The differentiation capacity of isolated loach (Misgurnus fossilis L.) blastoderm

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Experiments on the capacity of the isolated teleost blastoderm to undergo development when cultivated in vitro have revealed species differences in the time of initiation of blastoderm differentiation. Thus the Fundulus blastoderm acquires the ability to develop after being separated from the yolk at the stage of 32 blastomeres (Oppenheimer, 1936) while the blastoderms of pike and of salmon differentiate only after isolation at the blastula stage (Devillers, 1947). These differences have been interpreted as the result of a different ratio between the volume of the cytoplasm and the yolk (Tung, Chang, & Tung, 1945); the initiation of the capacity of the blastoderm to develop in vitro being interpreted as a result of the passage of some substances required for differentiation from the yolk into the blastoderm (Oppenheimer, 1936). This hypothesis of Oppenheimer was substantiated by other authors (Tung et al. 1945; Tung, Wu & Tung, 1955; Devillers, 1952), although the nature of these hypothetical substances, the time and the mode of their passage from the yolk into the blastoderm is still unknown.

This paper describes work designed to discover the minimum age at which cultured blastoderms of loach (Misgurnus fossilis) could differentiate and to elucidate the factors controlling their capacity to do so.

These factors could either be changes in the properties of the blastoderm cells as development proceeds, or substances in the yolk playing a morphogenetic role. It seems very likely that both factors act together. Consequently a blastoderm that is incapable of differentiating acquires after a certain time the capacity to develop in vitro in the absence of yolk. Changes in the potencies of the loach blastoderm in the course of its development in vitro during early embryogenesis and the influence of the culture media were also studied. The blastoderms were explanted at successive stages of cleavage, blastulation and gastrulation.

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The eggs were obtained from loach females after the injection of gonadotrophic hormone choriogonin according to Neyfakh (1959) (G. Richter, Budapest, Hungary). After the injection of choriogonin solution in balanced solution or distilled water, fertilized eggs were incubated at 21.5 °C. The blastoderm was isolated from the yolk by the method of Kostomarova & Neyfakh (1964). The procedure is as follows. A batch of eggs from one female is placed into 1 % trypsin solution made up in phosphate buffer (pH 7.4–7.5), where the egg shells dissolve in several minutes. Trypsin solution with the remains of shells is washed from the eggs with double-strength Holtfreter’s solution (isotonic for the loach eggs). After that the eggs are put into test-tubes with double-layered sucrose solution. The lower layer is 14 ml of 0.75 M, while the upper one is 8 ml of 0.5 M. In such density gradient the eggs sink down to the border between the layers. Then they are centrifuged at 6000 g for 2–3 min. Such acceleration is sufficient to isolate the blastoderm from the yolk. Isolated blastoderms float to the upper layer of sucrose while the denser yolk precipitates to the bottom of the test-tube. The isolates are transferred by pipette into Petri dishes and washed free of sucrose with Holtfreter’s solution.

It should be specially mentioned that the range of optimal temperatures for loach eggs is from 13–14 to 20–21 °C (Ignatieva & Kostomarova, 1966) so that all the reagents and media applied for the blastoderm cultivation should be kept at corresponding temperatures.

This procedure for the isolation of loach blastoderms from the yolk has some advantages when compared with the surgical method of blastoderm isolation of telolecithal eggs applied in experimental embryology (Oppenheimer, 1936, 1947, 1963; Devillers, 1947, 1961; Spratt, 1947, 1950, 1958; Spratt & Hermann, 1962; Trinkaus, 1953; Trinkaus & Drake, 1956; Tung et al. 1945; Tung et al. 1955, and others). Centrifugation in the sucrose gradient provides a large amount of material at the same stage, while applying the surgical method of isolation produces a relatively small amount of experimental material heterogenous in age. To test our method: (1) blastoderms were isolated surgically and cultivated in the same media as the blastoderms obtained by centrifugation and no differences in their differentiation were observed; (2) the action of centrifugation upon eggs in shells was studied. The eggs in shells were centrifuged at 6000 g for 2–3 min. in the same density gradient. It turned out that such an exposure increased the percentage of abnormal embryos (by 8–10 % at hatching) but 73–83 % of the hatched embryos were normal.

The isolated blastoderms were cultivated in Niu–Twitty’s solution modified by Steinberg (Steinberg, 1956) and in nutrient media: medium 199 by Morgan, Morton and Parker (Paul, 1960), and medium 199 with the addition of 2 % bovine serum. The pH of the culture media was 7.2–7.6. Penicillin and streptomycin (no more than 50 γ per ml of solution) were added to the media. To make
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them isotonic, the salt solution contained a double concentration of Na, K and Ca (compared to the initial one), while the medium 199 was two-fold diluted.

