The correlation of the processes of proliferation and determination in the morphogenesis of iris and ciliary body in rats

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In spite of the fact that causal embryology dealt extensively with the development of the eye in vertebrates, the iris attracted the attention of investigators only as a source of lens restitution in the course of Wolffian regeneration (Reyer, 1954, 1962; Goss, 1965; Scheib, 1965; Yamada, 1966). This is shown by the absence of sections on iris development in recent reviews devoted to the experimental analysis of eye development (Twitty, 1955; Lopashov & Stroeva, 1961; Coulombre, 1961, 1965a, b). The present communication reports a study of the development of the neural layers of iris and ciliary body in rat embryos.

Previously it was shown (Stroeva, 1963) that the lens epithelium was an inductor of the common iris-ciliary body rudiment and that at the stages of 12.5 and 13.5 days of pregnancy in rats any region of the inner layer of the eye cup could give rise to this rudiment. Having been cultivated for 25-28 days in the anterior eye chamber, induced irises transformed into strongly pigmented unilayer structures which had a tendency to fold if not firmly connected to the lens. As a rule, the folded unpigmented zone—the rudiment of the ciliary epithelium—represented an intermediate structure between retina and pigmented iris (Stroeva, 1963). Some observations confirmed the inductive role of the lens in iris development in mammalian embryos (Giroud, 1957), chick embryos (Genis-Galvez, 1966) and anuran tadpoles (Dabagian, Stroeva & Sheresheva, 1966).

The further analysis of this phenomenon could follow two paths: (1) the study of the molecular mechanism of induction and the inductive substances, or (2) investigations of the processes which take place in the reacting system as a result of induction and of the early events of differentiation in the iris-ciliary body rudiment, the time of its determination and segregation of the common rudiment into iris and ciliary body areas. I have chosen the second path, believing that knowledge of these events is necessary for a more efficient approach to the first.

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The attempt to find specific antigens at the beginning of iris formation in chick embryos (Maisel & Harmison, 1963) was concentrated on the detection of antigens common for lens and iris, but not at their relations to the particular stages of the iris development. It was found that the antigen corresponding to the lens α-crystalline was the first detectable antigen to appear in iris (at the stage of 72 h incubation); the antigens which correspond to the β- and α-crystallines were detected in the 8-day-old iris extracts. The first specific iris antigen was found only in the 10-day-old extract, i.e. at the stage when muscles were already being differentiated in iris. It was thought that these antigens might be synthesized in the iris much earlier, but were present at first only at low concentrations undetectable by the method used (Maisel & Harmison, 1963).

It was necessary to look for another approach to the detection of the first specific synthesis in iris rudiment cells. It was possible that the presence of a competitive relationship between the synthesis of specific proteins and the ability of nuclei to synthesize DNA (Stockdale & Holtzer, 1961; Stockdale, Abbot, Holtzer & Holtzer, 1963; Eisenberg & Yamada, 1966) is a general feature of the process of differentiation. If this is true not only the accumulation of specific proteins in cells, but also the cessation of DNA synthesis, i.e. the removal of cells from the reproductive cycle, may serve as a convenient criterion for studying the early processes of differentiation (Zhinkin, 1965). Differentiating systems were characterized not only by the number of cells proceeding from the autosynthetic interphase to the heterosynthetic one, but also by the composition of the population of cells which continued proliferation. As the process of differentiation proceeded the duration of the generation cycle increased, mainly due to change in the presynthetic (G1) phase. At the same time initially homogeneous cell populations became heterogeneous in the duration of the G1-phase in different cells. A heterogeneous population is characterized by a low and distended second spike of the curve of labelled mitoses after a single injection of ³H-thymidine (Dondua & Dondua, 1964; Zhinkin, 1965). The differences arising in the number of reproducing cells and the alterations in the parameters of the mitotic cycle may allow us to detect the beginning of divergent differentiation in rudiments which give rise to several tissues before the appearance of sharp differences detected by other methods. An attempt was made to use this method for the analysis of the time of differentiation of the iris-ciliary body rudiment within the inner layer of the eye cup.

