

## **A Re-Examination of Mitotic Activity in the Early Chick Embryo**

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**Summary.** As a result of extensive mitotic index analysis in colchicine-arrested chick embryos during gastrulation, it was ascertained that the primitive streak is a region of elevated mitotic index as compared to the surrounding tissue. Along the cephalo-caudal axis, the embryo displays two large peaks of mitotic index, one at the posterior end of the primitive streak and the other just anterior to Hensen's node. The length of the various phases of the mitotic period was determined *in vitro* by time-lapse filming, and the colchicine-arrested mitotic indices *in vivo* and *in vitro* were determined and compared for various regions. Some observations regarding the orientation of mitotic spindles and abnormal mitosis *in vitro* are also included, and the relevance of the above observations to early embryonic development is discussed.

**Key words:** Mitotic index – Chick embryo – Cell division – Spatial organisation.

### **Introduction**

Several authors have already examined the question of whether there are, in the early chick embryo, regional differences in mitotic activity. Initially, these studies were directed towards providing an answer to the rather old problem of the origin of mesoderm and endoderm, and the role of the primitive streak (PS). (For reviews see Nicolet, 1971 and Stern, 1978).

Derrick (1937) was one of the first investigators to look specifically at this question in the early chick embryo, finding that there were indeed some regional differences in late PS embryos. The mitotic index (MI) was high on either side of, but close to, the PS on the presumptive medullary plate. She also found that the MI was lower at Hensen's node than in the axis of the rest

of the PS. Pasteels (1942a, b) pursued the examination of this question, obtaining nearly the opposite result, and concluded that there were no differences in the MI of different regions of the trout, canary or chick embryo. However, close examination of his tables reveals that his samples are very small, comparing populations of approximately 8,000 cells each, and he does not specify how these populations were selected. His standard errors are also extremely large (e.g. Pasteels, 1942a, Table 2b showing MI values for the PS of 2.6% and the standard error amounts to no less than  $\pm 10.52\%$ ). Solomon (1957) determined the population doubling time, finding it to lie around 8.9 h for embryos during 'embryogenesis' (presumably meaning early axis formation) and around 6.5 h between stage 4 (Hamburger and Hamilton, 1951) and limb bud. Emanuelson (1961) found no significant MI variations in untreated chick embryos, but some regional differences became clear after mitotic accumulation with oestradiol, colchicine and heat shocks. Bellairs (1955, 1957) also found small regional variations in un-arrested embryos. Ozato (1969) found a peak of MI at the anterior end of the PS, while Dondua et al. (1966) found a peak of MI in the posterior part of the PS at stages 3–4, and at Hensen's node at stage 5. They also observed an elevated rate of proliferation in the mesoderm from stage 4 (see also review by Woodard, 1948).

It was this series of apparent contradictions in the literature in this field that compelled me to undertake a repeated and more detailed study of the MI in early chick embryos, using cell populations much larger than those used in previous studies. These studies are by nature extremely slow and tedious, and these were limiting factors in the present study, as it would take several years to produce a body of data large and reliable enough to follow regional MI differences with development. However, a detailed study was made of the embryo at stage 4 (all cells counted in two embryos at this stage) which comprised analysis of over 2,000,000 cells, as well as studies of transverse, selected sections through earlier chick embryos. Some studies were also made of the MI and other parameters of the same cell types in tissue culture, which had not been performed in the past.

Some studies were also undertaken of cells from similar embryos *in vitro*, partly as a measure of normality of the cultured cells, partly as a basis for future experimentation on the control of mitotic division in early embryonic development. If the whole cell population is cycling, then a combination of MI and duration of mitosis gives an estimate of cell cycle time.

## Materials and Method

For experiments to map the mitotic index (MI) in various regions of the early chick embryo, White Leghorn and Rhode Island x Light Sussex embryos were exposed *in ovo* to  $5 \times 10^{-7}$  M colchicine (BDH) for 2 h at 38° C, which had been injected into the latebra of the yolk to facilitate rapid diffusion. The embryos were then dissected, fixed in Bouin's fixative or ethanol/acetic acid (3:1) for 24 h, and then embedded in paraffin wax, sectioned at 8  $\mu$ m and stained with haematoxylin and eosin. Sections were taken perpendicular to the axis of the primitive streak (PS). In two embryos at stage 4 (staging in Arabic numerals for late stages according to Hamburger and Hamilton, 1951 and in Roman numerals for early -pre-streak- stages according to Eyal-Giladi and Kochav,

1976) every cell in the *area pellucida* was scored for mitotic figures (i.e. metaphases) proceeding from posterior to anterior sections. Apart from these two embryos, two embryos each at stage IX (unincubated), stage 1, stage 2, stage 3 and stage 5 were scored. In these, selected sections were counted, chosen by a pseudo-random number generator from a computer. 20 sections were scored in each of these embryos. Only unincubated embryos with clear antero-posterior polarity were used for this study.

