Changes in the rate of cell divisions in the course of early development of diploid and haploid loach embryos

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The period of development preceding gastrulation can be divided into two stages. The first is characterized by rapid synchronous cell division. True interphase, which is characterized by the fusion of karyomers and the occurrence of a nucleolus, is absent at this stage. During the second stage the rate of cell division decreases and divisions are asynchronous.

The process of cell division is antagonistic to genetic activity of nuclei, as nuclear synthesis of m-RNA appears to cease during mitosis. Consequently, one can suggest that the increase of the length of interphase is necessary for the onset of morphogenetic nuclear function, which ensures gastrulation and subsequent development (Neyfakh, 1959).

The present investigation was designed first to determine exactly the time of the appearance of the changes in the rate of cell division and to compare it with the time of onset of morphogenetic nuclear function.

Secondly, we tried to elucidate the causes of the decrease in the rate of cell division as early development proceeds. We assumed that one of the causes might be the establishment of a new, specific quantitative ratio between the nucleus and cytoplasm in the course of cleavage. The total volume of an embryo is known to be constant during cleavage. Accordingly, cell volume must be halved at each division. As the DNA content per nucleus remains constant (Sze, 1953), the ratio of nuclear DNA to other cell substances continuously increases. One can suggest that rapid cell divisions are possible until this ratio reaches some definite threshold value and subsequent divisions can proceed only after a period of synthesis for which an increase in the duration of interphase is necessary. To check on this suggestion we compared changes in the rates of cell division in haploid and diploid embryos. Haploid embryos have half the quantity of DNA per nucleus as compared with diploid ones, the initial amount of other cell substances being the same. Therefore any given quantita-
tive ratio between nuclear DNA and other cell substances must be achieved in haploid embryos one division later than in diploid ones. If our suggestion concerning the causes of the decrease of the rate of cell division is correct, then this decrease in haploids would occur one division later and result in the increase of the number of cells as compared with diploids, as observed by many authors before gastrulation (Boveri, 1905; Fankhauser, 1938; Rotmann, 1940; Graham, 1966).

MATERIALS AND METHODS

Experiments were carried out on embryos of the loach *Misgurnus fossilis* L. because we know the time of the onset of morphogenetic nuclear function for this species (Neyfakh, 1959), and also the duration of the mitotic cycle during synchronous cell divisions (Ignatieva & Kostomarova, 1966) and changes in the intensity of m-RNA synthesis (Kafiani & Timofeeva, 1964; Timofeeva, Neyfakh & Kafiani, 1967). Mature eggs were obtained 38–40 h (temperature 16–18 °C) after injecting females with chorionic gonadotropic hormone (150–250 rat units). Artificially inseminated eggs were incubated in an ultrathermostat at a temperature of 21.0 ± 0.1 °C. Each developmental stage is referred to by the number of hours of development required to reach it at this temperature. Haploid embryos were obtained by means of irradiation of spermatozoa before fertilization with a heavy dose of X-rays (60–70 kr, 200 kV, 15 mA, no filter).

The duration of the mitotic cycle was estimated from the equation

\[ T = \frac{\log 2 (t_2 - t_1)}{\log N_2 - \log N_1}, \]

where \( T \) is the mean duration of mitotic cycle at time interval \( t_2 - t_1 \), \( N_1 \) and \( N_2 \) the total number of cells of an embryo at time \( t_1 \) and \( t_2 \), respectively. Counts of the total number of cells were made by counting the number of nuclei in histological preparations. For this purpose the eggs were fixed in 3:1 alcohol:acetic acid fixative. The chorion was removed and blastodisks with a part of the yolk were embedded in melted 3% agar. A block of agar with 2–4 blastodisks was embedded in 54°C paraffin. Serial sections were cut at 10 μ and stained with Heidenhain’s, Regaud’s or Delafield’s haematoxylin. Nuclei were counted using a graticule microscope eyepiece. Separate tallies were kept for cells in mitosis (e.g. in prometaphase, metaphase and anaphase). We counted nuclei at each 5th section in embryos up to the age of 7 h and at each 10th section in those of more than 7 h; 10–20 embryos were taken for each stage. On the basis of data obtained the total number of nuclei for a given embryo was calculated. A correction was introduced for fragmentation of nuclei using the method of Marrable (1965). Then we estimated the mean number of nuclei for a given stage, and the mitotic index was calculated as a percentage ratio of the number of cells in mitosis to the total cell number. We considered as mitotic phases only those where individual spiralized chromosomes could be seen, as there is no precise criterion for the identification of interphase during cleavage.
RESULTS

