Properties of the primary organization field in the embryo of *Xenopus laevis*

IV. Pattern formation and regulation following early inhibition of mitosis

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**SUMMARY**

Visual observation of early blastulae, and counting of cell suspensions from the late blastula and subsequent stages of *Xenopus* development, have shown that exposure to either colcemid or mitomycin C can sustain blockage of the mitotic cycle, and hence prevent the normal increase in cell number. Such blockage is essentially complete within a period, following introduction of the drugs, which is short compared with the cell-cycle time normal to post-blastular stages. Although the cell-counting data do not allow a statistical demonstration, there is a suggestion that mitomycin C allows a greater proportion of cells to divide once, after its introduction, than does colcemid. This is in accord with its putative mode of action, in causing only intrachromatid cross-linkage within chromosomes, but there are no data on the status of DNA replication within nuclei of the non-dividing cells under either drug.

If blockage is begun not earlier than stage 10+, some 20 min after dorsal lip formation, essentially normal morphogenesis ensues up to tail-bud stages around 27, including histodifferentiation of ectodermal structures and notochord, and twitching of somitic muscle. Such embryos are described. In describing those arresting at earlier stages following blockage in blastulae, the possibility is mentioned that morphogenesis may fail for mechanical reasons due to low cell number and large cell size, rather than to lack of a normal field as such.

Cell counting reveals that blocked embryos with qualitatively normal pattern formation by stages 22–24, in having a total cell number appropriate to stage 10+ or 10½, show about a seventh or an eighth of the number of cells normal to their stage. Between then and stage 27 when histogenesis, particularly of twitching muscle, is more clearly seen, the direct method of estimating cell number can no longer be used, but histology reveals no mitotic metaphases in any blocked embryos.

In the same experiments, operations of two types described in previous papers are performed on early gastrulae so that regulation is required in the embryonic field after the cessation of mitosis. The results are as seen in the normal presence of the cell cycle, revealing that, in response to changes of positional information value, host material may change its presumptive differentiation tendency and final commitment, in the absence of cell division as such.

There is some indication that the proportional numbers of cells assigned to different structures in an individuation field may deviate from normal when overall cell number is limiting.

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INTRODUCTION

There is much evidence that the processes of pattern formation during development of regulative eggs do not involve cell counting as such. Instead, fields seem to be set up across sheets of cells, within whose boundaries appropriate zones of material become committed to the development of particular differentiations by the cells within them. A classical example of the size independence of a field among normally sized cells is the observation that, following removal of large amounts of the uncommitted territory of an amphibian blastula, a normally proportioned small neurula may result. Recently, Hamilton (1969) has shown that when the spatially rhythmic process of somite formation is compared in haploid and in normal diploid *Xenopus*, an appropriately larger but highly canalized number of the smaller haploid cells is assigned to each somite, the somites themselves being approximately normal in number and size.

There have been suggestions (e.g. Flickinger, Miyagi, Maur & Rollins, 1967; Holtzer, 1964) that whilst particular numbers of cells may not be required for morphogenesis, the continual process of the normal cell cycle is required for development in some other sense. These suggestions are of two types. One of them supposes that variations in the timings and overall rates of various phases in the cell cycle, which are observed to develop in a repeatable way in the different presumptive regions of embryos, are themselves a causal antecedent of the gradual commitment of various lineages of cells to their special patterns of macromolecule synthesis, and hence differentiation. The other proposes that some part of the normal ongoing cycle that results in mitosis is necessary, in order that one or both daughter cells shall change their state of determination, i.e. become members of new differentiation compartments, either in the normal course of the emergence of new cell types in development, or else in response to regulative changes in their position in an embryonic field. A less universal suggestion of the latter type has recently been made by Lawrence, Crick & Munro (1973), for an insect system where there is evidence that the cell cycle is required for the adjustment of the remembered position-value in epidermal cells, in response to changed local gradients caused by operations. The morphallactic head regeneration process in *Hydra*, however (Wolpert, Hicklin & Hornbruch, 1971), appears to provide a counter-example, in that it occurs successfully among cells effectively prevented from division by prior irradiation.