The results were analysed as indicated in the legend to Fig. 1.

For experiments *in vitro*, embryos were dissected and explants prepared according to the procedure described by Sanders et al. (1978) and grown on either glass coverslips or plastic (Falcon) culture dishes as sitting drop cultures. Tissues exposed to colchicine were grown on culture dishes for 24 to 36 h until attachment of the explant to the substrate was complete, and then exposed to  $5 \times 10^{-7}$  M colchicine for 2 h, after which period the explants were fixed in formal saline for 24 h and stained with Harris' haematoxylin. The total number of cells in each explant was subsequently scored for mitotic figures. Each explant comprised some 500 cells.

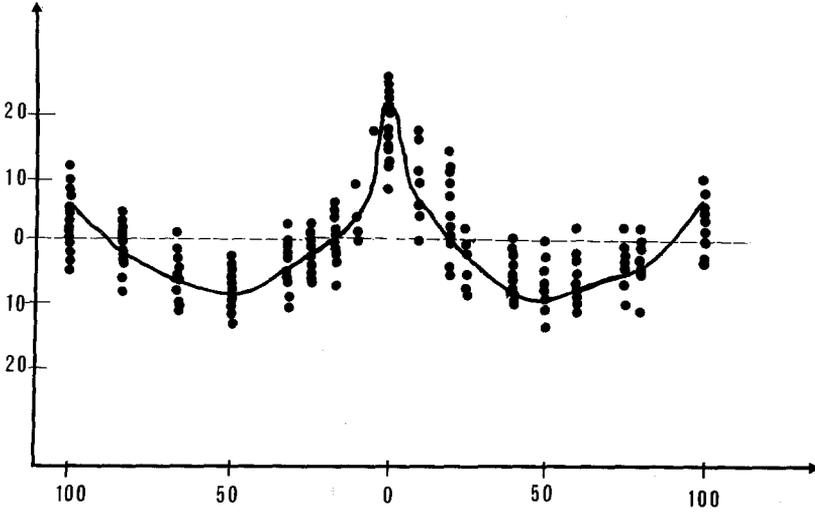
The procedure used for time-lapse filming and the equipment used were described previously (Sanders et al., 1978). The intervals between frames were 10 or 12 s, using a  $16 \times$  phase contrast objective giving a film magnification of  $38 \times$ .

In all the present experiments, the MI was defined as the number of metaphases per 100 nuclei. Cell density was determined by counting the number of cells in a given  $125 \times 125$   $\mu\text{m}$  eyepiece grid square, each of which contained some 100 cells in the case of epiblast.

To avoid counting mitotic figures twice in adjacent sections, a spot check was conducted where the locations of mitotic nuclei were compared with the positions of those in the preceding and subsequent sections, and also by applying the calculations described by Abercrombie and collaborators (see Abercrombie et al., 1968 and references therein), to obtain a measure of the degree of overlap.