Changes in duration of mitotic cycle

Figure 1 shows the number of cells as a function of age. The y-axis gives the logarithm of the cell number, but x-axis (time) is an arithmetic scale. One can see that between $5\frac{1}{2}$ and 6 h the angle of the slope of the curve for diploid embryos (I) coincides with that of the theoretically calculated step-like line (III). While plotting the latter we considered the duration of the mitotic cycle as equal to 31 min at 21 °C (Ignatieva & Kostomarova, 1966), cell divisions to be synchronous (Neyfakh & Rott, 1958) and the first cleavage division to occur at 75 min after fertilization.

After 6 h, i.e. in the 11th cell generation, the angle of the slope of line I deviates from that of curve III owing to a decrease in the rate of cell divisions in diploid embryos. This decrease can be seen more clearly as an increase in the duration of the mitotic cycle (Table 1) which begins at the 6th h in diploid embryos.
From Fig. 1 one can see that in haploid embryos at early developmental stages cell number is equal to that in diploid ones and coincides with the theoretically expected values. The increase in the duration of the mitotic cycle appears in haploids at 6½ h after fertilization, i.e. one cell generation later than in diploids. For some time thereafter the duration of the mitotic cycle in haploids remains shorter than in diploids of the same age, but at 7½ h after fertilization this ratio is reversed.

Table 1. The duration of the mitotic cycle and its phases (min)
in diploid and haploid loach embryos

<table>
<thead>
<tr>
<th>Time after fertilization (h)</th>
<th>Mitotic cycle</th>
<th>Interphase</th>
<th>Mitosis</th>
<th>Prometaphase</th>
<th>Metaphase</th>
<th>Anaphase</th>
<th>Mitotic cycle</th>
<th>Interphase</th>
<th>Mitosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>6–6½</td>
<td>49:6</td>
<td>34:1</td>
<td>15:5</td>
<td>4:5</td>
<td>7:5</td>
<td>3:3</td>
<td>31:0</td>
<td>17:4</td>
<td>13:6</td>
</tr>
<tr>
<td>6½–7</td>
<td>50:0</td>
<td>35:8</td>
<td>14:2</td>
<td>2:0</td>
<td>5:8</td>
<td>6:3</td>
<td>40:6</td>
<td>25:7</td>
<td>14:9</td>
</tr>
<tr>
<td>7–7½</td>
<td>51:0</td>
<td>36:3</td>
<td>14:7</td>
<td>2:1</td>
<td>5:7</td>
<td>6:9</td>
<td>46:1</td>
<td>33:9</td>
<td>12:2</td>
</tr>
<tr>
<td>7½–8</td>
<td>58:9</td>
<td>41:4</td>
<td>17:5</td>
<td>2:5</td>
<td>8:0</td>
<td>6:5</td>
<td>61:3</td>
<td>45:4</td>
<td>15:9</td>
</tr>
<tr>
<td>8½–9</td>
<td>82:7</td>
<td>65:3</td>
<td>17:4</td>
<td>2:8</td>
<td>7:8</td>
<td>6:8</td>
<td>85:7</td>
<td>67:0</td>
<td>18:7</td>
</tr>
<tr>
<td>9½–10</td>
<td>92:3</td>
<td>80:4</td>
<td>11:9</td>
<td>2:0</td>
<td>5:8</td>
<td>4:4</td>
<td>149:8</td>
<td>124:8</td>
<td>25:0</td>
</tr>
</tbody>
</table>