Opportunity to explore all these suggestions for amphibian development, as well as to investigate further the independence of spatial pattern and cell number, came with the finding that blockage of mitosis could be maintained with either of the drugs *colcemid* or *mitomycin C* in *Xenopus* blastulae and gastrulae, and that with blockage starting at the latter stages development could proceed in all visible respects fairly normally, up to tail-bud stages. The first object of this paper is to give a preliminary description of the nearly normal
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development seen after early gastrular blockage of cell division, and to present
the cell-counting data showing that there is essentially no cell division in
embryos under these drugs up to the stages where the presence of cell commit-
ment (i.e. mosaic development) can reasonably be assumed. It will be further
suggested that certain subsequent histodifferentiations also do not require prior
mitosis. Finally, experiments are described which make it seem highly probable
that changes of presumptive fate and final commitment to differentiation can
occur in cells in these embryos in response to regulative changes in the pattern
of positional information (Wolpert, 1969, 1971), without intervention of a
normal cell cycle.

In discussing the results, attention is paid to the limitation that DNA syn-
thesis in the nuclei of blocked cells cannot be examined with sufficient sensitivity
to counter suggestions that a minimal amount may be occurring, and be
necessary for the developmental changes observed.

MATERIALS AND METHODS

The general handling of embryos, and method of treatment during and after
surgical operations, has been given in detail in Paper I of the series (Cooke,
1972a). Embryos were exposed to either colcemid (CIBA), 0.05 %, or mito-
mycin C (Sigma), 40 μg/ml, in a layer of half-strength Holtfreter at pH 7.2,
equilibrated over a bed of 2 % agarose which has been found to cause less
neurectodermal ruckling and abnormal gastrulation than the agar previously
used. A rent was made with tungsten needles into the blastocoel to allow
access to all cells by the drugs at full concentration, and the embryos left for
about 45 min before transfer to 1/10 strength Holtfreter with a holding concen-
tration either of 0.01 % or 0.02 % colcemid, or 20 μg/ml mitomycin C. At this
time the rent had healed with minimal cell damage in the animal region. Subse-
quent development was in these solutions.

Operations were of two types, and were performed synchronously with the
onset of blockage of the cell cycle. In one, a second stage 10 organizer was
grafted into the marginal zone of a stage 10+ or 10½ gastrula, at an angle of
about 100° as described in Paper I. Typical results of such operation are
described in Paper II (Cooke, 1972b). In the other, the head organizer region
was simply excised, as from the donor in the previous operation, but from a
stage 10+ gastrula. A small control series showed the results of this procedure
to be qualitatively similar to those resulting from the same excision as performed
at stage 10, the earliest gastrula, which are described in full in Paper V (Cooke,
1973).

Control embryos at the time of blockage, and control and blocked, operated
embryos at stages 14-15 and stages 22-24, were disaggregated in pairs in Ca^{2+}-
and Mg^{2+}-free Holtfreter at pH 8.2, containing 150 mg/l EDTA. After dissecting
free the endodermal mass of large fragile and yolky cells from gastrulae, or
their well-known derivatives from later stages, the remaining cell sheets, incorporating neur ectoderm, mesoderm and a little head endoderm were transferred rapidly to 0.5 ml of the disaggregation solution per pair. After 10 min they were pipetted gently to a single-cell suspension with a Spemann pipette, and counted immediately. Embryos for histology were punctured, fixed overnight in Bouin, washed for 24 h in several changes of 70% ethanol, dehydrated and wax embedded, and finally sectioned at 12 μm and stained in haematoxylin/eosin.

Certain embryos, developing under cell cycle blockage, were explored by dissection.

