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Waves and periodic events during primitive streak formation in the chick

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SUMMARY

Morphogenetic movements occurring during formation of the primitive streak in the chick embryo are of a periodic nature, with a mean frequency of one pulse every 2·6 min. The period of the oscillatory movement is shown to be temperature-dependent. The onset of these pulses of movement can be seen as a slow wave starting at the posterior end of the embryo and making its way towards the anterior end. An interpretation of this behaviour is discussed.

INTRODUCTION

In the amphibian blastula, the region specialized in organizing gastrulation and neurulation is the grey crescent, and later the dorsal lip of the blastopore. (For reviews of induction in amphibia see Tiedemann, 1967, 1975.) The analogue of the amphibian dorsal lip of the blastopore in the chick is generally agreed to be the anterior portion of the primitive streak, including Hensen's node (Waddington, 1932; Hara, 1975; Gallera, 1971). This starts out as a condensation of cells at the posterior margin of the *area pellucida* (Koller's sickle). It then moves towards the anterior end of the blastoderm, cells condensing behind it to form the primitive streak.

This work is an attempt to identify the dynamic characteristics of the signalling process between Koller's sickle, or the anterior portion of the primitive streak, and cells in the blastoderm, resulting in the directed morphogenetic movements which give rise to primary axis formation.

The observations presented here represent the first direct evidence for the involvement of waves and periodic events in the organization of gastrulation in a vertebrate.

MATERIALS AND METHOD

Explantation and culture procedures

Embryos were usually explanted using the New (1955) technique and cultured on sterile plastic culture dishes (NUNC) over a small pool of thin albumen,

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which was changed every 8–12 h. In those cases where it was necessary to invert the embryo so that the epiblast was uppermost for marking, this was achieved by first freeing the edges of the *area opaca* with fine needles, then picking up the embryo with a wide mouth pipette and replacing it on the vitelline membrane in the desired orientation (see deHaan, 1967). Very young embryos, before they became attached to the vitelline membrane, were explanted by injecting a small bubble of air – or in some cases explantation saline – into the latebra of the yolk, then cutting the vitelline membrane around the edge of the bubble with scissors and allowing the membrane fragment with the embryo to float to the surface of the explantation saline, where it was picked up with a glass ring and forceps. Pannett-Compton saline (buffered) was used as explantation medium in all cases. Some of the embryos were marked with a few particles of powdered charcoal or carmine, in order to make cells easier to follow (Spratt, 1946), but wherever possible natural markers were used, such as yolk droplets or clumps of cells. In the case of charcoal, the particles were first passed through a sieve (53 μm grid) to separate single particles and small groups from large aggregates. Two reference points of the same marking material were placed outside the embryo, either on the *area opaca* or directly on the vitelline membrane, or, in one case, underneath the *area opaca* so that it was anchored to the vitelline membrane, but still in the field of view. Wherever possible more than one of these types of reference marking was used in the same embryo. Incubation was at 37·4 °C ± 0·2 °C (except for the temperature-dependence experiments) in a Gallenkamp incubator. A current of 5 % CO_2 in air was passed at all times through the incubation chamber.

Filming was done with a Vinten MK3 camera, Nikon EFM adaptor with time-lapse attachment, on 16 mm colour film, through a Leitz Diavert inverted microscope with two condensers arranged to give a shadow-casting effect (Hlinka and Sanders, 1970) and increase contrast and resolution. In one case, filming was done through a Leitz Elvar dissecting microscope, using the dark-field illumination method of Hara (1970). Intervals between frames ranged from 5 to 10 sec.

Analysis of films

The films obtained were analysed frame by frame by noting the positions of natural or artificial (charcoal or carmine) markers and the two fixed reference points, and analysing the behaviour of these markers with the computer programme described in detail in the Appendix. Additional checking of the periodicities was achieved by direct observation of the films while projecting at 8, 12, 16 and 24 frames/sec, and timing with a stopwatch. The periodicities of electrical apparatus in the room and adjacent areas were checked to eliminate the possibility of response of the embryo to interference from outside. Routine checks of focus, state of the embryo and culture medium, etc., were conducted randomly, about every 2 h. Filming was continued in general until the end

of gastrulation, but embryos remained in culture for as long as possible (they usually developed up to stages with one to four pairs of somites), to check their development for normality and correlate this with results obtained from the films. Films obtained from embryos which developed abnormally were disregarded in the analysis and not included in the results presented in this paper.