The blastoderms were isolated from the yolk at successive stages of cleavage, blastulation and gastrulation. It is extremely difficult to obtain isolates at early cleavage stages (8, 16, 32 blastomeres), since in the loach the first latitudinal furrow is formed at the stage of 32 blastomeres so that before this developmental stage the entire blastoderm is connected to the yolk. That is why the number of isolates obtained up to the stage of 32–64 blastomeres was no more than 10 successful ones per stage. Aseptic precautions were observed throughout. The time of cultivation was 2–3 days. Normally developing embryos of the same egg batches served as controls. The general appearance of the isolates was drawn with the aid of a drawing device. The objects were fixed in Bouin’s fluid, embedded in paraffin wax by the routine procedure (or passed through methyl benzoate). Sections of 5–6 μ thick were stained by the Azan–Heidenhein method. Developmental stages of the loach expressed in hours of development after fertilization are given at the temperature 21.5 °C.

RESULTS

1. The development in vitro of the loach blastoderm isolated from the yolk at the stages of cleavage and blastulation (Fig. 1, A–E).

1. Development of the blastoderm isolated from the yolk at the stages of cleavage and early blastulation (3–6 h of development). (Fig. 1, A–C).

One day of cultivation

(a) Steinberg’s solution. Blastoderms isolated from the yolk at the cleavage stages, starting from the stage of eight blastomeres up to the mid-blastula (6.5–7.5 h of development) undergo cleavage during several hours. After some hours of cleavage in vitro the explants acquire a blastula-like structure. Sometimes an eccentrically located cavity where the blastomeres are loosely arranged may be seen in explants. The outer layer of such explants is made of flattened blastomeres. The inner layers of explants are formed of blastomeres of homogenous structure. When the isolates attain the blastula-like stage of development, mitoses in them cease to occur, the blastomeres begin to fuse with each other; after that the isolates perish. In all experiments on the cultivation of the early blastoderm of the loach (up to the mid-blastula stage) no differentiation could be found in such isolates (Fig. 2 A, B; Fig. 3 A, B).

(b) Medium 199. When the isolates are cultivated in medium 199 similar pictures are observed on the first day. On the second day of incubation the explants perish in both the Steinberg’s medium and medium 199.

2. Development of the blastoderm isolated from the yolk at the stages of mid- and late-blastula (7–9 h of development) (Fig. 1 D, E).
Fig. 1. Stages of normal development of the loach (*Misgurnus fossilis L.*) at 21.5°C. A–C. Stages of cleavage and early blastulation (1–6h of development). D. Mid-blastula (7h of development). E. Late blastula (9h of development). F. Early gastrula (11h of development). G. Mid-gastrula (15h of development). H. Late gastrula (18h of development).
One day of cultivation

(a) Steinberg's solution. When cultivated in salt solution the embryos become elongated or vermiform. The ectoderm, mesoderm and endoderm differentiate. In the ectoderm the neural cord is induced; notochord is formed as a more or less regular form or as separate clusters of chordal tissue.

(b) Medium 199. Atypical head, trunk and tail regions appear in the embryos. Ectoderm, endoderm, notochord and the neural cord differentiate.

Fig. 2. A schematic representation of loach blastoderms isolated from the yolk at early blastula stage. Cultivation in Steinberg's salt solution (A) and medium 199 (B).
Control embryos have 23–25 pairs of trunk somites. Eye vesicles are transformed to eye cups; a rudiment of the heart appears.

Two days of cultivation

(a) Steinberg's solution. The embryos remain at the level of differentiation described above. Cells begin to migrate from the isolate surface. (Fig. 3 C).

(b) Medium 199. Explants develop further. Up to 11 pairs of somites are formed in the trunk mesoderm. Notochord cells undergo vacuolization. In the most advanced embryos atypical eye vesicles, mostly cyclopic ones, are formed. The embryos are able to perform irregular contractions of a quivering type (Fig. 4; Fig. 3 D–F).

In the control embryos the trunk mesoderm is fully segmented. The tail mesoderm has 16 pairs of somites. The tail is separated from the yolk.

Three days of cultivation

(a) Steinberg's solution. The embryos begin to perish.

(b) Medium 199. The cells start migration from the surface. The notochord cells undergo further vacuolization.

Fig. 4. A schematic representation of loach blastoderms isolated from the yolk at late blastula stage. Cultivation in the medium 199 for 48 h.
Control embryos hatch.

