campbell (1973) in *H. littoralis* and *H. oligactis* where the gastrodermis was also shown to heal before the epidermis. This sequence corresponds to the order of reflex coupling for the gastrodermal circular muscle extends the body and contraction is due to epidermal longitudinal muscle. Such an agree-

FIGURES 5 AND 6

Fig. 5. A *H. attenuata–H. attenuata* graft–host border after healing for 1 h at 18 °C. The cleft in the epidermis (E) marks the site of the border. M, Mesogloea; Ep, epithelial cell; I, interstitial cell. Scale bar = 10 μm.

Fig. 6. A *H. attenuata–H. attenuata* graft–host border after healing for 3 h at 18 °C. The epidermal epithelial cells (Ep) have undergone repair. Dense apical lateral membranes indicate the site of septate desmosomes (SD). Mit, Mitochondria; V, vacuole; SV, secretory vesicle. Scale bar = 1 μm.



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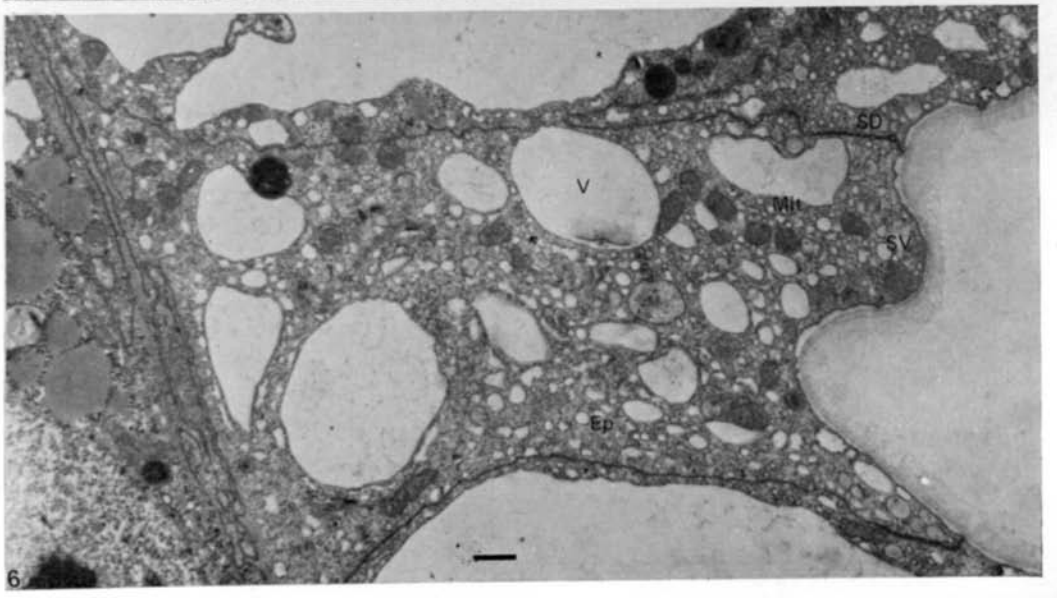

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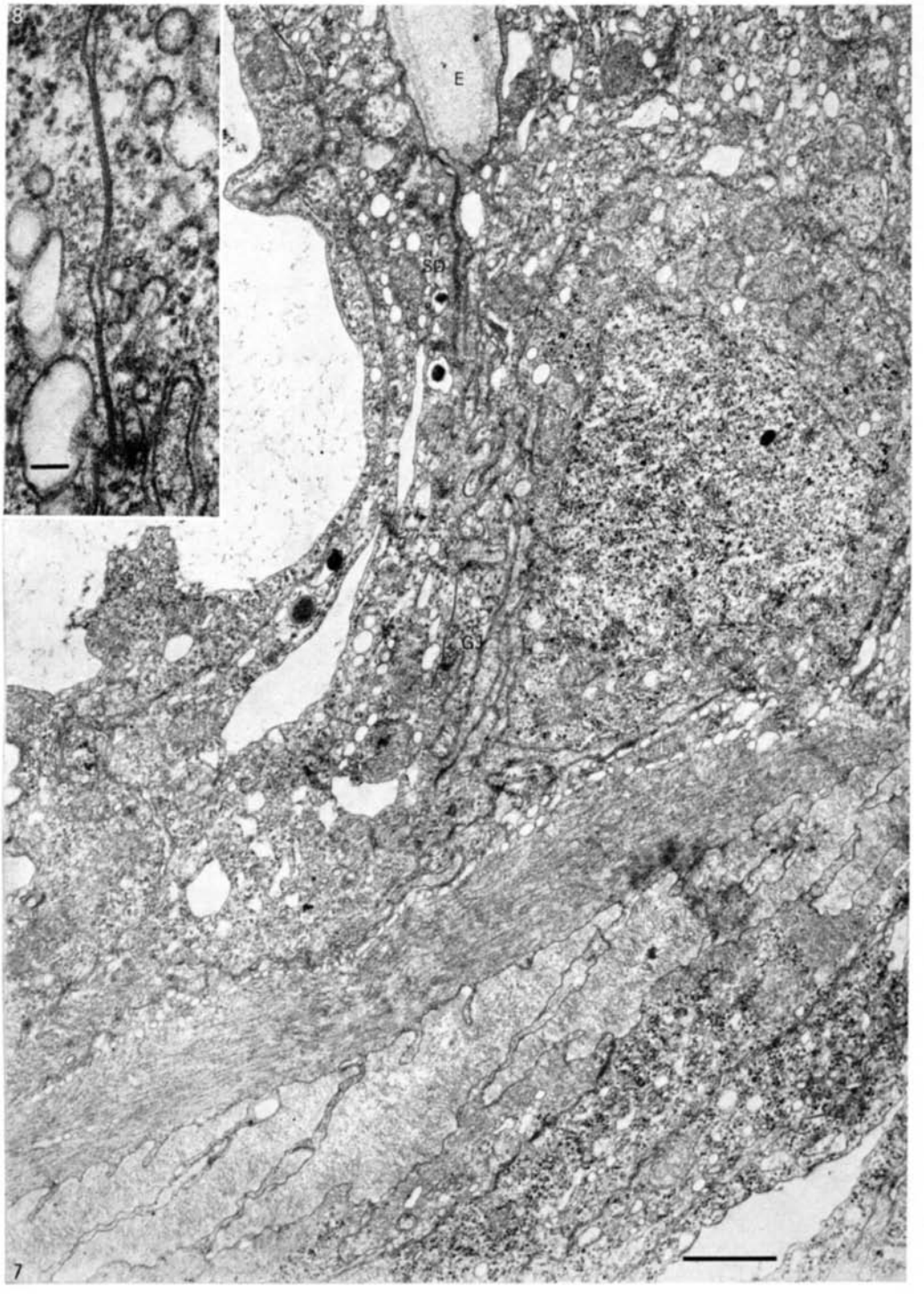
8
E
SD
GJ
7