RESULTS

After recording from 10 embryos at early, intermediate and late primitive streak stages (stages 0–2, 3 and 4 according to Hamburger and Hamilton, 1951), recording from 41 marked points and 20 reference points, the following results were obtained:

(1) There is a periodicity of cell movement with a period between 2·25 and 3·12 min at 37·4 °C (mean 2·6 min, S.E. = ±0·3).

(2) Each pulse of movement lasts for 12–16 sec, followed by a period of quiescence (see Fig. 2). The extent of movement per pulse varies with time, and also between embryos, but is of the order of 50 μm/pulse (i.e. approx. 7 cell diameters).

(3) Movements of all marks in one embryo have the same periodicity of movement at any one time, and each pulse lasts the same length of time. The period, however, fluctuates slightly over long periods of time, which may reflect small changes of temperature in the incubation chamber (see below).

(4) At the end of primitive streak formation, the amplitude (i.e. the extent of movement/pulse) decreases, but the frequency does not seem to be affected. The duration of each pulse appears to be similar to that visible earlier on, but the small distance the particles move makes it difficult to determine the length of this period accurately.

(5) The periodicity described can be seen in all tissues. In the hypoblast and mesoderm, however, there appear to be periods of no movement from time to time, apparently random in duration and time of occurrence.

(6) The oscillations first begin in areas adjacent to Koller's sickle (the posterior end of the blastoderm), whence a 'wave' of onset of movement can be seen directly, slowly progressing towards the anterior end of the blastoderm at a rate of approx. 200 μm/min.

By analysing the periodicities at various temperatures, it can be seen (Fig. 1) that there is a linear relationship between the $\log_e$ of the period and temperature. This is the same relationship as has recently been described for *Dictyostelium discoideum* by Wurster (1976) and by Nanjundiah, Hara & Konijn (1976). This correlation of the period of the oscillations with temperature affirms that the phenomenon observed is not an artefact of the experimental system.

In the films taken at slower speeds (5-sec interval between consecutive frames), it is possible to measure the time difference between the onset of movement in a pulse of two distant points on the same tissue. The distance between each of
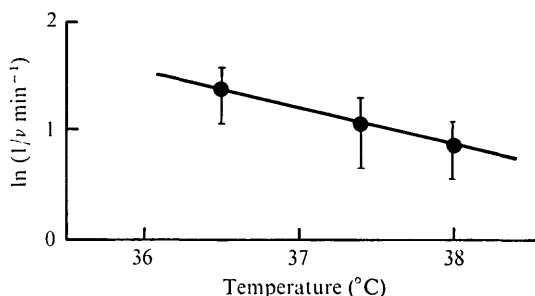
Fig. 1. Temperature dependence of the period of the oscillations. The $\log_e$ of the period is plotted on the ordinate and the temperature (°C) on the abscissa. Error bars represent the standard error, calculated on a linear scale, but plotted on a logarithmic scale.

these points and Hensen's node can then be measured (at the time of onset of the pulse) and used to calculate the speed of propagation of the inferred signal across the epiblast. This speed is between 25 and 40 μm/sec (mean 33·4 μm/sec, S.E. = ±4·2, n = 12 points in six embryos). This means that the signal here travels through the epiblast at a rate approx. 10 times faster than the wave of onset of movement described above.

The amplitude of the pulses does not decrease with distance from the node, which seems to rule out the possibility of a mechanical artefact, since a wave originating from a mechanical disturbance would damp out rapidly as distance from its point of origin increased.

The pattern of movements observed in all tissues was consistent with the detailed maps of the direction of cell movements in different regions of the embryo, contained in earlier reports (see Nicolet, 1971 for review).

