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Source: Zoological Science, 16(2) : 327-334

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.16.327

ZOOLOGICAL SCIENCE **16**: 327–334 (1999)

Development of Oriented Motion in Regenerating Hydra Cell Aggregates

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ABSTRACT—Hydra can regenerate its complete adult form from aggregates of dissociated cells. Aggregates of hydra cells are made by dissociating hydra tissue into a suspension of cells and then re-aggregating the cells by centrifugation. An aggregate formed in this way is a disorganized mass of individual cells and does not possess any regeneration polarity. In this study, we analyzed the development of motion in cell aggregates during the regeneration stages in which a new body axis was being established. Two perpendicular diameters (widths) of binalized projection images of an aggregate were continuously measured in order to detect changes in form, *i.e.*, motion. Between 30–35 hr, when the aggregates still appeared spherical, slight motion along a distinct axis was detected along with a simple expansion in the size of the mass. After that, quick twitches along a distinct axis, also seen in intact hydra, began to develop. The axis of the motion corresponded to the future body axis of the regenerated animal, and the future head-end of the body axis showed a larger degree motion than the foot-end. Motion in the aggregates made of cells from hydroxyurea-treated animals in which the stem cells of nerve cells has been eliminated, suggested that the slow one-directional motion observed was due to the epithelial cells, while the twitches were controlled by nerve cells. These results show that the development of motion could provide a useful index to the recovery of organization in the cell aggregates.

INTRODUCTION

Hydra, a fresh-water polyp, has a simple body plan and high regenerative ability. The animal has a head (consisting of a hypostome surrounded by 5–7 tentacles) and a foot (consisting of a peduncle and basal disc) on opposite ends of a tube-like body column which is composed of endodermal and ectodermal epithelial cell layers. Position-dependent features along the head (apical) – foot (basal) axis include polarity in morphallactic regeneration (Bode and Bode, 1984; Javois, 1992 for review). Under the control of the regeneration polarity, a piece of tissue excised from the body column is able to regenerate in such a way as to always form a new head on the apical end and a new foot on the basal end.

The intact hydra has a primitive, loose net of nerve cells which are differentiated from the interstitial stem cells. The nerve net maintains a steady state between the production and loss of nerve cells, due to the continuous expansion and turnover of the tissue (see Bode *et al.*, 1988; Koizumi *et al.*, 1990, for review). The nerve cells are not uniformly distributed throughout the epithelial cell layers; they are densely distributed in the head and foot, and less in mid-body. Such a pattern of distribution is controlled by the environment provided by the epithelial cells (Koizumi and Bode, 1986; Koizumi *et al.*,1988; Bode *et al.*, 1988; Koizumi *et al.*, 1990).

Hydra can regenerate complete polyps from an aggregate of cells. These cell aggregates are made by dissociating tissue into individual cells and then forcedly collecting the cells using a low-speed centrifuge (Noda, 1971; Gierer *et al.*, 1972). Therefore, an aggregate is a random cell mass in its initial state, where the original body organization has been completely destroyed. The polarity necessary to regenerate the body plan is established anew in an aggregate during the regeneration process. Similarly, a new nerve net is also formed in the aggregate (Itayama and Sawada, 1988; Sawada *et al.*, 1991).

It is still not clear how the position of the new head (or similarly the position of a new head-foot axis) is determined in the aggregate. The first phenomenon observed in the regeneration of aggregates is the sorting of the two kinds of epithelial cells, which is caused by tissue specific cell adhesion (Technau and Holstein, 1992; Sato-Maeda *et al.*, 1994). However, no obvious evidence for rudiments to generate new heads (*e.g.*, the sorting out of original head cells) was observed in this early stage (Sato *et al.*, 1992; Technau and Holstein, 1992). In the stage which follows the cell sorting, an inner cavity is produced in the aggregate composed of two

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epithelial cell layers. Then it becomes spheroidal and forms structures such as tentacles, feet and hypostomes.

In this study, we examined changes in the form and motion of aggregates during the period from when they first appear as spheroidal in form until they display an apparent body axis. Continuous image recording and analysis of aggregates during this period have demonstrated two types of motion: (1) slow, slight one-directional motion which occurs prior to the aggregate developing spheroidal form, and then (2) quick twitches along the newly developing body axis. It was suggested that the former motion was due to epithelial cells, while the latter motion was controlled by nerve cells. Results obtained in the present study showed that motion could be a useful index to define the level of organization of epithelial and nerve cells in cell aggregates.

MATERIALS AND METHODS

Preparation of cell aggregates

Hydra vulgaris, strain K9, was gifted from National Institute of Genetics, Mishima, Japan, and cultured in a hydra culture medium (HM: Muscatine and Lenhoff, 1965; Takano and Sugiyama, 1984). All animals used in the experiments were starved for 1 day before use.