RESULTS

Fig. 1A represents the control embryos at a stage near the maximum survival age in cases where cell cycle blockage is commenced soon after the start of gastrulation. This is the earliest stage of blockage which can be followed by qualitatively normal morphogenesis, and some histogenesis, up to a stage around 27 of Nieuwkoop & Faber (1956). Such an embryo is shown in Fig. 1D. The time schedule of development of embryos blocked with colcemid is delayed by several hours as compared with initially synchronous controls, over the 24 h period involved. Such a delay might be expected from the known involvement of microtubular structures in morphogenetic movements (Perry & Waddington, 1966; Tilney & Gibbins, 1969). Normal cement-gland induction, the outlines of brain and eye, somites and tail-fin formation can be seen externally.

The mitotic cycle is extremely rapid at most blastula stages in Xenopus, but slows down very markedly some short while before the onset of gastrulation (Graham & Morgan, 1966). Thus as the onset of blockage is pushed earlier, the number of cells involved in subsequent attempts at morphogenesis is sharply reduced. However, as seen in Fig. 1B, blockage at a mid-blastula stage allows significant invagination movements and animal epiboly, until arrest at a stage equivalent to a mid-gastrula with some mechanical abnormalities. Blockage at stage 9, the late blastula (Fig. 1C) leads to a neurula, often with the beginning of cement-gland induction apparent but with an abnormal foreshortened neural plate. Such neural plates, apparently unable to complete closure, have boat-shaped folds and a tendency towards radial symmetry of cell orientation within them, rather than the normal bilateral pattern.

When cell number is limiting to morphogenesis, the tendency is for the sizes of cavities both within and between structures in the embryos to be reduced, whilst in extreme cases they may be almost absent, even though approximately normal shaping has occurred. Cavities deriving from archenteron and neural tubes are all thus reduced, as seen from Fig. 2. In arrested half-gastrulae with very low cell number, dissection reveals that the bottle-shaped cells normally leading ingression beneath the dorsal lip cannot achieve normal shape. The animal cell sheet also contains too few layers of cells, which are so
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Fig. 1. Schematic drawings of the final stages attained by embryos having mitotic blockage imposed at various times. (A) Control stage 27. (B) Blockage begun at stage 7–8; gastrulation movement begun. (C) Blockage begun at stage 9; gastrulation complete and neurulation attempted, giving a foreshortened, boat-shaped neural plate. (D) Blockage begun at stage 10; morphogenesis qualitatively normal until ca. stage 27, though with small internal cavities and ill-organized somites.

large as to make the normal increase in its area, allowing epiboly, impossible mechanically.

In blocked stage 8 blastulae containing second organizers (see Cooke, 1972a) implanted close to their own site of dorsal lip formation, separate zones of cell orientation can be seen focused at each site, by the onset of the host's gastrulation attempts, even though the number of intervening cells is much less than normal by stage 10, and though no secondary pattern can finally be individuated.

Since normal embryos of given stages, from eggs of different females, are found to vary somewhat in cell number, cell counting results can only be presented by individual experiments. Table 1 shows the results of direct counting of cells, from two typical experiments, in which operations as described later were also carried out at the time of blockage. Fig. 3 gives the cell diameter distributions for the suspensions in another experiment. It is seen that both antimitotic agents cause effective blockage of cell division, without appreciable escape between the neurula (stages 14–15) and stage 22, and that such blockage must ensue when at most a small proportion of the cells present at time of introduction of the drug have divided once.

The somewhat higher stable cell number in the case of mitomycin-C-blocked embryos is usually seen in these experiments, though not statistically significant within the error of the method of estimation employed. As an inhibitor of
DNA replication, this agent is believed to act by causing intrachromatid cross-linkage so that the post-replicative chromosome may be partitioned at a subsequent mitosis, but thereafter cannot replicate again (see Kiehlman, 1966). Colcemid, however, might be expected to block the first subsequent mitotic spindle formation. Thus it may be that a rather higher proportion of cells (although still a small one) divides once after imposition of the mitomycin C block, but again, cell number thereafter seems to remain static.