DISCUSSION

The results of the time-lapse film analysis provide clear evidence for the occurrence of periodic movements in all tissues during primitive streak formation in the chick. The anterior part of the primitive streak, including Hensen's node at the definitive streak stage (st. 4), acts as a centre of organizing activity in the chick (see Gallera, 1971 for review, and Waddington, 1932, Hunt, 1931 and Hara, 1975). This tissue may be responsible for producing a periodic signal which propagates at a velocity of about 35 μm/sec, inducing cell movement towards the origin of the signal in much the same manner as that observed during aggregation in *Dictyostelium discoideum*. It thus appears that the organizing property of the anterior portion of the primitive streak is expressed as a pacemaker which leads to the periodic morphogenetic movements towards it.

These periodic movements are initiated by a slow wave propagating outwards from Koller's sickle at about 1/10th the velocity of the subsequent periodic

signals. We interpret this as a possible wave of competence after the passage of which cells become responsive to the morphogenetic signals. The slower speed of this wave suggests a more complex process than the waves leading to periodic movements, but there may be essential biochemical and physiological similarities underlying them.

The physiological nature of the processes underlying the observed periodicities of cell movement is far from clear, but the velocity of the waves suggests that some sort of ionic-electrical process may be involved. Preliminary experiments to test this have been conducted in our laboratory, by stimulating chick and amphibian (*Xenopus laevis*) embryos with pulses of electric current. It was observed in the chick that the direction of movement of cells in the epiblast could be distorted, the cells being attracted to the electrodes, and in *Xenopus* that a partial secondary blastopore could be induced between the two electrodes, and that secondary embryonic axes could therefore be induced.

A number of models have been suggested to explain the organization of the developmental process, generating spatial and temporal complexity from less ordered states. Among them Goodwin and Cohen (1969) suggested a model based on the action of oscillators and transmissible, self-propagating waves to generate multi-dimensional organization in a tissue. The idea of waves and periodic events accompanying the setting up of morphogenetic fields has also received experimental support. Novak and Bentrup (1972) showed that a pattern of periodic electrical spikes is produced at the cap end and travels down the plant in regenerating *Acetabularia mediterranea*. Goodwin (1974) described periodic events in *Tubularia* based on time-lapse film studies. Jaffe (1966) and Nuccitelli and Jaffe (1974) described periodic electrical events in eggs of *Fucus* and *Pelvetia*. More recently, Jaffe (personal communication) has found a travelling wave of free calcium originating from the point of sperm entry after fertilization of the egg of the Medaka. The classic example is, however, that of *Dictyostelium discoideum* amoebae during aggregation, where pulses of cyclic AMP act as an attractant and later as a trigger for differentiation and the establishment of spatial order (see Gerisch, Fromm, Huesgen & Wick, 1975; Konijn, Barkley, Chang & Bonner, 1968; Robertson and Drage, 1975). Cooke and Zeeman (1976) used the ideas of waves and oscillators to produce their model for the control of somite number and size in the amphibian embryo.

The properties of the interpretation outlined above are also being investigated theoretically, to characterize the model more precisely and allow more accurate predictions about the behaviour of the system under a variety of conditions.

Description of computer programme to analyse the data

The analysis of cell movement from the films is not designed to find out whether or not periodicities are present in the data, since this is obvious by direct observation of the films, but to calculate the period of the oscillations as accurately as possible.

The computer programme designed to carry out the analysis consists of three sections, namely:

(*a*) A section which corrects the coordinates of the points for drift of the film frame, etc. This is done by entering the values for the X and Y coordinates of the points and of the reference marks, and subtracting the values of each of the point coordinates from each of the reference coordinates in turn. The effect of this procedure is to move the frame of coordinates so that the coordinates of one of the reference points are 0, 0 at any time. The coordinate system is therefore shifted so that the reference point is always at the origin. The second reference point provides a safety factor, ensuring that the first reference point does not move with respect to it.

(*b*) A section which calculates and plots a linear regression for the positions of the points with respect to time. This is achieved by two major steps in the programme: (1) by a method of trial and error, the programme first selects a line which best represents the direction of movement of the points, so that the direction of movement can be represented primarily along one axis of the coordinate system; (2) the programme then applies a linear regression to the positions of the points along their new axis with respect to time, and plots this linear regression, alongside the actual data points. Fig. 2 shows a typical output from this part of the programme.