After removing the heads and the feet, tissue pieces of the gastric region of about 50 animals were collected to prepare cell aggregates using the following procedure (Flick and Bode,1983). First, the tissue pieces were minced into small fragments and then dissociated mechanically by repeated pipetting into a cell suspension in a hyperosmotic medium: 5 mM $CaCl_2$, 1 mM $MgSO_4 \cdot 7H_2O$, 2.8 mM KCl, 11 mM TES, 0.67 mM Na_2HPO_4, 0.44 mM KH_2PO4, 5 mM sodium pyruvate, and 5 mM sodium citrate. After filtration through a 45 μm mesh, cells were aggregated by low-speed centrifugation (～150g). The hyperosmotic medium was gradually diluted with HM according to the degree of regeneration. In the present study, the last dilution into HM was performed at 22 hr after the aggregate formation, at which point we started the video-recording of aggregates.

Sato and Sawada (1989) have shown that the number of structures formed depends on the size of the aggregate. Therefore, we carefully adjusted the size of aggregates so that only a single head would be formed. Eight aggregates, 0.53±0.06 mm in diameter at 20～24 hr, were analyzed in this study.

Continuous recording and image analysis

We recorded the regeneration process of aggregates in agar-coated (0.04% in HM) dishes. Twenty-two hours after aggregate formation, aggregates were placed in a small hole made on the agar base. The aggregates remained calmly without rotation for the first 60 hr.

The dish was set on the stage of a bisecting microscope (Nikon, SMZ-U). Projection images of the aggregates were recorded with a time-lapse video recorder through a CCD video camera, at a rate of one image per second from 22 hr to 72 hr. The temperature was kept at 18°C.

The outlines of the aggregates were obtained by the binalization of intensity of the images using a image processor (Perceptscope, HAMAMATSU PHOTONICS). Analysis was performed on data received until 55 hr, because after that the regenerated hydra moved actively and eventually stood upright.

Two measures of the aggregate forms were used in the present study. (1) Two perpendicular diameters of the outline of the binalized image (widths, W1 and W2). One of the diameter was set within the range of 45° against the future body axis (Fig.1A). The future body axis was determined by surveying the later images recorded on video. (2) Relative positions of the two points P-1 and P-2 (Fig.1B). These points P-1 and P-2 represent the two ends of the future body axis. They were recorded as coordinates, *i.e.*, (x_{P-1}, y_{P-1}) and (x_{P-2}, y_{P-2}).

Aggregates of hydroxyurea treated cells

It is known that hydroxyurea treatment selectively kills interstitial cells, the stem cells of nerve cells in hydra (Bode *et al.*,1976). Animals were exposed to 10 mM hydroxyurea (Sigma) for 3 days, fed daily, and then allowed to recover in HM for 3 days. After checking the cell composition of a few animals using the a maceration method (David, 1973), the rest of the animals were dissociated into single cells and re-aggregated as described above. Three hydroxyurea-treated aggregates were examined in the present study.

RESULTS

Motion of aggregates detected by “time sections” of the form

Aggregates were made of cells originating from the gastric region of hydra (see MATERIALS AND METHODS). The regeneration process of this type of aggregates has already been reported (Gierer *et al.*, 1972; Sato and Sawada, 1989; Sato *et al.*, 1990). Briefly, after a cyst stage, the aggregate becomes elongated, and forms tentacles and a hypostome at one end of the ellipsoid. Fig. 2 shows binalized images of an aggregate at each time obtained from 33.8 hr (Fig. 2A; spherical form) to 51 hr (Fig. 2F; a ellipsoidal form).

Continuous measurement of W1 and W2 (Fig. 1A) during the regeneration process provided information on the changes that occurred in the form of the aggregates (Fig. 3). While W1 and W2 maintained similar values, we considered the aggregate to be in a spherical form (Fig. 3–1A and B). If W1 and W2 were significantly different, the aggregate was considered to be no longer spherical (Fig. 3–1C).

Accumulation of such “time sections” of the changing form also provides information on the motion developing in the aggregate (Fig. 3–2). A sudden decrease of W1 and W2 would indicate a sudden contraction (Fig. 3–2A), and a change in W1 and W2 with a negative correlation (*i.e.*, one increases, while the other decreases) would indicate a slow axial contraction and relaxation (Fig. 3–2B) or a series of fast contractions and relaxations, *i.e.*, twitches (Fig. 3–2C).

Development of one-directional motion

Fig. 4 shows a typical example of the changes in form and motion that occurred in an aggregate. Several phases can be detected in the changes. First, slow isotropic expansion was observed, with the aggregate maintaining a spherical form (29–34 hr). Next, the aggregate became spheroidal, and intense contractions and relaxations were repeated along its long axis (34–39 hr). Finally, twitching started. These phases varied in each sample, but always appeared in this order.

A marked drop in W1 and W2 was observed at 34 hr (Fig. 4). By checking the video record of the regeneration process of the aggregate, we confirmed the partial rupture of the cell layers at this time: the aggregate discharged internal fluid into the outside medium and reduced its size temporally.