It was shown in an autoradiographic study using ³H-thymidine (Zavarzin & Stroeva, 1964) that between the 15th and 18th day of pregnancy in rats several cell populations can be detected in the eye rudiment, each of them being characterized by its own level of proliferative activity. Already at 15 days of pregnancy the outer layer of the iris-ciliary body rudiment differs from the pigment epithelium of the retina by the greater number of dividing cells and by the lower heterogeneity of this part of cell population as to duration of.
Rat iris and ciliary body

The differences between the internal layer of the iris-ciliary body rudiment and the peripheral area of the retina at this stage are expressed only as an insignificant decrease of the number of cells capable of proliferation. At 18 days of pregnancy the differences between these areas of the inner eye cup layer become detectable by the proportions of labelled nuclei, as well as by the form of the generation cycle curves (Figs. 2, 3). It is necessary to find out how the time of differentiation determined in this way is related to the time of rudiment determination detected by the other methods. The present study was undertaken to solve this problem.

It was shown earlier (Stroeva, 1963) that at the time of sphincter differentiation in the outer iris layer of control rudiments (whole eye rudiments cultivated with the surrounding tissues), no muscle differentiation occurred in irises induced by the lens in the inner eye cup layer. This suggests that under the influence of lens epithelium the formation of the inner iris layer and ciliary body only took place. As the character of the lens influence upon the differentiation of the external iris layer remains unknown, in the future analysis of the phenomenon of iris formation under lens influence the system ‘lens: inner layer of iris-ciliary body rudiment’ will be used, the pigmented state of the iris rudiment being a criterion of its differentiation.

MATERIALS AND METHODS

The investigation was made on grey rats of the line described earlier (Stroeva, 1960, 1963). To establish the time of determination of the whole inner layer of the iris-ciliary body rudiment, the lens was removed from the eye rudiments at 14-5, 15-5, 16-5 and 17-5 days of pregnancy and the inner eye cup layer was cultivated in the anterior chamber of the eye of adult animals (series A) by the method described earlier (Stroeva, 1960). The lens rudiment was removed surgically after the eye rudiments had been trypsinized (1·5 %) for a period of 3–5 min at 37 °C with previous and subsequent washing in three changes of Ca²⁺ and Mg²⁺-free Tyrode solution. The tissues were fixed in Zenker for a period of 6 h with subsequent washing in tap water for 24 h, followed by iodination. They were then dehydrated in alcohol-chloroform and the anterior segment of the host eye with the grafted eye rudiment was embedded in paraffin.

In order to establish the time of determination of the rudiments of iris and ciliary body within the common rudiment (series B) an attempt was made to increase the pigmented iris area at the expense of the ciliary body and peripheral retina areas. For this purpose female rats at 14-5, 15-5 (series B I—Plate 1, fig. A), 17-5 (series B III—Plate 1, fig. B) and 19-5 (series B III—Plate 1, fig. C) days of pregnancy were exposed to a single 400 r dose of X-rays under the following conditions: 195 kV, 15 mA, 0·5 mm Cu + 0·7 mm Al filter and 247 r/min. Several hours before birth (22nd day of pregnancy) embryos were taken from the uterus and their heads were fixed in Bouin fluid. After fixation the
eyes were removed, dehydrated in alcohol-chloroform and embedded in paraffin. To determine the differential role of lens and irradiation in the increase of the pigmented iris area within the inner eye cup layer some irradiated eye rudiments were cultivated with or without lens in the anterior chamber for 5 days (series BII). Some irradiated embryos were sacrificed 5 h after irradiation in order to determine the areas of damage in the eye rudiment. The eyes of untreated newborn rats served as a control. All cases were stained in 8 μ serial sections by Heidenhain's azan method.