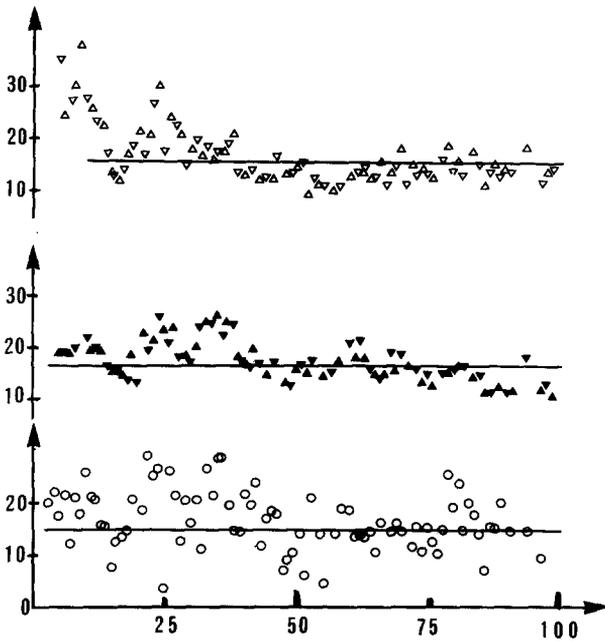
## Results

Figs. 1–3 summarise the results obtained for the two embryos at stage 4, clearly showing (Fig. 1) an elevated mitotic index (MI) at the primitive streak (PS), falling to a minimum at about the middle of the epiblast on either side of it and slowly rising again to a local maximum near the boundary between the *area opaca* and the *area pellucida*. When mapping the MI along the cephalo-caudal axis, Fig. 2 shows no striking correlation between position of the section on the axis and MI in the epiblast, mesoderm or lower layer (comprising hypoblast, definitive endoblast and junctional endoblast as defined by Sanders et al., 1978). When mapping the PS along the cephalo-caudal axis, two large peaks of MI become apparent (Fig. 3). One of them coincides with the posterior end of the PS and the other lies just anterior to Hensen's node. The posterior peak can also be found in the lower layer (Fig. 2). Since several tissues come together at the level of the PS, all of them contribute to the MI in the results. The effect this has on overall MI values on the epiblast transverse sections is thus less than that apparent in the lower layer, due to the greater cell density in the former than in the latter. In other words, the proportion of cells which form part of the PS in a single transverse section is greater in the lower layer than in the epiblast, and that reflects itself in the MI for the whole section in regions where the PS shows sharp peaks of MI. The anterior peak, however, does not appear in the mesoderm or the lower layer since the former is not a coherent sheet of tissue and is almost absent in the anterior PS regions, and in the case of the latter the width of the *area pellucida* at this level is



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**Fig. 1.** The mitotic index in a single stage 4 embryo. Each point represents the MI of one grid square of the eyepiece graticule. The difference between the MI of that grid square and the average MI for the whole section is plotted on the ordinate, and an index of distance (% distance from the PS to the right or left) is plotted on the abscissa. The curve passes through the arithmetic mean of each column of points. The total number of cells encompassed by this graph is approximately  $2.5 \times 10^5$ , and includes all cells from the caudal end of the embryo to the anterior end of the PS including Hensen's node. The 0 level is about 18% MI



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**Fig. 2.** Mitotic index in each of the three layers of a stage 4 blastoderm. The upper graph represents the lower layer, the middle graph the epiblast and the lower graph the mesoderm. Section number (counted from the caudal end of the embryo) shown on the abscissa, MI (%) on the ordinate. The solid lines represent a linear regression (least squares method) applied to each population of points

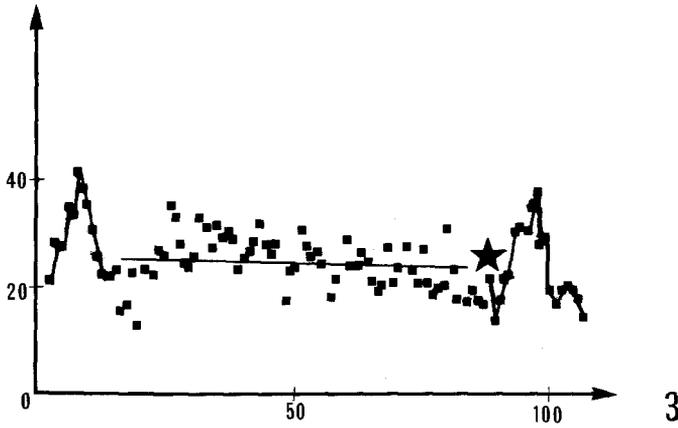


Fig. 3. Mitotic index along the PS of a single stage 4 blastoderm. Section number on the abscissa, MI(%) on the ordinate. The horizontal line represents a linear regression excluding the peaks. The star marks the position of Hensen's node