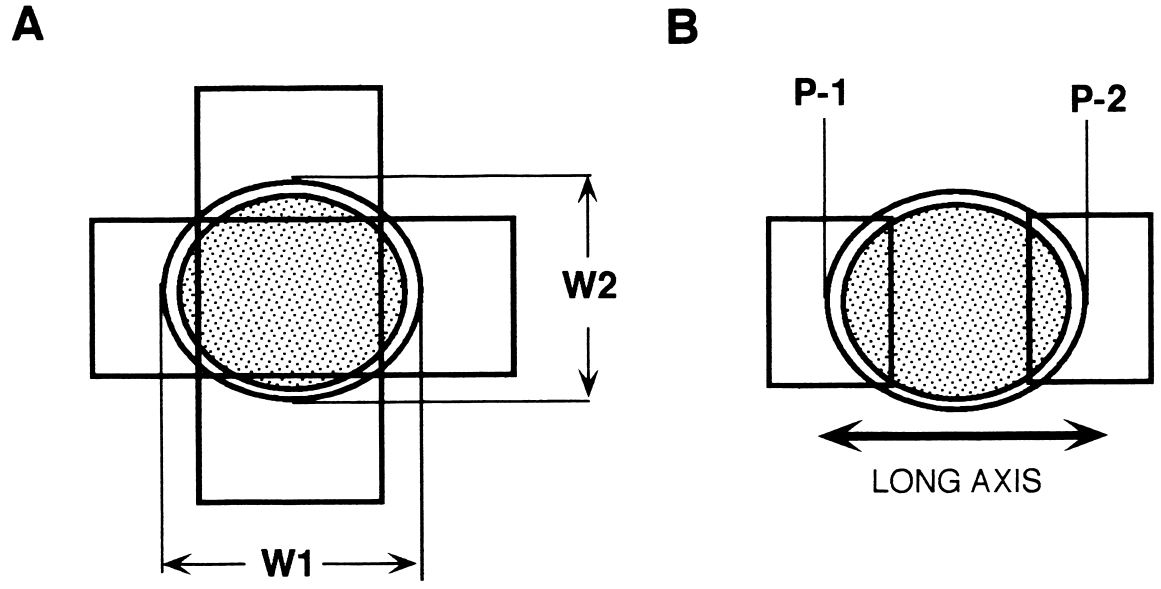

Fig. 1. Schematic representation of the two measures used in this study. **A**: two perpendicular diameters of the outline of the binalized image (widths, W1 and W2). **B**: relative positions of the two points P-1 and P-2, the two ends of the future body axis . They were recorded as coordinates, *i.e.*, (x_{P-1}, y_{P-1}) and (x_{P-2}, y_{P-2}). The future body axis was determined by surveying the later images recorded on video. See text.