As follows from the data in Table 1, the initiation of the capacity of the isolated loach blastoderm to differentiate occurs at the mid-blastula stage. From this moment on, up to the end of the late blastula, the number of differentiated embryos rapidly increased. As can be seen from Table 1 and Fig. 5, the

Table 1. Change in the number of differentiated loach embryos after isolation of the blastoderm at the stages of cleavage and blastulation

<table>
<thead>
<tr>
<th>Development stage (h)*</th>
<th>Steinberg’s solution</th>
<th>Medium 199</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total no. of isolates</td>
<td>Differentiated</td>
</tr>
<tr>
<td>5</td>
<td>53</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>93</td>
<td>29</td>
</tr>
<tr>
<td>8</td>
<td>37</td>
<td>15</td>
</tr>
<tr>
<td>9</td>
<td>421</td>
<td>321</td>
</tr>
</tbody>
</table>

*Stages expressed in hours of the development after fertilization are given for $t = 21\text{-}5°C$.
†Equivocal cases are included in the column ‘undifferentiated’.

Fig. 5. The dependence of differentiation in loach embryos on the stage of isolation from the yolk. Solid line = % of differentiated embryos in Steinberg’s salt solution, broken line = in medium 199.
Table 2.* The development in vitro of loach embryos in different culture media after their isolation from the yolk during gastrulation

<table>
<thead>
<tr>
<th>Stage of isolation of blastoderm from yolk (h of development after fertilization at 21.5°C)</th>
<th>% of differentiated isolates</th>
<th>Number of pairs of somites</th>
<th>Eye rudiment</th>
<th>Notochord cell vacuolization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steinberg's salt Medium / Medium 199 + bovine salt / Medium 199 + bovine serum / Medium 199 + bovine serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A Early gastrula (10-13h) 73 92 85 (First pair of somites appears in single isolates) 6-11 6-11 Absent, cyclopic in some cases Paired eye cups with lenses Non-vacuolized Strongly vacuolized Strongly vacuolized</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Mid-gastrula (14-16h) 36 88 90 8-17 21 27 Eye cups Eye cups with lenses Eye cups with lenses Strongly vacuolized Completed Completed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Late-gastrula (17-19h) Practically all the isolates differentiated The trunk and tail mesoderm segmented 30 30 Eye cups Eye cups with lenses Eye cups with lenses Strongly vacuolized Completed Completed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The time of cultivation was 3 days.
cultivation of the isolates in nutritive culture medium did not shift the onset of blastoderm differentiation to earlier developmental stages when compared with cultivation in salt solution. Therefore, the stage at which the loach blastoderm becomes able to undergo development, is internally determined and does not depend on the culture medium. The nature of the medium affects the percentage of differentiated explants obtained at more advanced developmental stages.

Fig. 6. A schematic representation of loach embryos isolated from the yolk at early gastrula stage. Cultivation in Steinberg's salt solution for 24 h (A, B) and in medium 199 (C).

**II. The development in vitro of the loach blastoderm isolated from the yolk at gastrulation stages. (Table 2)**

1. Cultivation of the blastoderm isolated from the yolk at the stages of transition to gastrulation and early gastrula (10–13 h of normal development) (Fig. 1F).
One day of cultivation

(a) Steinberg's solution. Of 493 cultivated isolates 358 differentiated. When cultivated in salt solution, the embryos lose their spherical form and become elongated. Atypical head, trunk and tail regions are formed in the embryos. Ectoderm, endoderm and mesoderm are developed, the neural cord, notochord or separated clusters of chordal tissue are differentiated. In some cases the first trunk mesoderm somites are formed. (Fig. 6A).

(b) Medium 199. Of 177 isolates 163 differentiated.
During the first day of cultivation atypical head, trunk and tail regions form in the embryos; the embryos also form ectoderm, endoderm, mesoderm and Anlagen of axial organs. In some embryos the trunk mesoderm segments (up to six pairs of somites).

(c) Medium 199 with bovine serum. Of 214 isolates 183 differentiated.
The form of the embryos changes as the head, trunk and tail regions develop and axial organs differentiate. The trunk mesoderm segments: up to six pairs of somites can be seen.
In control embryos 23–25 pairs of somites can be seen. The segmented mesoderm begins to contract. Eye vesicles transform to eye cups; lens placodes are formed.

Two days of cultivation

(a) Steinberg’s solution. The embryos remain at the stage of differentiation described. Cells begin to migrate from the surface of the embryos.

(b) Medium 199. The embryos develop further: the trunk mesoderm segments (up to 11 pairs of somites). The embryos perform contractile movements; the cells of the notochord are vacuolized; encephalomers appear; eye rudiments and ear vesicles can be distinguished. In some embryos atypically formed cyclopic eyes can be distinguished.