Changes in mitotic index

To calculate the mitotic index in the period of rapid cell division, when cells divide for the most part synchronously, we fixed embryos in the course of one mitotic cycle, i.e. for 31 min at 5 min intervals. The ratio of the number of cells in mitosis to total number of cells in all the embryos fixed is the mean mitotic index for a given period of development, equal to 43·9 % (Fig. 2). At subsequent stages, when the divisions are asynchronous, we calculated the mitotic index from 15–30 eggs fixed simultaneously (i.e. 5000–15000 cells) for each stage.

From Fig. 2 and Table 1 one can see that the decrease in mitotic index appears in diploids at 6 h and in haploids at 6½ h after fertilization. Thus in both cases this decrease coincides in time with the increase in the duration of the mitotic cycle. After 7 h (at the late blastula stage) there is a small rise in mitotic index which does not correspond to any major morphogenetic process. Figure 2 shows that the graph for haploids is similar to that for diploids, but is shifted to the right by approximately 30 min (i.e. by one mitotic cycle).

Changes in duration of mitosis and of interphase

We thought it would be of interest to follow changes in the duration of mitosis and interphase, which could be calculated from the data obtained. The length of interphase (including prophase and telophase) was estimated by subtracting
the length of mitosis from that of the whole mitotic cycle. The mean duration of mitosis (i.e. prometaphase + metaphase + anaphase) for a given time interval was calculated from the equation $M = T \times \text{m.i.}/100$, where $M$ is the duration of mitosis, m.i. = mean mitotic index (in percentage) for a given time interval, and $T$ is the duration of mitotic cycle. The approximate duration of individual mitotic phases (prometaphase, metaphase and anaphase) was calculated in a similar way. The results obtained are summarized in Table 1. One can see that the increase in the length of mitotic cycle is due mainly to the increase in duration of interphase. The duration of mitosis and of its individual phases remains roughly constant. In the period from 6 to $7\frac{1}{2}$ h the duration of interphase in haploid embryos is less than in diploid ones. During subsequent development this ratio is reversed.

![Fig. 2. Changes in mitotic index in diploid (I) and haploid (II) loach embryos.](image)

**DISCUSSION**

*Changes of the rate of cell division in diploid embryos*

In diploid embryos the rate of cell division remains constant during the first 6 h after fertilization at 21 °C, the duration of the mitotic cycle being 31 min. This result is based on the work of Ignatieva & Kostomarova (1966). They found that the duration of the first three divisions in loach embryos equals approximately 31 min. The data presented in this paper show that the duration of the 10th mitotic cycle (between $5\frac{1}{2}$ and 6 h) is also equal to 31 min. The mean duration of mitotic cycles between the 4th and 9th divisions must also be equal to 31 min. This is proved by the identity of the experimentally determined value for cell number at $5\frac{1}{2}$ h and the theoretically estimated one assuming a cell
doubling time in this period of 31 min. From the 6th h, i.e. from the 10th cell generation, cell division slows down. Thus the decrease in the rate of cell division coincides with the onset of morphogenetic nuclear function, detected in loach embryos at 6 h after fertilization (Neyfakh, 1959). This suggests a causal relation between these two phenomena. In fact, the decrease in the rate of cell division is caused mainly by the increase in the duration of interphase, which is known to be the time when genetic nuclear activity occurs. This decrease in the rate of cell division before gastrulation has been observed by many authors. Sze (1953), studying embryos of *Rana pipiens*, has demonstrated the linear dependence between cell number and age of embryo throughout the period studied, i.e. from the beginning of cleavage up to the tail bud stage; the tangent of the angle of the slope sharply decreases at the mid-late blastula stage from 0-18 to 0-0067, i.e. by 26 times. Thus, the decrease in the rate of cell division in *Rana pipiens* is much more pronounced than in the loach, although this difference may be false as the time intervals between successive counts of cell number in Sze’s work were much longer than in our experiments.