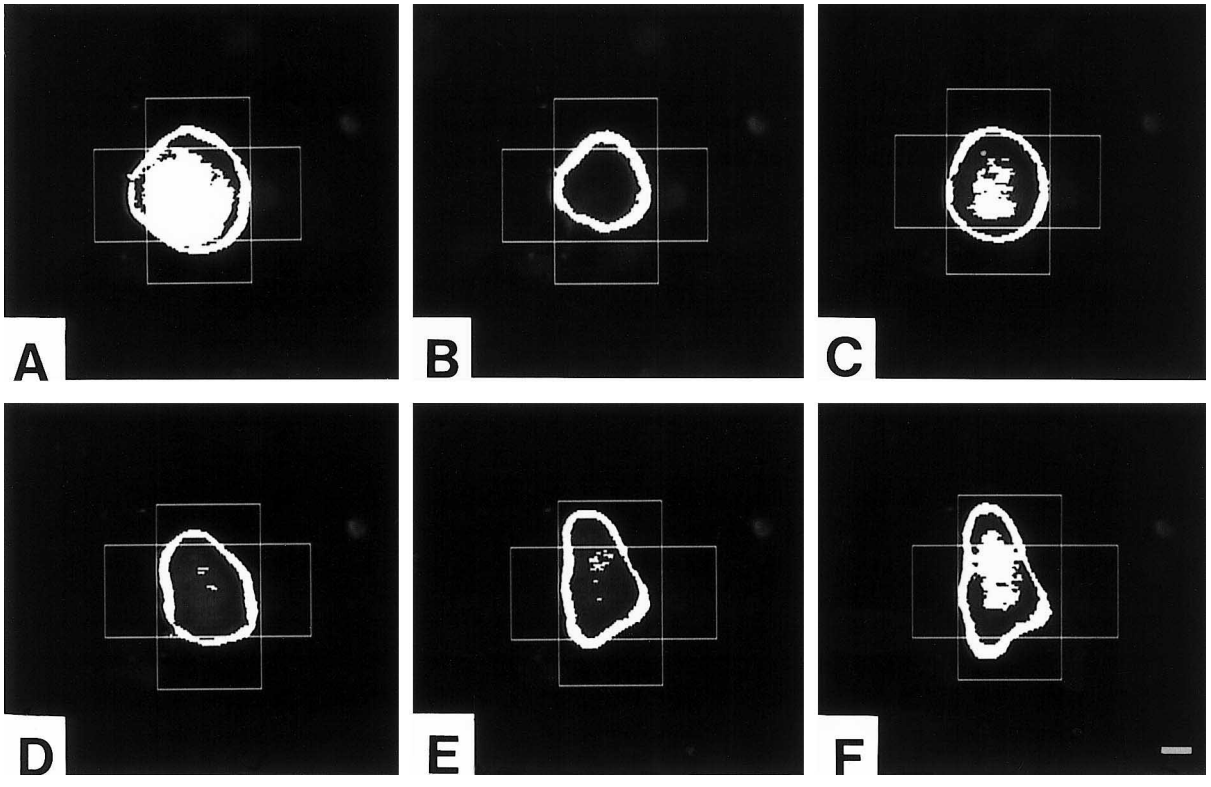

Fig. 2. Changes in form of an aggregate. Binarized images at A; 33.8 hr after aggregates formation, B; 34 hr, C; 38 hr, D; 42 hr, E; 50 hr, and F; 51 hr. Scale bar shows 0.1 mm.

Details of the development of the one-directional motion observed in Fig. 4 are shown in Fig. 5. The aggregate showed no apparent polarity of form or motion between 29 hr and 31 hr (Fig. 5A), nor for the period before 29 hr (data not shown). At around 33 hr, however, a slight elongation in one axis was observed, though the form of the aggregate remained in spherical (Fig. 5B). From about 46 hr (see Fig. 4), the aggregate showed obvious sudden motion along a single axis (twitches)

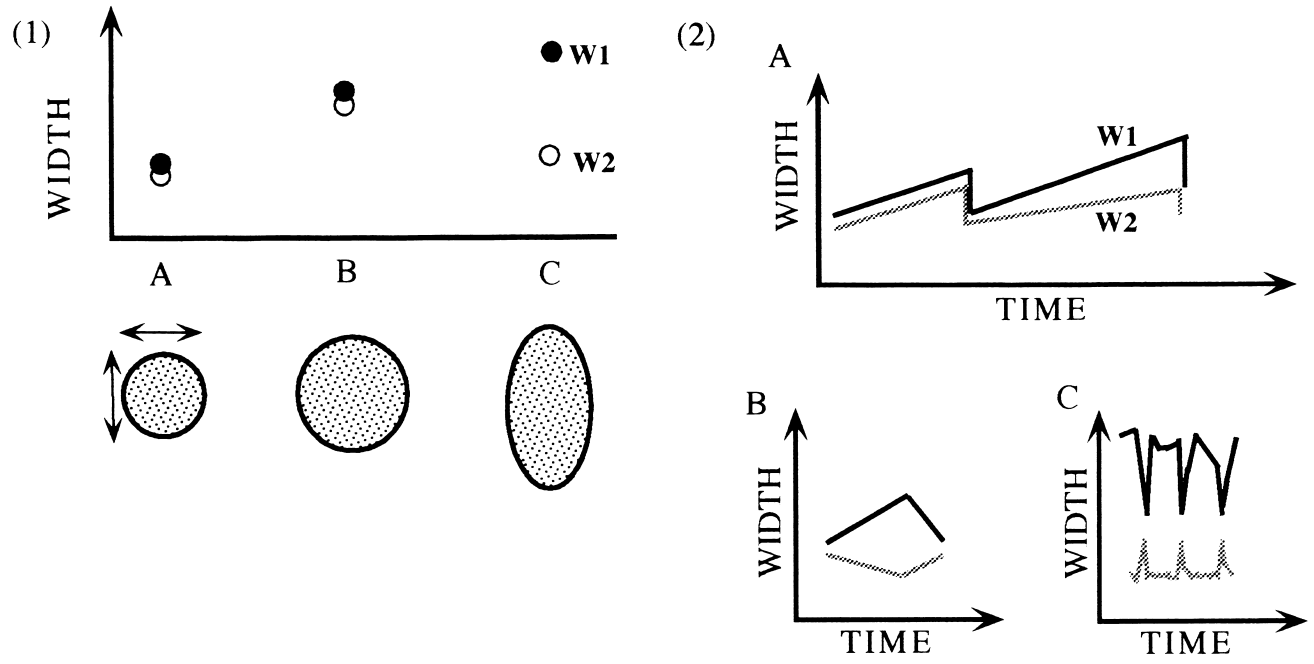

Fig. 3. W1 and W2 shown in Fig. 1A provides information on the changes in the form and motion of the ggregates. (1): the changes in the form. A and B: W1 and W2 maintained similar values, the aggregate was in a spherical form. C: W1 and W2 were different, the aggregate was not spherical. Open circles ;W1. Closed circles; W2. (2): the changes in the motion. A; sudden contraction, B; relax and contraction, C; twitches.

