Artificial metaplasia of pigmented epithelium into retina in tadpoles and adult frogs

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SUMMARY

The present work was aimed at investigating the possibility and conditions necessary for the artificial transformation of one tissue into another. Experiments were carried out with the pigmented epithelium of the eye in tadpoles and adult frogs of Rana temporaria. Following the removal of the mesenchyme envelopes (or their exfoliation during the experiment), pigmented epithelium transformed into retina under the influence of retina from tadpoles of the same species. This phenomenon was observed both under the cultivation of a piece of retina in a sandwich of pigmented epithelium and the transplantation of pigmented epithelium layers into the eye cavity of tadpoles. Such transformation did not occur in the absence of retinal influence. Metaplasia requires the removal of the mesenchyme envelopes, the action of the retinal agent, as well as preservation of the integrity of the pigmented epithelium layer and subsequent proliferation of its cells. The character of general control mechanisms both maintaining the stability of cell types and leading to their transformation into other cell types is discussed.

INTRODUCTION

The problem of stability of tissue differentiation remains one of the little-studied problems of developmental biology. Having attained a certain level of differentiation, cells of different tissues of vertebrates steadily keep this differentiated state. Differentiating and dying cells are only replaced by descendants of progenitor cells such as epidermal or pancreatic cells (Wessels, 1964), or else some reversible changes of differentiation occur with respect to the environment in vitro (Coon & Cahn, 1966; Cahn, 1968). But these changes are limited by cell type. Very few reliable cases of artificial metaplasia (Fell, 1961) do not suggest any definite rule. Apart from the phenomena of stable differentiation, only far-reaching tissue transformation proceeds during regeneration in urodelan amphibians. This ability of true metaplasia as well as its absence in other vertebrates should be based on some general laws which can be studied in experiments on artificial metaplasia.

The pigmented epithelium of vertebrate eyes appears to be the most suitable tissue for such studies. Following the removal of the retina in newts, a new retina

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is regenerated from the pigmented epithelium (Colucci, 1891; Wachs, 1920; Stone, 1950; Hasegawa, 1958; Mitashov, 1968, 1969; Reyer, 1971), but its differentiation is stable in all other vertebrates, including adult anuran amphibians (Stroeva, 1956). It has been clearly shown that the pigmented epithelium changes its differentiation (pigmentation, cell shape) \textit{in vitro} with respect to some environmental conditions but, given the initial conditions, returns to its original type (Doljanski, 1930; Cahn & Cahn, 1966; Cahn, 1968; Whittaker, 1968). The transformation of pigmented epithelium into retina has an advantage: these tissues differ sharply from each other in their differentiation, thus allowing the exact identification of developing changes.

Our attempts to produce artificial metaplasia were based on the following general prerequisites. The process of differentiation during development is due not only to the suppression of alternative ways or activation of one particular way of differentiation but to both of these phenomena (Lopashov, 1968; Nieuwkoop, 1968). At the same time influences stimulating one way of differentiation can suppress other ones. To obtain the transformation of pigmented epithelium into retina, we considered it necessary, at least: (a) to remove from the pigmented layer the mesenchyme envelopes which can fix its flattened state and, hence, its differentiation; (b) to introduce in its cells a lacking factor of retinal differentiation which is most probably accumulated in retina by the time of its transition to the final differentiation.

Some data have already suggested the possibility of transformation of pigmented epithelium into retina in other than urodelan amphibians. Following the removal of retina and iris in tadpoles of \textit{Bufo viridis}, pigmented epithelium is not usually transformed into retina; but if it comes to lie between the margins of regenerating retina it is transformed into retina (Lopashov, 1949). Following the removal of retina in 4-day chick embryos, it is not restored from the pigmented epithelium, but if a piece of retina from a chick embryo or a mouse embryo of the same age is transplanted into the cavity of such an eye, islets of retina arise in the pigmented epithelium (Coulombre & Coulombre, 1965, 1970). While interpreting these results a possibility to be excluded is that at these stages the pigmented epithelium can transform into retina simply as a result of accumulation of its cells due to delamination of mesenchyme envelopes; retina can arise from the outer eye layer in 4-day chick embryos both during its \textit{in vitro} cultivation (Dorris, 1938) and \textit{in vivo} following the exfoliation of mesenchyme envelopes under oxygen deficiency (Mushett, 1953).