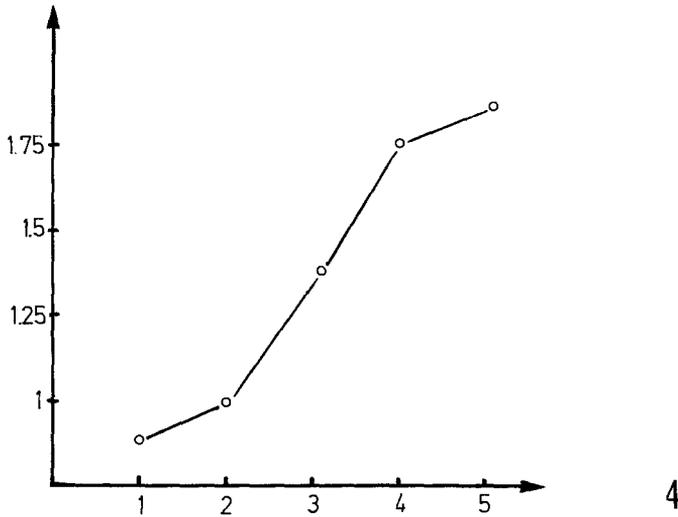
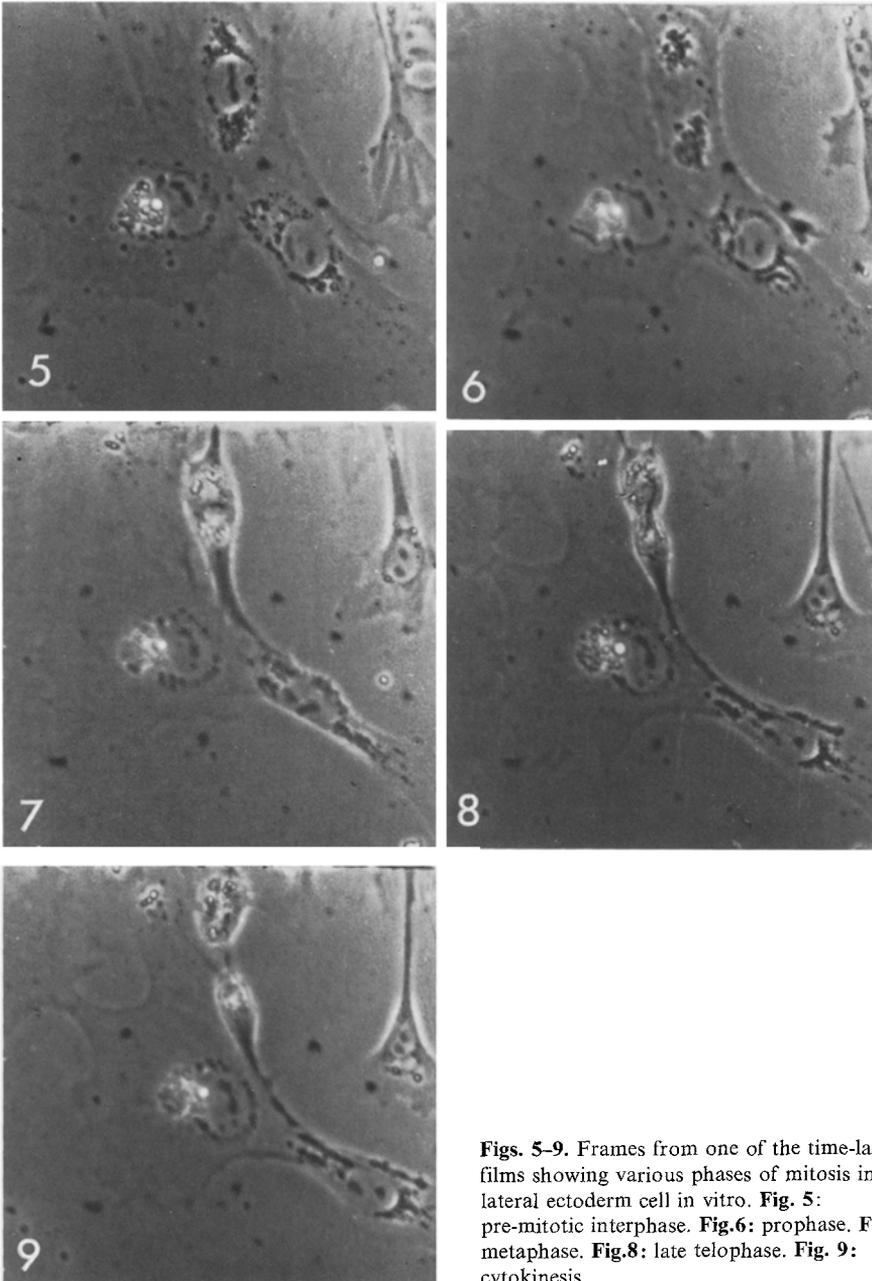


Fig. 4. Correspondence between the height of the anterior peak of MI with age of the embryo. Stage number plotted on the abscissa, and an index of height of the peak in arbitrary units on the ordinate. The index of height is computed by dividing the maximum value of the MI at the peak over the average MI for the PS excluding the peak regions

sufficiently great to balance out the effect of a high MI in the relatively small PS region.