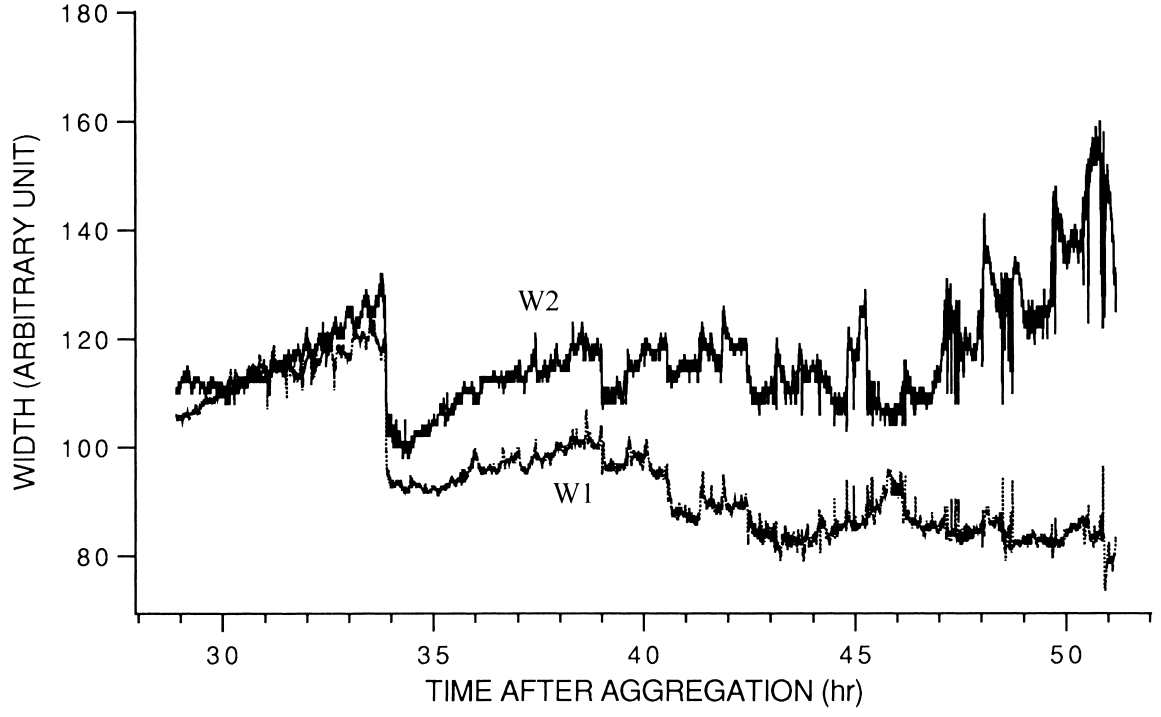

Fig. 4. Changes in W1 and W2, from 29 hr after aggregate formation to 51 hr. In this sample, small amount of cells expelled at the time before 29 hr and prevent the analysis. Here, we showed the result of continuous measurement after removing such expelled cells.

while also showing repeated slow contraction-relax motion (Fig. 5C). The twitching of the aggregate increased in amplitude and frequency after about 40 hr (Fig. 4). Examination of the video record of the aggregate confirmed that the direction of the oriented motion observed in Fig. 5B and that of the twitches observed in Fig. 5C were similar, *i.e.*, within the range of 90°, and that the orientation of the motions coincided with that of the future body axis in the eight aggregates examined.