We investigated the possibility of artificially stimulating the transformation of pigmented epithelium into retina at two stages of development in the common frog \textit{Rana temporaria}, early tadpole and adult frog, by combining the removal of mesenchyme envelopes and the effect of retina from a tadpole at the stage when it has just attained the differentiated state. Some of these results have already been published elsewhere (Sologub, 1968; Lopashov & Sologub, 1970).
MATERIALS AND METHODS

Experiments were performed on eyes of tadpoles (stage 41 after Cambar & Marrot, 1954; stage III-IV after Taylor & Kollros, 1946; Rugh, 1962) and adult frogs of Rana temporaria. The following experiments were carried out to study the potencies of pigmented epithelium (Fig. 1):

I. Cultivation of pigmented epithelium of tadpoles and adult frogs in the empty orbit of a tadpole eye.

II. Cultivation of a sandwich of pigmented epithelium of tadpoles and adult frogs with a piece of tadpole retina inside within the empty orbit of a tadpole eye.

III. Cultivation of pigmented epithelium of tadpoles and adult frogs in the lensless tadpole eye.

Prior to the operation, tadpoles were anaesthetized by 1% urethane; in adult frogs, eyes were removed without narcosis. To remove the eye from the orbit in tadpoles, the anterior margin of the outer cornea was cut through, the eye was pressed out by needles and the nerve and vessels cut by iridectomy scissors. All manipulations were performed in double Holtfreter solution with antibiotics. Mesenchyme envelopes of the eye (scleral and choroid) were removed with steel needles and a sharp-edged knife starting at the posterior eye pole. Remnants of the choroid coat were removed by rolling the eye on to Millipore filter HA.

In the present work an attempt was undertaken to remove the mesenchyme envelopes and Bruch’s membrane completely. However, the cells of the pigmented epithelium in tadpoles are so tightly connected with Bruch’s membrane that we rarely succeeded in obtaining the intact cell layer following the removal of Bruch’s membrane. It can only be successfully removed in adult frogs, in which it is not so tightly connected with cells of the pigmented epithelium. Attempts to remove this membrane by chemical methods proved to be unsuccessful (Sologub, 1968; Lopashov & Sologub, 1970), that is why it was removed surgically. With this aim after the removal of the sclera and cornea the eye knife was inserted between Bruch’s membrane and the cells of pigmented epithelium; Bruch’s membrane was then drawn off by forceps and torn, thus releasing a layer of pigmented epithelium.

In the first series of experiments the pigmented epithelium with or without the Bruch’s membrane was implanted into the empty eye orbit of tadpoles for cultivation (Fig. 1A).

In the second series of experiments, with sandwiches, only retina from tadpoles (at the above-mentioned stage) was used. With the mesenchyme envelopes intact, the eye was cut through around the limbus of cornea and the whole retina was removed. A piece with all layers (0.25 mm x 0.25 mm to 0.5 mm x 0.5 mm) was then cut out from its posterior pole. This piece was put in a sandwich of pigmented epithelium and placed through the cut into the orbit of
Fig. 1. Schemes of operations. (A) Isolation of pigmented epithelium with or without the Bruch’s membrane, wrapping up a piece of retina into it and its subsequent implantation into the empty eye orbit of a tadpole. (B) Implantation of pigmented epithelium with or without the Bruch’s membrane in the anterior chamber, pupil or posterior cavity of the tadpole lensless eye.
### Table 1. Transformation of pigmented epithelium of tadpoles and adult frogs into retina

<table>
<thead>
<tr>
<th>Series</th>
<th>Type of experiment</th>
<th>Total no. of fixed animals</th>
<th>Results</th>
<th>% of transformations</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Pigmented epithelium of tadpoles with mesenchyme envelopes or without them in the orbit of tadpoles</td>
<td>84</td>
<td>0/84</td>
<td>0</td>
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<tr>
<td>II(a)</td>
<td>Pigmented epithelium of tadpoles with a piece of retina in the orbit of tadpoles</td>
<td>90/74*</td>
<td>51/23</td>
<td>68.9</td>
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<td>III(a)</td>
<td>Pigmented epithelium of tadpoles in the lensless eye of tadpoles</td>
<td>92/87*</td>
<td>81/6</td>
<td>93.1</td>
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<tr>
<td>I</td>
<td>Pigmented epithelium of adult frogs without mesenchyme envelopes and Bruch’s membrane in the orbit of tadpoles</td>
<td>35</td>
<td>0/35</td>
<td>0</td>
</tr>
<tr>
<td>II(b)</td>
<td>Pigmented epithelium of adult frogs without mesenchyme envelopes and Bruch’s membrane and with a piece of retina in the orbit of tadpoles</td>
<td>90/75*</td>
<td>28/47</td>
<td>37.4</td>
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<tr>
<td>III(b)</td>
<td>Pigmented epithelium of adult frogs in the eye of tadpoles</td>
<td>169/99*</td>
<td>46/53</td>
<td>46.5</td>
</tr>
</tbody>
</table>