(c) Medium 199 with bovine serum. The embryos differentiate further (the average number of trunk somites is 8–10 pairs). The brain cavity is well developed. The paired eye rudiments with lens placodes and ear vesicles appear. Notochord cells are strongly vacuolized.

In control embryos the trunk mesoderm is completely segmented. Up to 16 somites are found in the tail mesoderm. The Kupffer vesicle is present.

Three days of cultivation

(a) Steinberg's solution. The embryos begin to perish.

(b) Medium 199. The embryos remain at the stage of differentiation described. Cells begin to migrate from the surface of the embryos.

(c) Medium 199 with bovine serum. The embryos differentiate further than in the preceding day of cultivation. The paired eye Anlagen are at the stage of the formed eye cup accompanied by lenses. The notochord cells differentiate further (stronger vacuolization, yolk granules disappear, the nucleus shifts to
Fig. 7. (A). Unicellular epithelial glands in the external layer of the epithelium of loach embryo isolated from the yolk at early gastrula stage. Cultivation in medium 199 with bovine serum for about 70 h (× 400). (B) Tangential section through the head part of loach embryo isolated from the yolk at mid-gastrula stage. Cultivation in medium 199 with bovine serum for about 70 h (× 400). 1 = unicellular epithelial glands, 2 = eye cup with lens, 3 = brain, 4 = ear vesicle. (C, D.) Longitudinal sections through the trunk parts of loach embryos isolated from the yolk at mid-gastrula stage. Cultivation in medium 199 with bovine serum for 24 h after preliminary exposure to Steinberg’s solution (C) or medium 199 with bovine serum (D). Vacuolization of the notochord cells incomplete (C), vacuolization of the notochord cells completed (D). (× 400).
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the basal portion of the cell). The connective tissue sheath of the notochord differentiates. In the epithelium unicellular mucous glands are formed. (Fig. 7A).

At this stage cell migration begins in the majority of explants.
All the control embryos hatch.

2. Cultivation of the blastoderm isolated from the yolk at the mid-gastrula stage (14–16 h of normal development). (Fig. 1G).

![Fig. 8. A schematic representation of loach embryos isolated from the yolk at mid-gastrula stage. Cultivation in Steinberg’s salt solution for 24 h.](image)

One day of cultivation

(a) Steinberg’s solution. Of 126 cultivated isolates 46 differentiated.
The head, trunk and tail parts are formed in embryos. The trunk mesoderm segments: 8–17 pairs of trunk somites on average. The embryos perform contractile movements. They differentiate axial organs and eye rudiments at the stage of eye cups. The notochord cells are strongly vacuolized.

(b) Medium 199. Of 162 cultivated isolates 144 differentiated.
The embryonic body is divided into the head, trunk and tail parts. Eye rudiments are present, ear vesicles appear. The trunk mesoderm segments: 9–17 pairs of somites can be found on the average (Fig. 8).

Axial organs are formed, the brain encephalomeres develop.

(c) Medium 199 with bovine serum. Of 80 cultivated explants 72 differentiated. The level of differentiation is similar to that attained by the embryos cultivated in medium 199. The head, trunk and tail parts develop. The trunk mesoderm has 21 pairs of somites of regular form. There are eye rudiments and ear vesicles. (Fig. 9A). The notochord cells are strongly vacuolized, chondrogenic
substance is deposited in the vacuoles. A rudiment of the gut is present in the form of a dense epithelial cord without cavity. Control embryos are at the prehatching stage.

Fig. 9. A schematic representation of loach embryos isolated from the yolk at mid-gastrula stage. (A) cultivation in medium 199 with bovine serum for 24 h. (B–C) Cultivation in medium 199 (B) and medium 199 with bovine serum (C) for 48 h. (D) cultivation in medium 199 with bovine serum for about 70 h.

Two days of cultivation

(a) Steinberg’s solution. The embryos remain at the developmental level described above.
(b) Medium 199. The embryos develop further. The number of trunk somites reaches 21 pairs (Fig. 9 B, C). The embryos are actively motile. The tail part of the mesoderm segments: up to six pairs of somites. In the notochord cells vacuolization is complete. Paired olfactory placodes, ear vesicles and eye cups with lens Anlagen develop. Many unicellular epithelial glands appear in the epithelium.

At this stage the migration of cells from the surface of embryos begins.
Differentiation of isolated blastoderm

(c) Medium 199 with bovine serum. The embryos develop like embryos cultivated in medium 199. The trunk and tail parts of the mesoderm segment (up to 27 pairs of somites). Olfactory placodes, eye rudiments and ear vesicles are present. The notochord cells are strongly vacuolized.