RESULTS

Series A. Cultivation of the inner layer of the eye rudiment without lens

After the cultivation of lens-free eye rudiments (158 cases) in the anterior chamber, the retina was well developed. In some cases the eye cup margins were necrotic, possibly as a result of the double damage of trypsinization and cutting of the outer layer. In other cases, the eye cup margins were healthy and in the absence of the lens the inner layer of the iris-ciliary body rudiment at 14-5, 15-5, and 16-5 days of pregnancy transformed into retinal rudiments in which mitoses could be seen (Plate 1, fig. D). The same result was obtained in the experiments on the removal of lens from eye cups of chick embryos at 3 and 5 days of incubation (McKeehan, 1961). In the rat eye rudiments taken from day 17-5 of pregnancy, in 4 of 22 cases, the inner iris layer was preserved, but had no typical structure and was not pigmented (Plate 1, fig. E). Ciliary body folds were not formed. In all cases where the lens was not completely removed and the eye cup margins were in contact with it, the pigmented iris was differentiated (Plate 1, fig. F), as in the experiments on lens removal from rat embryos in uterus (Woerdeman, 1963). The removal of lens in newborn rats does not prevent the differentiation of the pigmented iris (Stroeva, 1956).

Series B. The time of segregation of the common iris-ciliary body rudiment into iris and ciliary body areas

In order to establish the time of determination of the iris and ciliary body areas within the initially common rudiment an experimental increase of the pigmented iris area at the expense of the neighbouring areas of the ciliary body and retina was undertaken. The pregnant females divided into three age groups—14-5 to 15-5 (Series BI), 17-5 (Series BIII) and 19-5 (series BIII) days of pregnancy—were exposed to a single 400 r dose of X-rays. This attempt to increase the inner iris layer was based on the rates of DNA synthesis and the cell population kinetics found for the developing eye rudiment (Zavarzin & Stroeva, 1964). On the 15th day of pregnancy, as can be seen from the curves constructed from their data (Text-fig. 1), the nuclei of proliferating cells in the inner layer of the iris-ciliary body rudiment and in the peripheral retina area had similar generation times and phase durations in the mitotic cycle.
This is shown by the coincidence of the curves characterizing the changes in the proportion of labelled mitoses in both areas after a single \(^3\)H-thymidine injection. These areas, however, already differed from one another in the proportions of labelled nuclei (Text-fig. 3). At a similar generation time and the same specific period of the S-phase in both structures the difference in
of nuclei were labelled (Zavarzin & Stroeva, 1964). It was of interest to examine whether both areas of the inner eye cup at this stage had the same potencies and whether the peripheral retina would be able to differentiate into pigmented iris tissue under the influence of lens after an artificial decrease in cell numbers.

To test this more than half the retinal cells were damaged by 400 r X-irradiation (series BI). As the experiment continues for more than one day (28 h) after the single \(^3\)H-thymidine injection (Zavarzin & Stroeva, 1964) two neighbouring stages, 14.5 and 15.5 days of pregnancy, were studied. The animals were sacrificed just before birth, and their eyes were compared to those of normal newborn rats. An increase of the pigmented area of the inner iris layer in the experimental materials was expected. While most attention was paid to the structures at the margin of the eye, a brief description will be given of the damage elsewhere in irradiated eyes.

In the eye rudiments irradiated at 15.5 days of pregnancy and fixed 5 h after irradiation the external zone of the retinal rudiment was damaged most strongly (Plate I, fig. G). This area contained a mixture of necrotic cells, cell fragments and macrophages which phagocytosed them. Necrotic cells and cell groups can also be seen in the inner retinal areas, as was described by Rugh & Wolff (1955) for mice exposed to 350 r at 13.5 days of pregnancy. Damaged cells were also seen in the middle parts of the retina and in the base of the iris–ciliary body rudiment in close proximity to the equatorial lens area. The thickest retinal region, at the level of the optic nerve, was least affected. As is well known, the
most radio-sensitive cells in the eye rudiment are mitotic and differentiating cells at the stage of early neuroblasts; younger neuroepithelial cells are more resistant (Hicks, 1950, 1953; Hicks, O'Brien & Newcomb, 1954; Rugh & Wolff, 1955; Rugh & van Dyke, 1962). The thickness of the preserved retinal area varied in different eye rudiments, but on average it approximately equalled the thickness of the iris–ciliary body rudiment. In the pigment epithelium of the retina, lens rudiment, outer layer of the iris–ciliary body rudiment and in the marginal area of the inner iris layer itself no necroses were observed.