Pigmented epithelium was taken from two stages of development (tadpoles – stage 41– and adult frogs), retina was taken from tadpoles (stage 41).

+ Transformation of pigmented epithelium into retina.

− No transformation.

* Of the total number of fixed animals, only those were taken into account in the Results section which were fixed at the time when metaplasia became possible (see text).

Pigmented epithelium was taken from two stages of development (tadpoles – stage 41– and adult frogs), retina was taken from tadpoles (stage 41).

+ Transformation of pigmented epithelium into retina.

− No transformation.

* Of the total number of fixed animals, only those were taken into account in the Results section which were fixed at the time when metaplasia became possible (see text).

a tadpole from which the eye had been removed (Fig. 1 A). The empty orbit was used as a chamber for cultivation of implants.

In the third series of experiments with lensless eyes, an eye of a tadpole was pressed out from the orbit through a cut along the anterior margin in the outer cornea, nerve and vessels being intact. After zonules had been carefully cut, the lens was removed through the cut in the inner cornea. A layer of pigmented epithelium taken from another tadpole or adult frog was implanted in the anterior chamber, pupil, or the posterior cavity (vitreous chamber) of the lensless eye by means of a needle and a knife (Fig. 1 B). The eye with its implant was then cautiously pushed back under the outer cornea, which healed very rapidly.

Operated animals were fixed at intervals of 1 h, 1, 3, 5, 7, 10, 15, 20 and 30 days following the operation by the Bouin’s fluid. Sections 6 μm thick were stained by azocarmine after Heidenhain.
RESULTS

I. Cultivation of pigmented epithelium of tadpoles and adult frogs in the orbit of tadpoles

A sheet of pigmented epithelium of tadpoles implanted in the orbit did not transform into retina even after a long period of cultivation – 15–20 days after operation (Table 1). Cells of pigmented epithelium remained densely pigmented during the whole period of cultivation (Fig. 2). The layer of pigmented epithelium in most cases closed and it could be suggested that no metaplasia occurred because it was closed. To keep the margins of the pigmented epithelium free, we placed into it a disc cut out of the polyester film ‘Melinex O’. However, in this type of experiment all cells of pigmented epithelium remained unchanged as well.

When cultivated in the orbit without mesenchyme envelopes and Bruch’s membrane, the pigmented epithelium of adult frogs did not change its differentiation. In most cases, the layer became dissociated into individual cells dispersed in the orbit. In others, pigmented epithelium remained in the form of sheet or gave rise to groups of cells (Fig. 3). No cases of transformation into retina were recorded (Table 1).

Fig. 2. Pigmented epithelium of the tadpole eye 15 days after the implantation in the eye orbit.
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Fig. 3. Pigmented epithelium of the adult frog eye without the mesenchyme envelopes and the Bruch's membrane in the orbit, 15 days after the implantation.

Fig. 4. Pigmented epithelium of the tadpole completely closed around the piece of retina in the orbit. Ten days after the implantation. No changes in the pigmented epithelium. ir, Implanted retina.
II(a). Cultivation of pigmented epithelium as a sandwich with a piece of retina inside the tadpole eye orbit