Marrable (1965) found the first decrease in the cleavage rate in *Danio rerio* at the 197th cell stage at 153 min after fertilization at 25-27 °C. Cell doubling time increased from 15-5 to 21 min. It should be noted that the ratio between the time of the first decrease of the rate of cell divisions (2 1/2 h after fertilization) and the onset of gastrulation (4 h) in *Danio*, is approximately the same as in the loach (6 and 10 h respectively).

Graham & Morgan (1966) observed an increase in the length of the mitotic cycle at the transition from mid-late cleavage (st. 7) to early gastrula (st. 9) in the toad *Xenopus laevis*. According to Chulitskaia (1967) the sharp decrease of mitotic index in *Rana temporaria* embryos coincides with the onset of morphogenetic nuclear function. All these data support our suggestion about a close time relation between the decrease in the rate of cell division and the onset of morphogenetic nuclear activity.

*Changes in duration of mitotic phases in diploid embryos*

At the stage of mid-late blastula, i.e. from 5 1/2 h to 10 h after fertilization, the duration of the mitotic cycle in diploid embryos increases from 31 to 97 min. This increase is caused mainly by the prolongation of interphase from 17-4 to 80-4 min. The duration of mitosis and of its various stages remains roughly constant throughout this period. In this respect our data differ from those of Graham & Morgan (1966). They found a 15-fold increase in total duration of metaphase + anaphase, from 2 to 30 min, at mid-late blastula stage (from stage 7 to 9) in *Xenopus laevis*.

For comparing the data on the duration of mitotic phases in embryos of different species of animals in the course of synchronous cleavage divisions Dettlaff (1964) suggested a dimensionless unit $\tau_0$. $\tau_0$ is the duration of one mitotic cycle in the course of synchronous cleavage divisions. Dettlaff (1963) and Skoblina.
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(1965), using this unit, found the durations of corresponding mitotic phases to be very similar in different species of sea urchins, fishes and amphibia. For instance, the duration of prometaphase in embryos of these animals is equal to $0.05-0.10\tau_0$, metaphase to $0.19-0.21\tau_0$ and anaphase to $0.13-0.15\tau_0$. In loach embryos the durations of these phases are equal to $0.07, 0.21$ and $0.15\tau_0$ respectively.

Comparison of changes in the rate of cell division
in diploid and haploid embryos

Comparison of haploid and diploid embryos in early embryogenesis allows us to elucidate the causes of the decrease in the rate of cell division in the period preceding gastrulation.

In haploid loach embryos the period of rapid cell division lasts until 6.30 h i.e. one mitotic cycle later than in diploid embryos. Thus cell divisions slow down in diploid and haploid embryos at the same threshold ratio between quantity of nuclear DNA and other cell substances. This confirms our suggestion about the role of this ratio in the regulation of the rate of cell divisions. There is also the following indirect evidence supporting this suggestion. According to Graham (1966) during the later developmental stages of *Xenopus laevis* embryos cell number in haploids becomes twice as great as in diploids. Consequently, the ratio between nucleus and other cell substances becomes equal in haploid and diploid embryos of the same age. At these stages the duration of the mitotic cycle and interphase are the same in these embryos. Thus the ploidy itself does not play any role in the regulation of the rate of cell division.

Comparison of changes in cell number in diploid
and haploid embryos

Due to the prolongation of the period of rapid cell division cell number in haploid embryos becomes a little higher than in diploids. This results in some compensation of total amount of genetical substance per embryo. Further compensation would be possible if the rate of cell divisions in haploids was higher than in diploids at subsequent stages. However, throughout the period studied (up to the onset of gastrulation), mitotic indices in these embryos change in a similar way. Consequently the ratio of cell number in haploids and diploids remains approximately constant ($1.2-1.6$). Accordingly, the total amount of nuclear DNA in haploids remains less than in diploids ($0.6-0.8$) throughout the period studied.