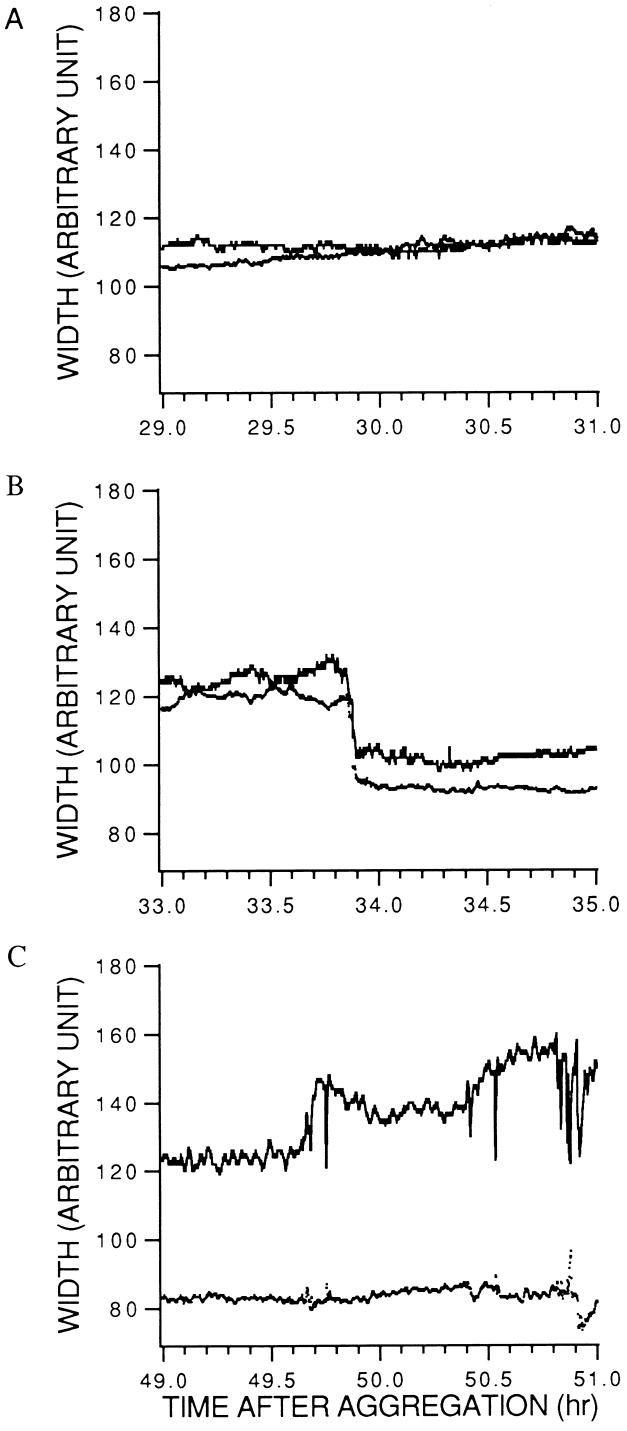

Fig. 5. Details of the development of the one-directional motion observed in Fig. 4. A: from 29 hr to 31 hr. W1 and W2 were almost same. B: from 33 hr to 35 hr. A slight elongation in one axis was observed. C: from 49 hr to 51 hr, when twitches along a single axis were observed.

Difference in motion of the two ends of the developing body axis

One of the two ends of a developing body axis in an aggregate will become the new head, while the other becomes the foot. We examined whether there is any difference in the motion of the two ends of the future body axis (Fig. 6). At first, no significant difference was detected between the two ends (Fig. 6A). However, one end, P-1, began to show more frequent contractions with larger amplitude (Fig. 6B). In the aggregate shown in Fig. 6, P-1 showed 7 contractions with an amplitude of more than 35 μm during the period of the observation, while P-2 showed only 2. Eventually, a hypostome with two tentacles and a foot were regenerated in the aggregate, and the head was formed at P-1. In the eight aggregates we examined, it was confirmed that one end of a developing body axis showed more frequent contractions with larger amplitude than the other, and that in each case the head developed at the end showing greater motion.

Slow one-directional motion in hydroxyurea-treated cell aggregates

Before commencing our experiments, we examined the cell composition of hydroxyurea treated animals (see MATERIALS AND METHODS) and confirmed the complete elimination of interstitial stem cells (data not shown). Fig. 7 shows the development of motion in an aggregate made from hydroxyurea-treated animals. Since that interstitial stem cells were eliminated by hydroxyurea treatments, no nerve cells would differentiate in the hydroxyurea treated cell aggregates, and no new nerve net would form (Itayama, personal communication). Observation revealed that theses aggregates showed slow asymmetric motion (Fig. 7A) similar to that observed in Fig. 5B, and a big contraction at around 29.7 hr (Fig. 7A). However, we did not detect any twitches, even in the later stages of regeneration in this type of aggregate (Fig. 7B).

DISCUSSION

In this study, we were able to detect changes in form and motion in hydra cell aggregates during the regeneration stages as a new body axis was being established. Slow oriented motion was observed before the apparent elongation of form in aggregates, and the direction of the axis of the motion corresponded to the future body axis. Also, a difference in motion between the two ends of the axis was detected before the formation of head structures. In aggregates made from hydroxyurea-treated cells in which the old nerve net had been destroyed and no new nerve cells would differentiate, the twitching motion was absent, though slow one-directional motion was observed. The results showed that the several types of motion observed were strongly correlated to the establishment of the body axis and nerve net formation. The development of motion observed here could provide a useful index of the recovery of organization in both nerve and epithelial cells.

It is known that the contraction and following relaxation of the body column constantly observed in intact hydra is due to ectodermal and endodermal epithelial cells, while the contraction itself is triggered by the nerve cells (Passano and McCullough, 1964, 1965; Josephson, 1967; Kas-Simon, 1972).