In earlier embryos, the overall pattern is similar to that described above, but the steepness of the peaks is much less pronounced. Fig. 4 shows a correlation between the slope of the anterior peak at the PS with age of the embryo between stages 1 and 5.



**Figs. 5-9.** Frames from one of the time-lapse films showing various phases of mitosis in a lateral ectoderm cell in vitro. **Fig. 5:** pre-mitotic interphase. **Fig. 6:** prophase. **Fig. 7:** metaphase. **Fig. 8:** late telophase. **Fig. 9:** cytokinesis

The following general features were also observed while scoring sections:  
 (a) when a section has a low overall MI, then the one immediately posterior as well as the one immediately anterior frequently show a high MI, and there is a predominance of spindles oriented with their long axis parallel to the axis of the embryo (68.8%);

**Table 1.** Comparison between the MI in vivo and in vitro in various stage 4 tissues. The last column is the ratio of the in vitro MI to the in vivo value

Tissue	MI in vitro %	MI in vivo %	Mliv/MIio
Epiblast	10.24	18.0	0.569
Hypoblast	10.03	12.1	0.829
Endoblast	9.41	16.5	0.570
Junctional	9.11	28.8	0.316
Germ wall	9.17	22.3	0.411
Posterior PS	10.39	39.9	0.260
Mid. PS	10.65	21.7	0.491
Hensen's node	9.87	28.8	0.343

**Table 2.** Duration of the various phases of mitosis in cells which divided normally in time-lapse films. Only one value was determined for telophase as its beginning and end are difficult to determine from the films

Phase	Average duration	% of total
Prophase	12'35" $\pm$ 3'05"	45.12
Metaphase	6'52" $\pm$ 3'18"	24.6
Anaphase	3'08" $\pm$ 2'08"	11.23
Telophase	1'36 (one observation)	5.74
Cytokinesis	8'38" $\pm$ 4'30"	30.96
Total	27'53" $\pm$ 7'24"	117.65

(b) in the lower layer there is a shallow gradient of cell density with its lowest point under the PS in most regions;

(c) in the epiblast the gradient of cell density is the opposite of that in the lower layer (i.e. it follows the MI, see Fig. 1);

(d) in the anterior portions of the PS most of the spindles are oriented at 90° to the plane of the section, whereas in other regions of the epiblast to either side of the PS the major axis of the spindle tends to be perpendicular to the antero-posterior axis of the embryo (83%, 72.4%).

Table 1 compares the average MI values for the various tissues from stage 4 embryos in vivo and in vitro. It can be seen that on average, the in vitro values are considerably lower than the corresponding in vivo values, and that the differences between the MI of the various tissues are less in vitro than in vivo.

Table 2 summarises the results obtained by measuring the duration of the various mitotic phases from time-lapse films of in vitro cells. No significant difference was found between the duration of the various phases with the type of tissue studied (Chi<sup>2</sup> and *t*-test both showed *p* > 0.1).

Approximately 18% of the mitoses visible in the films were abnormal, leading to the production of binucleate cells or of daughters of very different size to each other. Four different causes of binucleate formation were observed:

(a) failure of daughters to separate completely; they rejoin after some time following partial separation;

(b) fusion of daughter cells after what appears to be complete separation. It is interesting that fusion of nonsister cells was never observed by me in the films;

(c) direct division of a single nucleus into 3 daughter nuclei of unequal size, containing an uneven and variable number of nucleoli. The result of this is one mononucleate and one binucleate cell;

(d) anisometric cytokinesis, plus both daughter nuclei remaining in one of the daughter cells. The result is one binucleate and one anucleate cells.

Although mitosis in binucleate cells was never observed in the films, cells containing more than two nuclei (up to 5) can be observed in older cultures (see Brodsky and Uryvaeva, 1977).

Regarding the morphology of mitosing cells *in vitro*, I have observed that cells round up and apparently diminish their degree of adhesion to the substrate during mitosis, and more especially so during and after metaphase (Figs. 5–9). Cells can often be seen blebbing rapidly during mitosis, probably an indication of the poor attachment of the cells to the substrate or to other cells (see Harri and Low, 1975 for an SEM study of the morphology of mitosing cells in the PS of chick embryos, and Vasiliev and Gelfand, 1977).