The transformation of the pigmented epithelium of tadpoles into retina involves depigmentation of its cells, their proliferation and, then, formation of layers in the newly formed retina. If one follows the course of metaplasia, characteristic sources of retina formation are disclosed. Transformation of pigmented epithelial cells begins either on its free margins or in breaks through the sheet. When the sheet of pigmented epithelium closed around the piece of retina, no new retina formed (Fig. 4). In other cases, beginning from the 3rd day after the operation, cells of pigmented epithelium depigmented and the flattened ellipsoid cell nuclei became rounded, with a distinct nucleolus (Fig. 5). On the 5–7th day after the operation iris-like regions arose and their intensive proliferation proceeded with the formation of retina (Fig. 6A). In the iris-like region, cells had large light-stained nuclei and one or two nucleoli brightly stained (Fig. 6B). By 10–15 days after the operation, large masses of newly formed retina appeared. Later (25 days after the operation) the newly formed retina became subdivided into main layers: ganglionar and inner nuclear ones, layer of visual cells and reticular layers (Fig. 7). The frequency of transformation of the pigmented epithelium of tadpoles into retina amounted to 68.9 % (Table 1).
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Fig. 6. Pigmented epithelium of the tadpole eye regenerating into retina on the 7th day after the implantation into the orbit. (A) General view. (B) A region of A at a greater magnification. Cells of pigmented epithelium are depigmented simultaneously in two regions on both sides of the break in it. dir, Depigmented iris-like regions; epg, extruded pigment granules; m, mitoses in developing retina; pe, pigmented epithelium; n, nucleoli.

II(b). Cultivation of the pigmented epithelium of adult frogs without Bruch's membrane together with a piece of retina in the orbit of tadpoles

A sandwich of the pigmented epithelium of adult frogs with the retina of tadpoles implanted in the orbit broke rather frequently in many places and in these cases a layer of pigmented epithelium was dissociated into individual cells not surrounding the piece of retina and the cells of the pigmented epithelium remained unchanged. Only in 28 cases (Table 1) did the sandwich remain intact, and the transformation of pigmented epithelium into retina proceeded in the same way as in the experiments where the pigmented epithelium and retina of tadpoles were combined. Later (13–15 days after the operation, Fig. 8) one could see the transformation into retina of nearly half of the pigmented epithelium layer (Fig. 9). A large number of mitosing cells could be seen in the newly forming retina (Fig. 10). Metaplasia of pigmented epithelium in these experiments occurred in only 37.4% of cases (Table 1), due to the wide separation of cells devoid of connecting envelopes.
Fig. 7. Pigmented epithelium of the tadpole eye with the retina completely regenerated from it. Twenty-five days after the implantation in the orbit. nr, Newly formed retina; ir, implanted retina; mz, zone of mitoses; pe, pigmented epithelium.

Fig. 8. Pigmented epithelium of the adult frog without mesenchyme envelopes and the Bruch’s membrane 15 days after the implantation in the orbit with a piece of retina. nr, Newly formed retina; ir, implanted retina; pe, pigmented epithelium.
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Fig. 9. Retina developed from the pigmented epithelium of the adult frog eye. Twenty days after the implantation in the orbit. *nr*, Newly formed retina.

Fig. 10. A region of retina developed from the pigmented epithelium of the adult frog eye. Twenty days after the implantation. Many mitosing cells are seen.
Fig. 11. Pigmented epithelium of the tadpole in the anterior chamber of lensless eye. Twenty days after the implantation. No changes in differentiation. *ic*, Inner cornea; *hir*, host iris; *pe*, pigmented epithelium.

Fig. 12. Pigmented epithelium of the tadpole in the posterior cavity of the lensless eye. Three days after the implantation. Onset of transformation into retina. *dpe*, Depigmented cells of pigmented epithelium.
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Fig. 13. Pigmented epithelium of the tadpole in the pupil of the lensless eye. Ten days after the implantation. *pe*, Pigmented epithelium; *nr*, newly formed retina, not divided into layers; *ilm*, membrane on its surface corresponding to the inner limiting membrane; *hir*, host iris.

**III(a). Cultivation of pigmented epithelium of tadpoles in the lensless eye of tadpoles**

These experiments were performed by the method used by Ikeda (1935), Mikami (1941), Sato (1953), Reyer (1956), Sologub (1972) and others: the lens was removed from the eye and, instead of iris, a layer of pigmented epithelium was implanted in the lensless eye. In this series of experiments an isolated sheet of pigmented epithelium stuck to the outer cornea and remained in the anterior chamber or it came to lie in the pupil or in the posterior cavity of the eye. No transformation of pigmented epithelium into retina proceeded in the anterior chamber (Fig. 11). In those cases where the pigmented epithelium remained in the pupil, the whole process of metaplasia of the pigmented epithelium could be followed. Depigmentation of cells began on the 3rd day after the operation (Fig. 12) followed by an intense proliferation of depigmented cells. Nucleoli became distinct. On the 7th day after the operation the region of epithelium adjacent to the posterior cavity was already two- or three-layered rather than monolayered. Depigmentation and proliferation of cells of the pigmented epithelium began in the area where Bruch’s membrane was absent or broken. On the 10th day after the operation a mass of newly formed retina appeared which was surrounded by a membrane corresponding to the inner limiting membrane of normal retina (Fig. 13). By 25–30 days after the operation the formation of the main reticular and nuclear layers began. On the surface of the new retina
adjacent to the pigmented epithelium and corresponding to the outer nuclear layer, visual cells began to form (Fig. 14). In some cases three layers of pigmented epithelium with different degrees of transformation into retina could be seen in the pupil. The upper layer (1st layer) adjacent to the anterior chamber does not develop into retina; the layer of pigmented epithelium adjacent to the posterior cavity (2nd layer) transforms into retina partially; and the portions which moved in the posterior chamber (3rd layer) transformed into retina completely (Fig. 15).