All the control embryos hatch.

*Three days of cultivation*

(a) Steinberg’s solution. The isolates begin to perish.

(b) Medium 199 and medium 199 with bovine serum. The level of differentiation of the embryos cultivated in medium 199 with serum is similar to that of embryos cultivated in medium 199. Up to 32 pairs of somites can be distinguished in the embryo (Fig. 9D). The tail part of the mesoderm segments. Areas of the unpaired fin fold develop. All parts of the brain form. The cavity appears in the rudiment of the gut. Vacuolization is completed in the notochord cells. Eyes are of regular form, with lens, and they differ little in their development and size from those of normal (control) embryos (Fig. 7B). There are many hatching glands in the head epithelium of the embryos; in the gland cells clusters of secretory granules are seen.

3. Cultivation of the blastoderm isolated from the yolk at the stage of the late gastrula (17–19 h of normal development). (Fig. 1H).

*One day of cultivation*

(a) Steinberg’s solution. The level of differentiation of the embryos is similar to that of the embryos isolated from the yolk at the stage of 15–16 h. The embryos divide into the head, trunk and tail parts. Axial organs form, the brain parts differentiate. The mesoderm segments in the trunk and tail parts. Eyes and ear vesicles are present. Notochord cells are vacuolized to a considerable extent.

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Fig. 10. A schematic representation of loach embryos isolated from the yolk at late gastrula stage. Cultivation in medium 199 and medium 199 with bovine serum for 24h.
(b) Medium 199 and medium 199 with bovine serum. The embryos in these culture media attain later stages than those cultivated in Steinberg’s solution. The number of somites increases to 30 (Fig. 10). The embryos have a well-developed brain and neural tube with cavity. The lenses are formed in the eyes; ear vesicles and olfactory placodes differentiate.

Control embryos are at the prehatching stage.

Since the isolates were cultivated until the hatching of the controls which occurs after two days of cultivation, no further cultivation of the explants was carried out.

As follows from the data summarized in Table 2, when cultivated in Steinberg’s solution embryos isolated from the yolk at early gastrula stages develop atypical head, trunk and tail parts, ectoderm, mesoderm and Anlagen of axial organs. Such embryos are unable to develop further. When isolated at mid and late gastrula stages differentiation of the isolates is considerably advanced. The trunk mesoderm segments (and after isolation at the late gastrula stages the tail mesoderm also segments), eye rudiments and ear vesicles appear, brain parts (encephalomes) differentiate, the embryos acquire the capacity to perform contractile movements.

When cultivated in nutritive media (medium 199 and medium 199 with serum) the level of differentiation of the isolates was rather similar in both media. The embryos attain a higher level of differentiation than those cultivated in Steinberg’s solution. When isolated at early gastrula stages the embryos develop head, trunk and tail parts. The trunk mesoderm segments, and when isolated at the mid-gastrula and late gastrula stages the tail mesoderm also segments. Sense organs, brain encephalomes, Anlage of the gut, areas of the unpaired fin fold develop. In the epithelium of the embryos many hatching glands and unicellular secretory glands appear.

III. Passage of nutritive substances into the isolated blastoderm

In studying the role of culture media particular attention should be given to the problem of the extent and the time during which the embryos isolated from the yolk are permeable to nutritive substances. It is suggested that the main passage of substances occurs into the isolated blastoderm through its coat-free surface. It seems that the exchange between the blastoderm and the culture medium proceeds through the cell layer bordering the yolk at least where the free edges of blastoderm do not close.

To check this hypothesis the following experiment was performed. At the stage of 14 h of development (mid-gastrula) some isolates were placed into Steinberg’s solution until the edges of the wound closed; then they were put into nutritive solution: medium 199 with serum. Other isolates were transferred from the nutritive medium into Steinberg’s solution after closure of the wound. Isolates placed into salt or nutritive solutions without changing media served as controls. The time of cultivation in all experiments was 24 h.
Differentiation of isolated blastoderm

The experimental results obtained are presented in Table 3; this table summarizes the criteria of level of differentiation, namely, the number of pairs of somites and the extent of vacuolization of notochord cells (See also Fig. 7C, D). These criteria are good indices of the difference in the structure of the isolates. As may be seen from Table 3, the isolates can be divided by the level of their development into two groups, I–II and III–IV. The isolates of the III and IV groups attained more advanced developmental stages than those of the I and II groups. It follows from the data presented that the passage of nutritive substances into the blastoderm occurs mainly during the time when the surface of the blastoderm remains open (nearly 8–9 h) and allows nutritive substances to enter through the naked surface from the surrounding medium.