One can suggest that the deficiency of genetical substance in haploids may be compensated for by intensification of genetic activity of chromosomes. Such a phenomenon, known as a gene dosage compensation, was found by Muller (Muller, Leage & Offerman, 1932) in *Drosophila*. He found that the genetic activity of a single *X*-chromosome in the male is higher than that of either of two *X*-chromosomes in the female. These data are in agreement with those of Mukherjee & Beermann (1965) who observed a higher intensity of m-RNA syn-
thesis in the $X$-chromosome of the *Drosophila* male as compared with that of the female. Investigations are in progress to determine whether such an intensification of m-RNA synthesis occurs in chromosomes of haploid loach embryos as compared with diploid ones.

The results outlined above suggest that insufficient activity of chromosomes is one of the causes of the prolongation of interphase in haploids as compared with diploids, observed from 7.5 h after fertilization. Perhaps in this way an increase of intensity of morphogenetic nuclear function is achieved in haploids at these developmental stages (Neyfakh & Rott, 1967). However, later on, after 8 h, the prolongation of interphase in haploids results in a decrease of the ratio of their cell number to that of diploids (Table 1), and this reinforces the genetic deficiency of haploid embryos.

**SUMMARY**

1. In the course of the first 6 h after fertilization the cells divide in diploid loach embryos at a constant rate. The cell doubling time or mitotic cycle duration is shown to be 31 min. This period is known as the period of rapid cleavage divisions.

2. After 6 h, in the 11th cell generation, the rate of cell division slows down, the duration of the mitotic cycle and interphase increases and the mitotic index decreases. All these changes coincide in time with the onset of morphogenetic nuclear function.

3. The duration of mitosis and that of its individual phases remains roughly constant throughout the period studied. The duration of mitotic phases as a proportion of the length of the whole mitotic cycle in the course of synchronous cell divisions in loach embryos is shown to be similar to that of other animal species.

4. In haploid embryos the decrease in the rate of cell division occurs after 6.5 h, i.e. one cell generation later than in diploids. This suggests that the cause of this decrease may be the establishment of a definite threshold ratio between nucleus and cytoplasm.

5. Owing to the prolongation of the period of rapid cell division, the cell number in haploids becomes greater than in diploids after 6 h. In this way partial compensation for the lower amount of genetic material is achieved in haploids.

6. The paper discusses the possibility of further compensation of genetical substance in haploids by intensification of chromosome activity and by increase of interphase duration.
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Резюме

Изменения скорости клеточных делений в ходе раннего развития диплоидных и гаплоидных зародышей вьюна

1. В течение первых 6 час. после оплодотворения клетки диплоидных зародышей вьюна делятся с постоянной скоростью. Продолжительность клеточного цикла, определенная как время удвоения числа клеток, составляет 31 мин. Это период быстрых делений дробления.

2. После 6 час., т.е. на 11-м клеточном поколении, клеточные деления замедляются, продолжительность митотического цикла и интерфазы увеличивается, и митотический индекс уменьшается. Все эти изменения совпадают по времени с началом морфогенетической функции ядер.

3. Продолжительность митоза и его отдельных фаз остается приблизительно постоянной в течение всего исследованного периода. Показано, что продолжительность отдельных фаз митоза, отнесенная к продолжительности всего митотического цикла в период синхронных делений, у зародышей вьюна близка к таковой для других видов животных.

4. У гаплоидных зародышей уменьшение скорости клеточных делений происходит после 6 час. 30 мин., т.е., на одно клеточное поколение позднее, чем у диплоидов. Можно предположить, что причиной этого уменьшения может быть достижение определенного порогового отношения между ядром и цитоплазмой.

5. Благодаря продлению периода быстрых клеточных делений число клеток у гаплоидов после 6 час. становится больше, чем у диплоидов. Таким образом обеспечивается некоторая, хотя неполная, компенсация генетического материала у гаплоидов.

6. В статье обсуждаются возможные пути дальнейшей компенсации генетического материала у гаплоидов посредством интенсификации активности хромосом и увеличения продолжительности интерфазы.

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