The twofold difference in means of the size-distributions of spherical suspended cells, as seen between normal stages 10½ and 22, is in agreement with the normal approximately eightfold cell number difference, since each doubling would be expected to reduce diameter of the daughter cells by about $\sqrt{2}$. Thus some three rounds of the cell cycle occur on average between these stages in the
Fig. 3. Diameter distributions of disaggregated cells from control and blocked embryos at early gastrula stages and during subsequent morphogenesis. For the preparation of cell suspensions, see text. Each size-class (in μm) represents a half division of a microscope calibration. (A) Control at stage 10½ and at stage 22. (B) Stage 22 blocked since stage 10½ with colcemid. (C) Stage 22 blocked since stage 10½ with mitomycin C.

mesoderm + neurectoderm, although the actual cell counts of Exp. 1 are more normal in this respect, those for the later control stages in Exp. 2 being somewhat low.

Initial morphogenesis, though little histodifferentiation, has occurred for the whole primary axial organization by stage 22, the latest used for direct cell counting. We can be confident that cell commitment has by then occurred for each of the principal zones of differentiation tendency within the mesoderm/endoderm and much of the induced axial nervous system and accessory ectodermal structure. Indeed it is probable that a general degree of mosaicism has been established within the closing neural plate, by the earlier neurula stages 14–15.

Histodifferentiation within the mesodermal mantle, with accompanying secretion of matrix by notochord and other cells, makes the direct method of cell-number estimation extremely difficult between about stage 24 and death
Table 1. *Inhibition of cell multiplication during Xenopus morphogenesis*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Controls at time of operation + blocking</th>
<th>Control</th>
<th>Stage 14–15</th>
<th>Stage 22</th>
<th>Blocked</th>
<th>Stage 14–15</th>
<th>Stage 22</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. 1 (colcemid block only)</td>
<td>4300 (300) (Stage 10+)</td>
<td>17800</td>
<td>30600</td>
<td>4600</td>
<td>4700</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. 2 (mitomycin C and colcemid block in same egg-batch)</td>
<td>5700 (400) (Stage 10½)</td>
<td>18200</td>
<td>28900</td>
<td>6500</td>
<td>6200</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cell numbers are the averaged results of counting four haemocytometer samples from each of two cell suspensions, each made with a synchronous pair of embryos at the morphological stages shown. Values are to nearest 100 cells.

Table 2. *The numbers of cell diameters measured across some structures in normal and in mitotically inhibited embryos at stage 27, in transverse sections*

<table>
<thead>
<tr>
<th></th>
<th>Epidermis from dorsal-ventral midline at level of anus</th>
<th>Half circumference of midbrain at level of greatest eyecup area</th>
<th>Greatest eyecup circumference</th>
<th>Paraxial somitic material (greatest dimension)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal embryo</td>
<td>66</td>
<td>46</td>
<td>55</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>59</td>
<td>42</td>
<td>56</td>
<td>47</td>
</tr>
<tr>
<td>Embryos with mitosis inhibition from stage 10½</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (colcemid)</td>
<td>32</td>
<td>25</td>
<td>41</td>
<td>20⁺</td>
</tr>
<tr>
<td>2 (mitomycin C)</td>
<td>35</td>
<td>23</td>
<td>38</td>
<td>20⁺</td>
</tr>
<tr>
<td>Ratio</td>
<td>1·9</td>
<td>1·8</td>
<td>1·4</td>
<td>2·0</td>
</tr>
</tbody>
</table>
of the experimental tail-bud stages at about stage 27. However, the cytological appearance of the large muscle cells in their somites is approximately normal, despite derangement at the histological level. Normal onset of myogenic twitching has been observed in embryos under both types of blockage, where cell counting in the same experiments reveals a static cell number up to stage 22. The notochord is normal in histodifferentiation apart from its large cell size.

Histology of blocked embryos at these later stages reveals no mitotic spindles in ectoderm or endoderm although the mitotic index in controls at this time is appreciable. Thus it is probable, though less certain, that the special protein synthesis involved in certain terminal histodifferentiations can also be achieved without intervention of the cell cycle as such.