Table 3. Effect of the changes of the culture media upon the development in vitro of isolated loach blastoderms

<table>
<thead>
<tr>
<th>Level of differentiation of the isolates</th>
<th>Steinberg's solution (control)</th>
<th>Medium 199 with serum after preliminary exposure to Steinberg's solution</th>
<th>Medium 199 with serum (control)</th>
<th>Steinberg's solution after preliminary exposure to medium 199 with serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of embryos in the experiment</td>
<td>37</td>
<td>27</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>No. of mesoderm somites</td>
<td>6 pairs</td>
<td>4–5 pairs</td>
<td>20 pairs</td>
<td>17–20 pairs</td>
</tr>
<tr>
<td>Vacuolization of notochord cells</td>
<td>Incomplete</td>
<td>Incomplete</td>
<td>Completed</td>
<td>Completed</td>
</tr>
</tbody>
</table>

DISCUSSION

The investigation was chiefly aimed at the elucidation of (1) the time in loach embryogenesis when the blastoderm acquires the capacity to differentiate in vitro, and (2) the factors related to the initiation of the process. As follows from the data, the initiation of the capacity of the loach blastoderm to differentiate occurs at the mid-blastula stages (6·5–7·5 h) of development at 21·5 °C. The beginning of this process coincides in time with the onset of morphogenetic activity of the nuclei of loach blastoderm (Neyfakh, 1959, 1961, 1964, 1965). The biochemical nature of this event was studied in several works (Spirin, Belitsina & Aitkhozhin, 1965; Kafiani & Timofeeva, 1964; Timofeeva, Kafiani & Neyfakh, 1967).

Primary embryonic differentiation is known to be related to the passage of m-RNA into the cytoplasm and to the subsequent protein synthesis on ribosomes. Kafiani & Timofeeva (1964, 1965), Timofeeva et al. (1967),
Kafiani et al. (1969) succeeded in revealing two periods of d-RNA (including m-RNA) synthesis in the nuclei of the loach blastoderm, differing in their intensity. The intensity of the synthesis was low at the stages of synchronous cleavage (up to 6 h of development), after which a drastic increase of synthesis was observed. All (or almost all) RNA produced by the nuclei at the period of the onset of morphogenetic activity was shown to be d-RNA; therefore, activation of the genetic nuclear apparatus corresponds to the beginning of the morphogenetic function of nuclei. According to the data obtained by Spirin et al. (1965) m-RNA in loach blastoderms is synthesized during this period in the form of 'informosomes'. Thus it can be suggested that intensification of the synthesis of m-RNA in blastoderm nuclei is the molecular basis of the morphogenetic function of nuclei and the initiation of the capacity of the blastoderm cells to differentiate in vitro is related to the activation of the genetic apparatus of nuclei. However, an intensification of m-RNA synthesis itself does not account for the fact that loach blastoderm isolated from the yolk at stages earlier than mid-blastula is unable to develop in vitro, i.e. to synthesize new proteins.

In connexion with this problem it would be appropriate to recall the Oppenheimer hypothesis (1936), according to which gastrulation and the appearance of embryonic structures in isolated blastoderms of Fundulus required penetration of some hypothetical substances from the yolk into the embryo. The yolk of teleosts seems to play a morphogenetic role as well as a nutritive one. This hypothesis was indirectly supported by Tung et al. (1955); they succeeded in obtaining by centrifugation just after fertilization 41% of differentiated embryos after isolation of the animal part of centrifuged eggs from the yolk. But without centrifugation animal parts isolated immediately after fertilization did not differentiate. Centrifugation seems to displace morphogenetic elements of the yolk towards the animal pole of the eggs. Devillers (1952) combined the salmon blastoderm from the morula stage with the yolk of the gastrula. In such artificially combined embryos epiboly proceeded normally but no differentiation occurred; at the stage of semi-epiboly the whole embryo formed only a deficient neural plate. This suggests that the yolk of the late gastrula of the salmon already lacks some formative substances required for the normal development of the embryo. Thus the early isolation of the blastoderm from the yolk, despite the activation of the nuclear genetic apparatus and the transfer of information to the cytoplasm, prevents the morphological expression of nuclear function.