In the posterior cavity, cells of the pigmented epithelium began to depigment on the 3rd day after the operation. By 15–20 days the layer of pigmented epithelium is completely transformed into retina. By 25–30 days a large mass of newly formed retina developed which sometimes filled the whole posterior cavity (Fig. 16). The frequency of transformation of pigmented epithelium of tadpoles in the lensless eye amounted to 93.1 % (Table 1).

**III(b). Cultivation of the pigmented epithelium of adult frogs in eyes of tadpoles**

Cells of the pigmented epithelium without Bruch’s membrane dispersed in the posterior cavity of the lensless eye did not transform into retina. They remained densely pigmented even when cultivated for a long time (Fig. 17). When a sheet of pigmented epithelium without Bruch’s membrane remained intact, depigmentation began on the 5–7th day after the operation (Fig. 18) followed by prolifera-
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Fig. 15. Pigmented epithelium of the tadpole in the pupil of the lensless eye subdivided into three zones (1, 2, 3) with different degrees of transformation into retina. Twenty days after the implantation.

tion of the depigmented cells. If the layer of pigmented epithelium remained in the pupil, metaplasia began in the region adjacent to the posterior cavity. When the layer moved to the posterior cavity by 25–30 days after operation, the complete transformation of pigmented epithelium into retina with the formation of visual cells on the surface of its inner cavities could be seen (Fig. 19).

To preserve the integrity of the sheet of pigmented epithelium, in a series of experiments Bruch's membrane was not removed. Being not so tightly connected with the cells of pigmented epithelium as in tadpoles, Bruch's membrane separated from the sheet of pigmented epithelium on the 3rd day after the operation (Fig. 20); the latter preserved its integrity and transformation into retina proceeded more frequently than in the preceding series. Some depigmented cells were seen in the implant on the 7th day after the operation and by 15 days large areas of depigmented cells proceeded to proliferation. The transformation of the whole layer of pigmented epithelium into retina was completed by 20–30 days after the operation (Fig. 21).

In seven cases the lenses were left in the eyes of tadpoles and the pigmented
Fig. 16. Complete transformation of the tadpole pigmented epithelium into retina in the posterior cavity of the lensless eye. Twenty-five days after the operation. Mitotic divisions can be seen in the mass of newly formed retina. hr, Host retina; hir, host iris; nr, newly formed retina; m, mitoses.

Fig. 17. Separated cells of pigmented epithelium of the adult frog in the posterior cavity of the lensless eye. Fifteen days after the operation. No changes. pe, Pigmented epithelium; hir, host iris.
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Fig. 18. Pigmented epithelium of the adult frog in the region of pupil of the lensless eye. Onset of transformation. Five days after the implantation. *dpe*, Depigmenting cells of pigmented epithelium.

Fig. 19. Retina developed from the adult frog pigmented epithelium in the cavity of the lensless eye. Twenty-five days after the operation. *nr*, Newly formed retina; *hr*, host retina; *ph.r.*, rosette with photoreceptors.

Epithelium of adult frogs, without Bruch’s membrane, was implanted under the lens inside the eye. By 25 days after the operation the transformation of pigmented epithelium into retina could be seen: its part adjacent to the normal retina had the retinal structure whereas that adjacent to the lens preserved its initial pigmentation (Fig. 22).
Fig. 20. Pigmented epithelium of the adult frog with the Bruch's membrane in the pupil of the lensless eye. Three days after the implantation. *bm*, Separated Bruch's membrane; *dpe*, depigmenting cells; *hir*, host iris.