Table 2 gives numbers of cell diameters, in sections such as those of Fig. 3, traversed in crossing four different parts of the structure of two control embryos and one embryo blocked from stage 10½ with each of the drugs. It can be seen that the overall ratio of these numbers, generally around two, is again as would be expected from the relative counts of cells (packed into three-dimensional space) in whole embryos. There is indication in the table that the eyecup incorporates relatively more of the available cells in the blocked embryos. This aspect of morphogenesis with lowered cell number has not yet been confirmed or explored in more detail.

In Papers I, II and V (Cooke, 1972a, b, 1973), operations on early gastrulae have been described which lead to regulatory changes in the prospective significance of large areas in these gastrulae, and the final individuation of extra, coordinated sets of structures within them. One of these is the implantation of an extra stage 10 organizer into the stage 10 marginal zone, leading in many cases to the production of a second set of anterior axial structures, fusing smoothly with those due to the host’s field posteriorly and incorporating mainly host cells. The second is the excision of the organizer region itself at stage 10, as in the donors of the previous operation, which can lead to a regulative restoration of head-producing information (and thus to a change of fate in cells around the excision site) and also to the formation of two sets of posterior axial structures. The mechanism underlying the latter effect is not understood, but it again represents a change in the configuration of positional information (Wolpert, 1969, 1971), and thus the assumption of new developmental trajectories by many cells in the embryo.

Both of these operations may be performed on stage 10* gastrulae with qualitatively similar results, so that it became of interest to perform them on embryos in which, simultaneously, the further increase of cell number was blocked with colcemid or mitomycin C. Operation and introduction of drugs were simultaneous, since after some time under colcemid, particularly, the processes of cellular readhesion after wounding were slowed somewhat. However the randomly phased cell cycle, in gastrulae at this time, must be some hours in length (Graham & Morgan, 1966), and healing in of a grafted organizer
Fig. 4. High-power photographs of the surface of control and blocked, living embryos after organizer implantations, showing new pattern formation.

(A) Ventrolateral view of a control implantation, into a stage 10+ host, showing the two cement glands, the host and secondary forebrain fields and the projecting outline of the secondary axis. Note the small surface cell size. ×2.

(B) Lateral view of an implantation into stage 10+ and simultaneous block of mitosis with mitomycin C. The host cement gland and eye are visible in side view, together with a frontal view of secondary cement gland and forebrain caused by an organizer implanted relatively close to the host’s own (75%). The surface cell size can be compared with (A). ×2.

(C) Dorsal and (D) ventrolateral views of an implantation into stage 10+ and simultaneous block of mitosis with mitomycin C. The dual anterior axis and in (D) the large surface cell size can be clearly seen.

takes some 25 min. Thus, consideration of the data of Table 1 leads to the conclusion that at most a very small percentage of the host cells could divide in these experiments after having experienced any changes in the embryonic field, caused by the operations.

Some 15 operations of each type were performed in conjunction with each mitotic inhibitor, in experiments where direct monitoring of cell number was also carried out as a control. Nineteen out of 28 organizer implantations produced apical (= cement-gland and forebrain-inducing) secondary individuation, with patterns and relative sizes similar to those in control, unblocked operations
where 10 out of 17 succeeded from the same egg-batches. Two operations disintegrated under each drug, whilst the remainder showed only small, unclear secondary structures. Fig. 4 shows photographs of a typical control and two mitomycin-C-blocked operations by tail-bud stages. The surface cell-size difference, and some of the accessory structures, are apparent.

Twenty-one out of 30 organizer excisions regulated to produce cement-gland induction, whilst 17 produced more or less profound accessory tail structures, including anus, spinal cord and tail-bud. The control incidence of these two events after operations are about 70% and 40% respectively, and again the spectrum of sizes and shapes of secondary axial parts was comparable.