The eyes after irradiation at 14-5 days of pregnancy were completely destroyed in half the cases by the time of birth and the orbit was filled up with the fragments of lens, pigment epithelium and other differentiated eye tissues. In the other cases the eyes were not destroyed and in structure were similar to those of the embryos irradiated at 15-5 days of pregnancy, differing from them only by their lesser dimensions and vacuolization of the pigment epithelium on the posterior pole (Plate 2, figs. A, B, C). In some cases after irradiation at 14-5 days the substance of the lens escaped from the ruptured lens capsule. The primary eye cavity was strongly hypertrophied and filled up with macrophages phagocytosing the fragments of dead cells. Cornea, mesenchyme eye coats, pigment epithelium and lens were differentiated.

Table 1. The ratio of the pigmented part to the total length of the inner layer of the iris–ciliary body rudiment in newborn rats (average data) in controls and after irradiation at 400 r at different stages of pregnancy

<table>
<thead>
<tr>
<th>Stage and number of eyes under measurement</th>
<th>Pigmented part (L) in μ</th>
<th>Total length (L) in μ</th>
<th>K = L/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn intact, 4 eyes</td>
<td>118.5</td>
<td>425.7</td>
<td>0.28</td>
</tr>
<tr>
<td>Irradiated at 14-5 days, 12 eyes</td>
<td>312.9</td>
<td>359.2</td>
<td>0.87</td>
</tr>
<tr>
<td>Irradiated at 15-5 days, 10 eyes</td>
<td>276.7</td>
<td>370</td>
<td>0.77</td>
</tr>
<tr>
<td>Irradiated at 17.5 days, 4 eyes</td>
<td>321</td>
<td>655</td>
<td>0.49</td>
</tr>
<tr>
<td>Irradiated at 17.5 days, 8 eyes</td>
<td>94.2</td>
<td>325</td>
<td>0.29</td>
</tr>
<tr>
<td>Irradiated at 19-5 days, 12 eyes</td>
<td>75.7</td>
<td>264</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Though the eye rudiments as a whole at 14-5 days of pregnancy were less resistant to X-rays than at 15-5 days, the reaction of the inner eye cup layer at both stages was the same. By the time of fixation the vitreous body cavity was always absent and the inner eye cup layer adjoined the lens which was covered with the lens epithelium along its whole surface. A major part of the inner eye cup layer had transformed into the intensely pigmented inner iris layer (Plate 2, figs. A, B, C). Only in the optic nerve area was undifferentiated...
retina preserved, having been of different thickness and length in different eyes (Plate 2, fig. B). The retina was built mainly of spindle-like cells with mitoses on the outer surface. Anywhere on the inner surface of these retinas a small number of cells of the future ganglion layer, with large round nuclei, was found. In two cases (one of each stage) the whole inner eye cup layer transformed into the pigmented iris layer (Plate 2, fig. A). To evaluate qualitatively an increase of the pigmented area in the experimental material the total length of the inner layer of the iris–ciliary body rudiment (L), from the pupil margin to the beginning of the retina, and its pigmented part (l) were measured. Then the ratio of these two values was calculated $K = l/L$. In intact newborn rats the ratio of the pigmented area of the inner iris layer to the total length of the iris–ciliary body rudiment, if the length was measured after straightening ciliary folds, equalled 0.28. In Table 1 the average data are given on the absolute and relative increase of the pigmented iris area in the experiments where the cases of the complete transformation of the inner eye cup layer into the pigmented one are not included. In this experiment $K$ equalled 0.87 and 0.77 for