Fig. 21. Retina developed from the adult frog pigmented epithelium in the eye cavity. Twenty days after the operation. *nr*, Newly formed retina with mitoses; *hr*, host retina.
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Fig. 22. Transformation of the adult frog pigmented epithelium in the posterior cavity under the lens. Twenty-five days after the implantation. nr, Newly formed retina; hr, host retina; ir, iris-like regions; pe, preserved pigmented epithelium; l, host lens.

In all experiments with implantation of the pigmented epithelium of adult frogs into the eye of tadpoles, metaplasia into retina was observed, on the average, in 46.5% of cases (Table 1).

**DISCUSSION**

1. **Significance of mesenchyme envelopes and lens to differentiation and morphogenesis of newly forming retina**

At first sight, the role of mesenchyme envelopes in the development of retina might appear unambiguous and purely negative. When mesenchyme envelopes surround the surface of the eye rudiment its cells develop into pigmented epithelium rather than retina (Lopashov, 1963; Lopashov & Stroeva, 1963). But under close examination their role proves to be more complicated. The pigmented layer develops from the cells of the eye rudiment when they are arranged in one thin layer as well (Lopashov & Hoperskaya, 1967). At the same time the mesenchyme envelopes (1) together with the enrolling and expanding retina promote the stretching and flattening of cells of the outer eye layer, and (2) becoming attached to the pigmented layer fix its differentiation. Consequently the formation of retina in the outer layer is possible after separation of mesenchyme envelopes at embryonic stages of all vertebrates. Artificial metaplasia at later stages of development is possible, as follows from our experiments, as a result of
the removal of envelopes or in those cases when mesenchyme envelopes become separated from the pigmented epithelium or slip down from its margins.

But the role of mesenchyme envelopes in the formation of the retina is not simply negative. If the retina develops \textit{de novo} in the absence of the mesenchyme envelopes – in the eye cavity (Results, §§ III(a), (b)) or in a sandwich of pigmented epithelium with a piece of retina (Results, § IIb) – it contains typical cell constituents but does not acquire a cup-like shape and regular ratio of its layers (Figs. 9, 16, 19). The cup-like shape of the retina (although without the pupil) arises only in those cases when it is formed from the margins of pigmented epithelium surrounded by the mesenchyme envelopes (Fig. 7); such a shape never arises in the absence of mesenchyme envelopes. Metaplasia gives rise only to the transformation of pigmented epithelium into retina at the cellular level but does not provide the formation of its functional cup-like shape and regular ratio of its constituents.

The role of the lens is also evident in the formation of the retina. During embryogenesis it provides the development of the initial cup-like shape of the retina (Lopashov, 1963). Later, together with the retina, it gives rise to intraocular pressure stretching the retina, pigmented epithelium and mesenchyme envelopes (Coulombre & Coulombre, 1964) and providing their normal morphogenesis. During regeneration of the eye the normal morphogenesis of the retina with the formation of a pupil proceeds only in the presence of a lens (Lopashov, 1949; Lopashov & Stroeva, 1963).

The role of the lens is also unambiguous. In the zone of direct contact with the cells of the pigmented epithelium, it prevents their transformation into retina (Results, § III(b), Fig. 22); they remain monolayered and preserve their pigmentation. Here, the role of the lens is similar to that in differentiation of the inner layer of secondary iris and ciliary body (Stroeva, 1963, 1967), where contact with the lens leads to flattening and pigmentation of the former.

Thus, one of the necessary conditions for the initiation of metaplasia of pigmented epithelium into retina is its liberation from the mesenchyme envelopes stabilizing its differentiation.

2. \textit{Role of retinal agent in the transformation of pigmented epithelium into retina}

During development the retina induces the formation of the lens from ectoderm, thus suggesting the presence of a lens-forming agent, but it could be expected that the development of the retina itself is directed by another agent as well (Lopashov, 1963). Indeed, at early stages of development the retinal \textit{Anlage} is able to induce both the formation of retina and lens in gastrula ectoderm, depending on the type of contact (Lopashov & Hoperskaya, 1970). In this case it is probable that a similar retinal agent is concentrated in the retinal \textit{Anlage} in the phase of its homotypic induction, its maximal concentration being attained by the beginning of its differentiation. It is possible that such an agent disappears
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from the pigmented epithelium during the development of animals incapable of regeneration. In this case the introduction of this agent from the retina would allow the metaplasia of pigmented epithelium into retina. The results of our experiments (Results, §§ II(a), III(b)) have shown that pigmented epithelium did transform into retina under the influence of retina, thus providing the second principal proof of the presence of a retinal agent. Since the direct contact of cells is not necessary for its effect, it appears to be an inducing substance.