It follows from our experiments and from those of other authors that the cultivation of isolated blastoderms of telolecithal eggs in nutritive media advances differentiation (Trinkaus & Drake, 1956; Spratt, 1958). We have shown that cultivation of isolated loach blastoderm in medium 199 and medium 199 with bovine serum advances its differentiation and increases survival of the isolates. However, when the blastoderm is isolated from the yolk at stages earlier than the mid-blastula differentiation can not be initiated. Hence, the stage at which the loach blastoderm becomes capable of developing in vitro depends upon the
Differentiation of isolated blastoderm blastoderm connexion to the yolk so that it is impossible to shift this stage by means of a culture medium. It is evident that the culture media fail to supply some yolk constituents of morphogenetic importance. Therefore, the initiation of loach blastoderm differentiation requires, apart from the morphogenetic function of nuclei as expressed in an intensification of the m-RNA synthesis, a contact with the yolk.

A study of the dynamics of ribosomal RNA in the loach embryogenesis carried out by Aitkhozhin, Belitsina & Spirin (1964) demonstrated that despite the constant level of the ribosomal RNA per egg, its concentration in the isolated blastoderms increased up to the stage of late blastula (9 h of development) remaining constant thereafter. Electron micrographs of sections through the basal region of cleaving blastomeres and the periblast at stages when the level of ribosomal RNA increases show that they are filled with ribosomes which seem to pass into the blastoderm from the yolk. Thus as a result of the passage of ready-made ribosomes from the yolk into the blastoderm the protein synthesis preceding morphological differentiation is made possible (Kostomarova, 1965). These ribosomes are reprogrammed later on by the new m-RNA (Spirin et al. 1965) which was shown to be synthesized by the maternal nucleus of the oocyte during amphibian oogenesis (Brown, 1964; Gurdon & Brown, 1965). So yolk contributes ribosomes to the developing embryo. The question as to how the ribosomes pass from the yolk into the embryo remains open. We think that this problem can be explained from the data of Svetlov, Bystrov & Korsakova (1962), who found a complicated cytoplasmic network penetrating the whole of the yolk in loach eggs. This network is formed by projections from the basal parts of the blastomeres. Time-lapse filming during early developmental stages reveals a sustained flow of cytoplasm from the vegetative to the animal pole. By means of this flow both the nutritive material of the yolk and its morphogenetic elements in form of ribosomes seem to be transported to the embryonic disc of the loach. After the formation of the periblast yolk particles are added to the blastomeres by periblast mitosis, as was shown in Brachydanio rerio (Thomas, 1968). Cytoplasmic projections extending from the periblast (and from the basal region of cleavage blastomeres prior to formation of the periblast) were also revealed in Fundulus (Lentz & Trinkaus, 1967).

In conclusion a schematic representation of the initiation of differentiation in the isolated loach blastoderm can be suggested. At the stage of mid-blastula the activation of m-RNA synthesis and therefore that of the genetic apparatus of the blastoderm nuclei takes place. The output of information coincides in time with the passage of ready-made ribosomes from the yolk to the embryo. Genetic information is expressed in the form of morphological differentiation in the course of blastoderm cultivation in vitro from the stage of mid-blastula. This scheme based on embryological and biochemical data could be applied to a study of the mechanisms determining the onset of blastoderm differentiation in other teleosts.
A discussion of the results concerning the nature and time of the passage of substances from culture media into the isolated blastoderm and the role of the culture media will be given briefly. The main conclusion to be drawn from this part of the work is that a nutritive culture medium seems to be an adequate substitute for the nutritive components of the yolk but lacks a morphogenetic component. Cultivation of isolated blastoderms in nutritive media allows further differentiation of the explants, an increase in the percentage of differentiated isolates, and longer survival in culture. These data are in good agreement with the data of other authors (Trinkaus & Drake, 1956; Spratt, 1947, 1950, 1958, and others), and do not need to be discussed in detail. On the other hand, the problem of how and when the nutritive substances pass from the culture media into the blastoderm can be explained by comparing our results with those of other authors. It was shown that nutritive substances enter the embryo chiefly during the time when the surface of the cells normally adjoining the yolk have not healed over. Substances of the surrounding medium are known to penetrate into intact embryos either after an artificial change of the permeability of the surface coat (Loeffler & Johnston, 1964), or when parts of the embryo are cultivated with the blastocoel surface open (Chibon, 1962). These data obtained with the aid of [3H]thymidine in amphibian embryos are in a good agreement with the results obtained in the loach, since the coat of both amphibians and fishes is impermeable to most constituents of culture media.

While discussing the data obtained it would be of great interest to compare the role of m-RNA in teleost and amphibian eggs and to discuss also the morphogenetic role of intracellular yolk in amphibian embryos. But in view of the enormous amount of factual material concerned with this problem we point only to comprehensive reviews by Brachet (1965, 1966) and publications by Karasaki (1959) and Flickinger (1957, 1961) devoted to these questions.