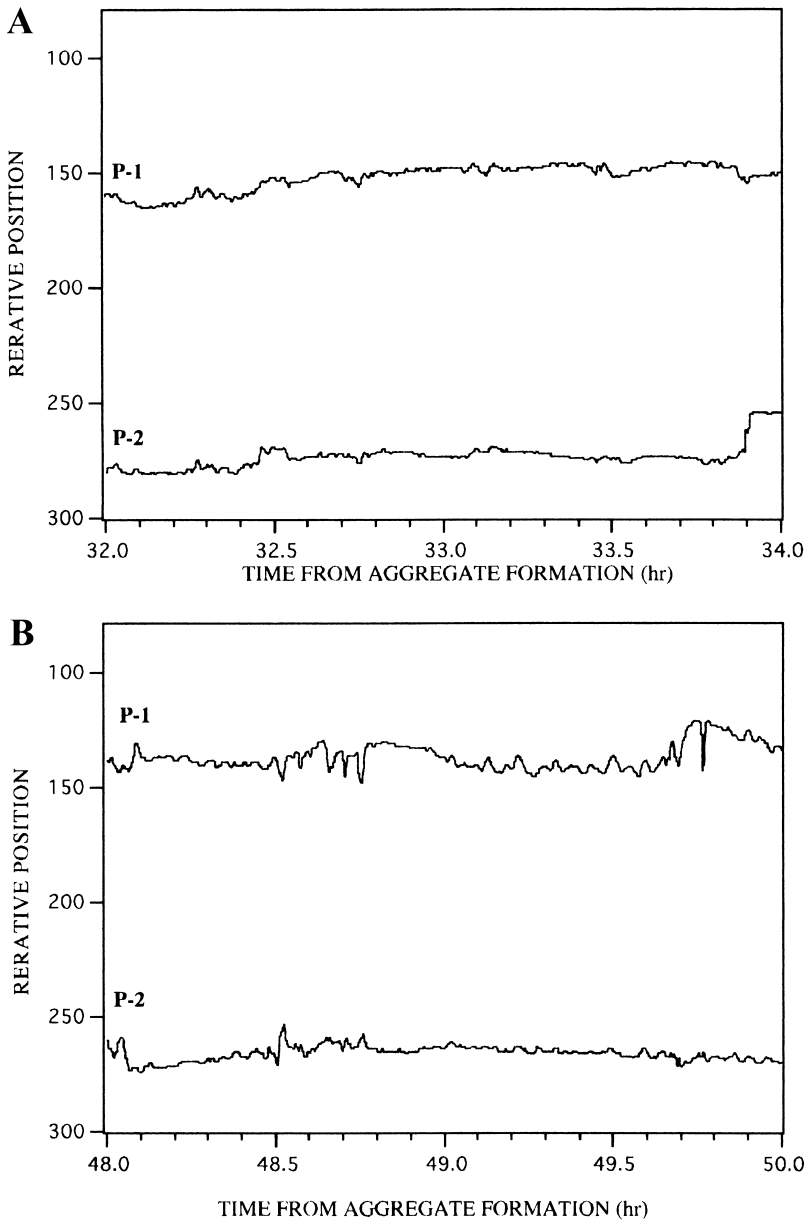

Fig. 6. The difference in the motion of the two ends of the future body axis (P–1 and P–2, see Fig.1B). A: from 32 hr to 34 hr. B: from 48 hr to 50 hr. The head was formed at P-1 in this aggregate.

Nerve-free hydra, known as “epithelial hydra”, show growth, budding, and even regeneration, but cannot feed by themselves (Marcum and Campbell, 1978; Sugiyama and Fujisawa, 1978). So, it is thought that epithelial cells play a major role in basic motion as well as in morphogenesis, while the nerve net controls quick and more highly-cooperative motion in intact animals.

In the aggregate, it is expected that the nerve net, as well as epithelial cell layers, would be reconstructed (Itayama and Sawada, 1988; Sawada *et al.*, 1991). In the present study, we observed the well-developed quick twitches after 40 hr of the aggregate regeneration. The timing strongly suggests a correlation to the formation of the nerve net. The following studies support this notion. (1): Gierer *et al.* (1972) found that though more than half of the original nerve cells are expelled by 24 hr after aggregate formation, the number of nerve cells in normal cell aggregates has doubled between 24 hr and 48 hr by differentiation from interstitial stem cells. (2): Itayama and Sawada (1988) and Sawada, *et al.* (1991) found that the average density and connection number of FMRF amide-like immunoreactive nerve cells starts to increase after about 40 hr. (3): Itayama and Sawada (1995) found that the number of electrical pulses greater than 1 mV increased in the period from 30 hr to 48 hr.