Histology after organizer implantation confirms, as in control material, that secondary individuations are usually too massive, and normally organized, to have been composed of redistributed and regulated graft material. Grafts are very small relative to the hosts, and vital staining had previously shown them to remain usually compact near the anterior of the secondary axes. In Fig. 5 the section passes through the trunk notochord/somite region of an apically incomplete though large secondary axis induced in a colcemid-blocked embryo. The distinctive histological appearance of differentiated notochord allows the presence of two of these structures to be made out in the photograph. As in other such fixed embryos, no mitotic spindles could be found although many of the nuclei were in typical arrested metaphase configurations (see Discussion).
These results constitute strong evidence that, in the absence of a normal cell cycle and thus of the genesis of new cells by mitosis, cells may proceed from a labile, pluripotent state to one of commitment, and of initial differentiation in behaviour and adhesive properties such as characterizes stage 22 in *Xenopus*. As well as participating in normal morphogenesis, such blocked cells can perceive newly imposed position values in an embryonic field and change their differentiation tendencies accordingly. It is also confirmed that, even where only half the normal number of cells is included along each dimension of a pattern, this may still finally be qualitatively normal, in the sense that all elements of the pattern are represented in cell behaviour, in the right spatial sequence. However normal proportionality, and particularly normal cavitation in cell sheets and tubes, may begin to break down as cell number becomes limiting.

Due to the lack of any reliable direct cell-counting method it is less certain, although probable, that some later histodifferentiated states such as those of early somitic muscle and notochord, can also be attained without intervention of mitosis.

In view of the evidence (e.g. Denis, 1964, and review in Davidson, 1968) for the necessity of some classes of RNA synthesis from the embryonic genome, to support the developmental programme in postgastrular stages of amphibians, the present findings were initially surprising. Particularly in the case of colcemid blockage of mitosis, the arrested metaphase with its extremely condensed chromosomes would not be expected to permit transcription, which is normally correlated with interphasic nuclear states such as are first widely seen at the very late blastula stage. Histological inspection has shown that, although for an hour or so after imposition of colcemid the nuclei in gastrulae all maintain typical arrested metaphase configurations, there is at all stages after this a large (though never near 100 %) incidence of interphasic-type nuclear states, with nucleoli visible. Since no mitotic spindles are seen, however, and the cell-counting data confirm the static cell number, we may have to conclude that some endogenous cycle with respect to chromosome state may run on in these nuclei, even though the formation of spindles and thus cytoplasmic and chromosomal partition are permanently impossible. Mitomycin C is considered not necessarily to affect the status of DNA as an RNA template (Kiehlman, 1966), and nuclei under prolonged block with this drug in the present work retain an interphase-like appearance.

It is important to realize that there is as yet no evidence as to the status of DNA synthesis in nuclei of these embryos, whether mitosis is blocked by colcemid or by mitomycin C. Thus either complete endoreplication of the genome or, in the case of mitomycin C, restricted DNA synthesis may be occurring in the cells at any time, and the presence of an amount of such synthesis, small in
terms of the content of the genome, might be difficult to establish biochemically. The suggestion (e.g. Gurdon, 1969) that a transient breakdown of the nuclear membrane, normally associated with mitosis, is necessary for developmental progression or 'reprogramming' in cell lineages, is also not excluded for the cells in these experimental embryos at the present time.

The present results thus do not refute any theory stating that some particular biochemical phase, normally associated with an ongoing cell cycle, must be traversed to allow any of the aspects of development and change in cells that have been mentioned above (e.g. Lawrence et al. 1973). Such a repeating cycle of events within individual cells may continue, dissociated from mitosis as such which produces new cells. It appears, however, that partition of cytoplasms or membranes, or separation of sets of chromosomes into different nuclei, are not prerequisites for change in developmental status or differentiation potency in the cellular material of amphibian embryos.

I thank Dr Alan Lehmann for help in the search for an antimitotic agent other than colcemid, and non-toxic to these cells over the periods involved.

REFERENCES


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