Another interesting feature of mitosing cells in culture which I observed in the films is that cells tend to divide such that the orientation of the mitotic spindle is parallel to the axis of elongation of the cell before entering mitosis, which is especially interesting in view of the observation described above that cells lose their elongation and become spherical and non-polarised (see Vasiliev and Gelfand, 1977) during mitosis. This observation was also made on other cell types by other investigators (Ivanova et al., 1976; Donachie et al., 1976; Curtis and Seehar, 1978 and see also Folkman and Moscona, 1978).

In un-arrested embryos at stages 5–7, it was found that only 12 out of 212 (5.7%) mitotic spindles analysed were oriented along axes other than cephalo-caudally, dorso-ventrally or medio-laterally. The majority of the spindles in the embryo are therefore oriented along one of these axes and intermediate orientations are conspicuously absent. 110 spindles (51.9%) were oriented along the medio-lateral axis (at 90° to the PS), 66 (31.1%) along the cephalo-caudal axis (PS axis), and 24 (11.3%) along the dorso-ventral axis. (See also Martin, 1968, who studied spindle orientation from stage 7 onwards in the chick).

## Discussion

The results presented above indicate that there are regional differences in the mitotic index (MI) in early chick embryos at definitive streak stage (stage 4): the primitive streak (PS) itself shows increased MI when compared to the surrounding tissue. Along the antero-posterior axis, two large peaks of MI are apparent: one at the posterior end of the PS and the other just anterior to Hensen's node. At earlier stages, the pattern of MI distribution is similar to that described above, but the peaks are less pronounced. In cells from tissues

of embryos at similar stages which were cultured *in vitro* there is a reduction in the MI as compared to the *in vivo* situation, and there is no significant difference between the various tissue types, as indicated in Table 1. The duration of the various phases of mitosis was determined, and some observations were made of cell morphology during mitosis and abnormal mitosis types were described. Some observations on the orientation of mitotic spindles are also included.

For the interpretation of the results using colchicine, I have made the assumption that the pattern of mitotic activity is directly related to the MI of that particular region. If there are no regional differences in terms of the penetration or effectiveness of the colchicine on the tissue, this is a reasonable assumption, but even if there were such differences the results would still be of interest as they would then reflect other regional properties of the tissues which are of obvious relevance to the study of the developmental process. It is also worth emphasizing at this point that other authors have observed consistent regional variations in the MI in untreated embryos (e.g. Derrick, 1937; Bellairs, 1955, 1957; Dondua et al., 1966; Ozato, 1969). It must be borne in mind, however, that there is a possibility that colchicine may be itself inducing some regional differences (Overton, 1958). Emanuelsson (1961) could only find regional differences in MI when the embryos were arrested using oestradiol, colchicine or heat shocks. Examination of cell shape and other morphological parameters in the conditions of these experiments (Stern, 1978 and unpublished observations) did not reveal any significant differences between treated and untreated embryos or *in vitro* tissues. Bancroft (unpublished observations) and Portch (1975) both showed that the morphology of individual cells, whole embryos and isolated tissues is only affected after prolonged exposure to much higher concentrations of colchicine than those used here to arrest mitosis. For these reasons I have assumed that these results are real and that they reflect mitotic activity in the embryo.

The regional differences described above confirm Emanuelsson's (1961) findings for arrested embryos, in that the node region in late PS embryos constitutes a region of high MI. The results also agree with those of Derrick (1937) in that the MI is high close to and to either side of the PS. Both the anterior peak found by Ozato (1969) and the posterior peak found by Dondua et al. (1966) are confirmed in the present study.

The results of Snow (1977) on the mouse embryo MI are an interesting comparison from another system, showing that this may be a general feature in vertebrate gastrulation. He found a peak of mitotic activity in that part of the mouse embryo which corresponds with Hensen's node or lies slightly anterior to the anterior end of the PS during gastrulation. It is also interesting that he finds this peak of mitotic activity at this position even before the node itself reaches this point. Further study will no doubt provide interesting results with respect to this feature in the chick embryo, as well as new insight into possible similarities between different organisms at comparable stages of development.

The findings presented above indicate that whereas there are marked regional differences in the whole embryo, these disappear when fragments are cultured

separately *in vitro*. To me, this suggests that mitotic activity in the embryo is under global control, as is probably also the case with morphogenetic movements (Stern and Goodwin, 1977). Further support for this hypothesis comes from the observation that both the pattern of MI and the orientation of mitotic spindles at stage 4 closely follow the pattern of electrical current flows at the same stage (Stern and Jaffe, 1979). Experiments to test for a possible causal connection between these two phenomena are now in progress.

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