(*c*) The last section consists of two parts: (1) a section which calculates by subtraction the deviation of each observed point from the linear regression fitted by the previous part of the programme. The second section then uses these deviations to calculate the autocorrelation coefficient given by the formula:

$$C_r = \frac{\sum\limits_{i=1}^{n} (x_i)\,(x_{i+r\tau})}{n}$$

where x_i is the above deviation of the position of the points from the 'expected' value if they were moving continuously, τ is taken $= 1$ and r is an integer which varies from 0 to $n-1$, n being the number of observations (or frames of the film). The programme then proceeds to plot C_r *versus* r (see Fig. 3). The calculation has the effect of multiplying each term first by itself ($r = 0$) then by the next term ($r = 1$), then by the next but one term ($r = 2$), etc. In this way, one gets a picture of how high the value is with respect to what the population of data is doing as a whole. The plot gives rise to a curve, the period of which (see Fig. 3) is an estimate of the actual period of the pulsations to be tested.
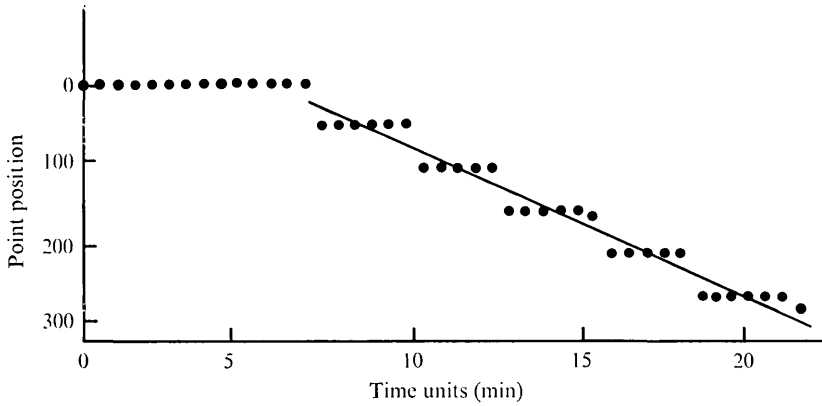
Fig. 2. Part of the primary output from the computer programme designed to analyse the data from the films. The points on the graph represent the distance of a single marker in one embryo, measured from its initial position (0), as a function of time. The line drawn is the regression line through the points after movement begins and serves as the basis for the calculation of the expected positions of the points if motion were non-periodic. For further details see Appendix.
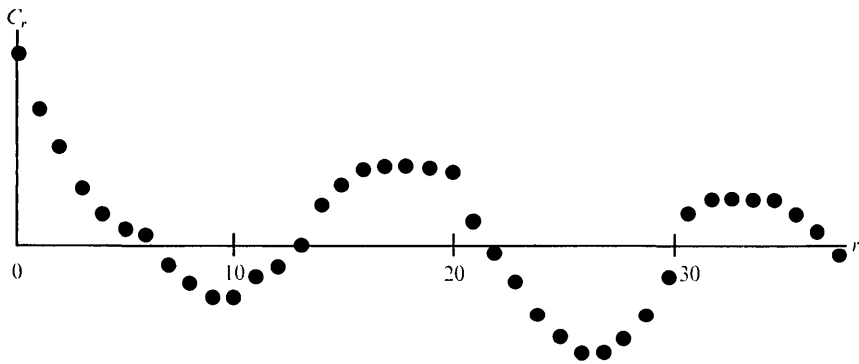

Fig. 3. Correlation coefficient (C_r) plotted against the value of the integer variable (*r*). The sampling time was 10 sec. For further explanations see Appendix.

A plot of C_r *versus r* then gives information about any periodicity in the original data by virtue of the fact that whenever $r\tau$ takes a value equal to a period in the data, C_r will have a local maximum. It is clear that $r = 0$ gives an absolute maximum simply because all terms in the sum are then positive. The frequency analysis is independent of the time interval between observations (in this case, the frame interval, so long as this is significantly shorter than the observed periodicity).

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