The retinal (R) agent is not the only agent formed by the retina. It forms a 'retina factor' as well which is necessary for regeneration of lenses from the newt iris (Stone, 1958; Reyer, 1962) and which we have designated as L_r-agent (Lopashov & Sologub, 1970). These agents differ from each other in some other peculiarities of their effects as well: R-agent induces the metaplasia of pigmented epithelium, exerting its effect both from the whole retina (Results, §§ III(a), (b)) and its pieces (Results, §§ II(a), (b)), whereas lens regeneration can be stimulated by the whole closed retina rather than by its pieces (Eguchi, 1967; Eisenberg-Zalik & Scott, 1969). Thus, the whole retina can stimulate both lens regeneration and metaplasia of pigmented epithelium into retina. Therefore we can conclude that the transformation of the pigmented layer of the tadpole eyes into retina in the posterior cavity of their eyes described by Sato (1953) is due to the effect of R-agent.

It is known that the primary lens-inducing influence of eye rudiments which is capable of inducing lens formation from the ectoderm of early embryos is not identical with the latest L_r-agent. In newts the lens-inducing ability of the retina decreases during the process of its differentiation and disappears at the time that the iris acquires its capacity for lens regeneration (Reyer, 1950, 1954; Takeuchi, 1963). The second step of the effect of the retina on lens-forming epithelium, following the initial induction of a lens, involves the stimulation of crystallin formation rather than polarization of lens-forming cells under the influence of mesenchyme (Muthukkaruppan, 1965; Eguchi, 1967; Philpott & Coulombre, 1968). Bearing in mind these changes in time, one can conclude that R-agents in the retinal Anlage and the differentiated retina are probably not identical. In any event, it is likely that the concentration of this agent increases with time since the differentiated retina of tadpoles can exert its effect at a distance on a more stable pigmented epithelium and not only on supersensitive gastrula ectoderm.

3. Role of cell proliferation and mass-effect in metaplasia of pigmented epithelium into retina

Apart from two initial factors which we varied in these experiments – removal of mesenchyme envelopes and introduction of retinal agent – two other factors were found to be important. First of all, cell proliferation. In its absence, no transformation of the cells of pigmented epithelium proceeded in experiments in vitro (unpublished), in spite of the fact that they surrounded a piece of retina. The importance of the second of these factors, mass-effect, is due to the fact
that the cells of pigmented epithelium dispersed in the posterior eye cavity never change their differentiation (Results, § IIIb). A certain minimal mass is necessary for the initiation of transformation into retina.

Does the effect of R-agent involve the stimulation of cell proliferation in the pigmented epithelium? A comparison of the behaviour of cells of pigmented epithelium in the anterior chamber and the posterior cavity makes this suggestion very unlikely. Both cavities may serve as good chambers for the cultivation of various tissues in vivo involving intensive processes of proliferation (eye tissues – Royo & Quay, 1959; Stroeva, 1960, 1967; ovaries – Noyes, Clewe & Yamate, 1961; nephrogenic mesenchyme – Grobstein & Parker, 1958, etc.). But the metaplasia of pigmented epithelium into retina proceeds only in the posterior cavity which is surrounded by retina (Results, §§ III(a), (b)). Proliferation of the pigmented epithelium itself in vitro (Cahn, 1968) does not result in such transformations.

However, this does not exclude the proliferation from playing a part in the metaplasia of pigmented epithelium into retina, although it has to be combined with the effect of the specific R-agent. Processes of embryonic induction develop in cell populations characterized by mitotic activity (gastrula ectoderm – Flickinger, Freedman & Stambrook 1967; epithelium of pancreatic rudiment – Wessels & Cohen, 1967; Rutter et al. 1968; chondrogenic cells of somites – Holtzer, 1968). The metaplasia of iris into lens in newts also involves the stimulation of the whole complex of phenomena of the cell cycle (Eisenberg & Yamada, 1966; Eisenberg-Zalik & Yamada, 1967; Yamada, 1967; Dumont, Yamada & Cone, 1970); the same is true for the metaplasia of pigmented epithelium into retina in newts (Mitashov, 1969). The phenomena of artificial stimulation of the transformation of pigmented epithelium into retina described here involve, besides intensive cell divisions, a steady increase in the size of nucleoli (Figs. 6B, 13).