**SUMMARY**

1. The development *in vitro* of loach blastoderms (*Misgurnus fossilis*) isolated from the yolk at successive stages of cleavage, blastulation and gastrulation was studied. The culture media were Niu-Twitty's salt solution modified by Steinberg, medium 199 and medium 199 with bovine serum.

2. The loach blastoderms were isolated from the yolk by means of centrifugation of shell-free eggs in double-layered sucrose density gradient at 6000 g for 2–3 min.

3. It was shown that loach blastoderms isolated from the yolk starting from the stage of 8 blastomeres up to the mid-blastula undergo cleavage in salt solution and in the nutritive media but are unable to differentiate in salt solution or in nutritive media.

4. Beginning from the mid-blastula stage (6.5–7.5 h of development) the
isolated blastoderms acquire the capacity to differentiate when cultivated in salt solution or in nutritive media.

5. When isolated from the yolk at early gastrula stages after cultivation in Steinberg's salt solution the level of differentiation of blastoderms was as follows: atypical head, trunk and tail regions were formed; ectoderm, endoderm and mesoderm and Anlagen of axial organs developed. In some cases the segmentation of trunk mesoderm began. When isolated at mid and late gastrula stages the level of differentiation of the isolates increased considerably, the trunk mesoderm segmented (and at late gastrula stages the tail mesoderm segmented as well) and Anlagen of sense organs developed.

6. Cultivation in nutritive media (medium 199 and medium 199 with bovine serum) promoted the differentiation of blastoderms isolated from the yolk at gastrula stages.

7. Substances contained in the culture media enter the explants chiefly during the time when the surface of the blastoderm cells normally adjoining the yolk is exposed to the medium.

8. A schematic representation of the initiation of isolated loach blastoderms to differentiation in vitro is suggested. At the stage of mid-blastula the synthesis of m-RNA intensifies and coincides with a passage of ready-made ribosomes from the yolk to the blastoderm through the channel of a cytoplasmic network connected to the blastomeres and the periblast. These events probably initiate new synthesis of proteins leading to the beginning of differentiation of the isolated blastoderm.

Резюме

Развитие изолированной бластодермы вьюна (Misgurnus fossilis L.)

1. Изучалось развитие in vitro бластодермы вьюна (Misgurnus fossilis L.), изолированной от желтка на последовательных стадиях дробления, бластулы и гастркулации. Использовался приготовленный солевой раствор Нью-Твитти в модификации Стейнберга; среда 199 и среда 199 с добавлением бовиной сыворотки.

2. Бластодермы вьюна отдаляли от желтка методом центрифугирования яиц, с предварительно удаленной оболочкой, в двухслойном градиенте сахарозы при 6000 г в течение 2–3 мин.

3. Показано, что бластодермы вьюна, изолированные от желтка на стадиях от 8 бластомер до средней бластулы, способны к дроблению в солевом растворе и питательных средах, но не способны к дифференцировке.

4. Начиная со стадии средней бластулы (6,5–7,5 часов развития), изолированные бластодермы приобретают способность к дифференцировке, как в солевом растворе, так и в питательных средах.

5. После изоляции от желтка на стадии ранней гастркулы при культивации в солевом растворе бластодермы достигают следующего уровня развития: у них образуются атипичные головной, туловищный и хвостовой отделы, формируются эктодерма, мезодерма и энтодерма и зачатки осевых органов. В единичных случаях начинается сегментация туловищной мезодермы. При изоляции на стадиях средней и поздней бластулы уровень дифференцировки изолятов
существенно повышается: сегментируется тулowiщенная мезодерма (а при изоляции бластодермы на стадиях поздней гаструлы и хвостовая), формируются зачатки органов чувств.

6. При культивации в питательных средах (среде 199 и среде 199 с добавлением бычьей сыворотки) бластодермы, изолированные на стадиях гаструляции, дифференцируются до более продвинутых стадий развития, чем при культивации в солевом растворе.

7. Питательные вещества, содержащиеся в питательных средах, проникают в изолят преимущественно в течение того времени, когда поверхность клеток бластодермы, граничащих с желтком, остается открытой (до смыкания краев яиц).

8. Была предложена следующая схема объяснения появления у изолированной бластодермы вновь способности к дифференцировке in vitro. На стадии средней бластулы в ядрах клеток бластодермы происходит резкая интенсификация синтеза iРНК, что совпадает по времени с переходом из желтка в бластодерму готовых рибосом по каналам цитоплазматической сети периплазма. В результате этих событий, вероятно, создаются необходимые условия для осуществления синтетических процессов, ведущих к дифференцировке изолированной бластодермы.

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REFERENCES


Differentiation of isolated blastoderm


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