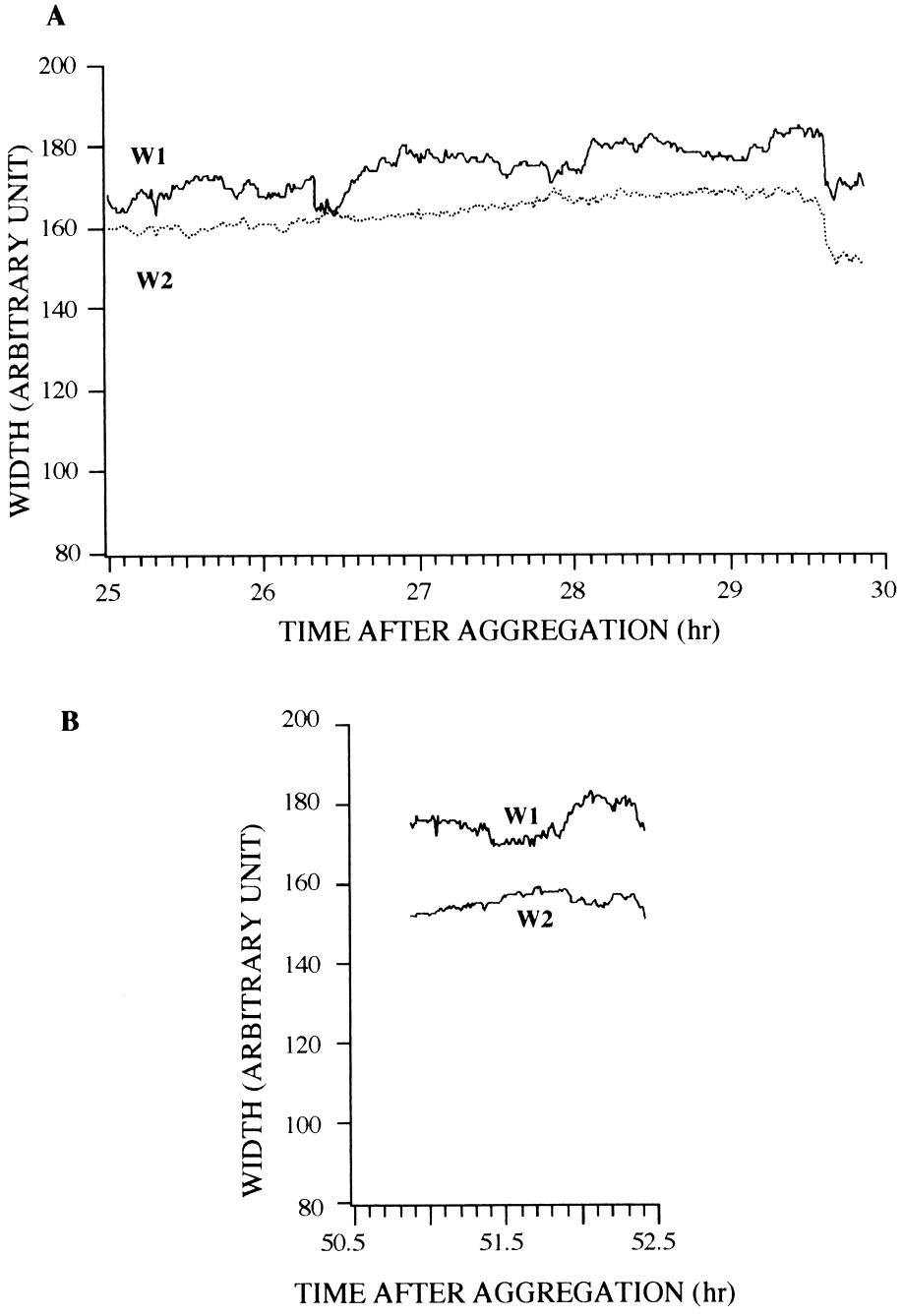

Fig. 7. The development of motion in the aggregate made from hydroxyurea-treated cells. A: from 25 hr to 30 hr. B: from 50.5 hr to 52.5 hr. Twitches were not detected even in later stages of the regeneration.

Taking into account the motion of hydroxyurea-treated cell aggregates in which the new nerve net was not formed, the slow one-directional motion observed in the aggregates appear to be controlled by the epithelial cells. On the other hand, the quick twitches should indicate that the formation of a new nerve net has been achieved. Since the nerve net pattern is controlled by the environment provided by the epithelial cells (Koizumi and Bode, 1986; Koizumi *et al.*,1988; Bode *et al.*, 1988; Koizumi *et al.*, 1990), it is thought that the nerve net was re-constructed in the aggregates as the body axis was being formed by the epithelial cells (*i.e.*, the positions of the head and the foot were being established), resulting in quick twitches like those observed in intact animals. Since the future head-end in the body axis showed the larger degree of motion during the stages we observed, it is possible that the epithelial cells and, therefore, the nerve net may be organized earlier in the future head-end than in the foot-end.

Epithelial cell layers composing the hydra body column

have muscle processes at their basal ends (see Campbell and Bode, 1984). In regenerating aggregates, the organization of these muscle processes should bring about the oriented motion observed before the apparent elongation of form. We can not exclude the possibility that it is this muscle processes organization which dominates the axis formation in an aggregate. In order to clarify this, histological analysis with molecular markers that can detect head-specified epithelial cells is needed.

Some experimental results showed that mesoglea, a natural extra-cellular matrix of the hydra, plays an important role in the morphogenesis of aggregates (Sarras *et al.*,1993). Also, the expression of a Hom/Hox homeobox gene *Cnox-2* suggested its involvement in axial pattern formation in the hydra (Shenk *et al.*, 1993). Recently, it was shown that *prdl-a*, encoding *paired*-like class homeodomains, may also be involved in apical (head) specification (Gauchat, *et al.*, 1998). It would be of interest to analyze the correlation between these results and the development of motion observed in this study, from the view point of the self-organization of cells.

ACKNOWLEDGEMENTS

We are grateful to Dr. O. Koizumi, Dr. Y. Tsukahara, and Dr. T.Maeda for their helpful comments on this work.

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(Received November 12, 1998 / Accepted January 14, 1999)