ment is further confirmation that the behavioural patterns correctly reflect the structural pathways. Gap junctions form rapidly between the hydra host and graft and after allowing for a delay for the repair of wound damage communication appears to return as quickly as in vertebrate cells (DeHaan & Hirakow, 1972; Goldfarb, Slack, Subak-Sharpe & Wright, 1974). Positional communication is resumed at the same time but the slow rate at which it is completed is in marked contrast to reflex coupling. The difference may lie in the nature of the early contacts which occupy a very small area compared to normal tissue (Hand & Gobel, 1972). Even so there appear to be sufficient junctional channels present to pass a current stimulating muscular activity. These gap junctions are produced by the aggregation of individual sub-units already present in the membrane (Johnson & Preus, 1973) but in time the synthesis of new sub-units (Decker, 1976) leads to larger and more regular structures such as those shown in Fig. 8. An additional seal against lateral leakage may also be made when the peripheral band that surrounds each junction is repaired (Hand & Gobel, 1972). It is possible therefore that normal positional communication requires a much larger junctional area than that which provides electrical coupling. The other intercellular junctions that are found in hydra, fascia adherens desmosomes and septate desmosomes, form at the same time as gap junctions (Bibb & Campbell, 1973) but are likely to be responsible for functions other than communication. Fascia adherens desmosomes resemble the intercalated discs of vertebrate cardiac muscle (McNutt & Weinstein, 1973) and anchor myoneme fibres to the membrane while septate desmosomes maintain the transepithelial potential (Macklin & Josephson, 1971; Filshie & Flower, 1977). Positional communication is independent of nerves for they are destroyed at the cut surface and do not regenerate from interstitial cells for up to 24 h (Bode *et al.* 1973). Furthermore, polarity reversal and the inhibition of head regeneration is unaffected by denervation with colchicine (Marcum, Campbell & Romero, 1978).

Several situations are known in which gap junctions form (Tupper & Saunders, 1972; Dulcibella, Albertini, Anderson & Biggars, 1975) and disappear (Dixon & Cronly-Dillon, 1972; Blackshaw & Warner, 1976) during normal embryonic growth but there is no evidence as yet that these changes play a causal role in development. The present work provides an experimental test of the dependence of positional communication upon gap junctions. The H12/12...F combination subjected to a temperature shift defines the minimum

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FIGURES 7 AND 8

Fig. 7. A *H. attenuata–H. littoralis* graft–host border after healing for 48 h at 22 °C. The apposed lateral membranes may be followed in from a deep indentation in the epidermis (E). GJ, Gap junctions; SD, site of septate desmosomes. Scale bar = 1 μm.

Fig. 8. (insert). Gap junctions at the site marked on Fig. 7. Scale bar = 100 nm.

period in which contacts must form if they are to pass positional signals between graft and host. As new gap junctions have formed at the border within this minimum period of 4–5 h, the case for gap junctions as a channel for positional communication is strengthened. However it has yet to be conclusively demonstrated that this correlation reveals a close functional dependence and is not the coincidence of two separate but simultaneous repair processes.

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