Proliferation appears to be indispensable for the formation or transformation of cell types because the effect of corresponding agents can be applied only to cells at a certain phase of the cell cycle. This viewpoint (Lopashov & Sologub, 1970) agrees with the ideas of Gurdon & Woodland (1968, 1970), according to whom cytoplasmic proteins responsible for long-term changes of cell differentiation can enter the nuclei only at certain phases of their cycle. The similarity between the most carefully studied inducing agents (Tiedemann, 1968) and non-histone proteins which is due to the derepression of specific DNA sequences in nuclear chromatin (Paul, 1971) allows one to suggest that similar agents may determine stable cell differentiation in all these cases. The phenomena of artificial metaplasia appear to be based on a similar mechanism which, in the case of induction, involves not only exchange of proteins between the nucleus and the cytoplasm but also between different cells. If so, the occurrence of inductive phenomena would depend on the longevity of the corresponding phase of cell cycle and the specificity of cell differentiation on the agents participating in the process of induction.
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But the mass-effect should precede cell proliferation and therefore cannot be reduced to its consequences. Mitoses resulting in the formation of the mass of retina in amphibians (Hollyfield, 1968; Straznicky & Gaze, 1971) can proceed only in its Anlage, which has a certain minimal mass, whereas they do not proceed in the pigmented epithelium (Lopashov, 1963; Coulombre, Steinberg & Coulombre, 1963; Sologub, 1968). The most likely significance of mass-effect to the transformation of cells of the pigmented epithelium consists in the fact that, following the effect of R-agent, subsequent processes of homotypic induction occurring in the mass of homogenous cells cannot occur in isolated cells. Meanwhile, it is in this phase (Discussion, § 2) that one-sided reproduction and concentration of R-agent can proceed, thus providing one-sided differentiation of retina and the development of its ability to induce the formation of retina in the gastrula ectoderm and the pigmented epithelium. The development of other Anlagen has similar phases, as witnessed by Cooper (1965) who has shown that cartilage at a certain period is able to induce the formation of cartilage. Phenomena of homotypic induction occurring at the phase of one-sided differentiation can be found in other Anlagen as well and the agents concentrating at this phase can be used to obtained directed differentiation and artificial metaplasia in some other tissues and Anlagen.

4. Control circuits of metaplasia and its stimulation in non-regenerating species

In those cases when animals are adapted to regeneration such as the ability to regenerate the eye in newts, an impression can be created that the damage of the eye or its parts with their subsequent destruction results itself in all other processes of regeneration acting as a trigger. But the experiments with artificial stimulation of metaplasia have clearly shown that for this a definite set of events has to be started. Thus, what looks at first sight as the 'stimulation' of metaplasia deserves in fact more thorough comprehension. Such stimulation requires a re-arrangement of the whole control circuit of the given process rather than any particular treatment.

To obtain metaplasia of the pigmented epithelium in frogs, a number of constituents of this circuit is to be reproduced: (1) liberation of pigmented epithelium from Bruch's membrane; (2) effect of R-agent which appears to act like inducing agents switching on (derepressing) certain syntheses which result in differentiation; (3) mass-effect which reflects the situation that any derepression in an individual cell leads to nothing for a mass of interconnected cells is necessary for their differentiation; (4) development of conditions for proliferation, other than the liberation from Bruch's membrane. If during derepression of certain DNA sequences, phenomena at the molecular level are included in the circuit, other constituents of the circuit naturally belong to the supercellular level. The removal of Bruch's membrane, influence of the R-agent and mass-effect should act together on groups of cells, otherwise they would be
inefficient. Therefore such control circuits should involve phenomena at different levels, otherwise they cannot operate.

It does not mean that the control circuit of retinal regeneration can be limited by the constituents of the control circuit of metaplasia. The formation of retina de novo requires some other above-mentioned factors (Discussion, § 1): the presence of mesenchyme envelopes, lens and intra-ocular pressure. Metaplasia and regeneration in newts are operated essentially by the same circuits, but the adaptivity of the process of regeneration in these species is manifested by the fact that these circuits are switched on automatically and in a certain sequence leading to the restoration of the eye. A study of the structure of such circuits is not too complicated and their knowledge does not allow one to reduce the processes of metaplasia and regeneration to the effects of a single ‘priming’ event, such as initial damage, inducing influence, or molecular information.

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REFERENCES


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