**Explanation of Plates**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>bl.</td>
<td>blood cells</td>
</tr>
<tr>
<td>c.</td>
<td>cornea of the cultivated eye rudiment</td>
</tr>
<tr>
<td>h.c.</td>
<td>the cornea of the host eye</td>
</tr>
<tr>
<td>h.i.</td>
<td>the iris of the host eye</td>
</tr>
<tr>
<td>i.</td>
<td>iris of the eye rudiment</td>
</tr>
<tr>
<td>i.i.c.</td>
<td>inner layer of the ciliary body area</td>
</tr>
<tr>
<td>i.i.i.</td>
<td>inner layer of the iris</td>
</tr>
<tr>
<td>l.</td>
<td>lens</td>
</tr>
<tr>
<td>l.e.</td>
<td>lens epithelium</td>
</tr>
<tr>
<td>m.</td>
<td>mitoses</td>
</tr>
<tr>
<td>n.</td>
<td>optic nerve</td>
</tr>
<tr>
<td>nec.</td>
<td>necrotized cells</td>
</tr>
<tr>
<td>o.l.e.</td>
<td>outer layer of the ciliary body area</td>
</tr>
<tr>
<td>o.l.i.</td>
<td>outer layer of the iris</td>
</tr>
<tr>
<td>p.e.</td>
<td>pigment epithelium of the retina</td>
</tr>
<tr>
<td>r.</td>
<td>neural retina</td>
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</table>

**Plate 1**

Figs. A–C. Stages of normal development of the marginal eye cup zone. Fig. A. At 15.5 days of pregnancy, morphologically undifferentiated iris-ciliary body rudiment. Fig. B. At 17.5 days of pregnancy, beginning of the thinning of the inner iris layer. Fig. C. At 19.5 days of pregnancy, beginning of pigmentation of the inner iris layers.

Fig. D. The marginal part of the inner eye cup layer from which the lens was removed at 17.5 days of pregnancy and cultivated for 10 days in the anterior chamber. The inner layer of the iris-ciliary body rudiment has thickened and has the structure of an undifferentiated retinal rudiment with mitoses on its outer surface. Its tip, as well as the retina in the bottom area, damaged at lens removal, contains necrotic cells.

Fig. E. Another case of inner eye cup layer from which lens was removed at 17.5 days of pregnancy and cultivated for 10 days in the anterior chamber; the unpigmented iris–ciliary body rudiment is preserved.

Fig. F. Differentiation of pigmented iris in the eye rudiment from which lens was not completely removed at 17.5 days of pregnancy and which was cultivated for 7 days in the anterior chamber.

Fig. G. Eye rudiment X-irradiated with 400 r in the female body at 15.5 days of pregnancy and fixed 5 h after irradiation. One may see the vast damage of the outer parts of the retinal rudiment, as well as groups of necrotic cells on the inner surface and inside it. No necroses are seen in the lens, pigment epithelium of the retina, outer layer of the iris-ciliary body rudiment and marginal zone itself of the inner iris layer.
PLATE 1

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14.5 and 15.5 days of pregnancy respectively. Thus at 14.5 and 15.5 days of pregnancy in rats the cells of the peripheral retina and in some cases all the retina cells that survived irradiation were capable of differentiation into the pigmented inner iris layer. The tissue of the ciliary folds at these stages was thus undetermined. The results of this experiment confirm the idea, suggested by mitotic studies, that the peripheral area of the retina and inner layer of the iris-ciliary body rudiment have similar potencies. The extent of the outer neural layer of iris and ciliary body development after irradiation at various stages of pregnancy is given in Table 2.

Table 2. Extent of the outer neural layer of the iris and ciliary body shown at the time of birth after irradiation of pregnant rats with 400 r at different stages of pregnancy

<table>
<thead>
<tr>
<th>Stage of irradiation (in days of pregnancy)</th>
<th>Absent</th>
<th>Present</th>
<th>Absent</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.5 days, 12 eyes</td>
<td>8</td>
<td>2</td>
<td>12</td>
<td>.</td>
</tr>
<tr>
<td>15.5 days, 10 eyes</td>
<td>5</td>
<td>1</td>
<td>10</td>
<td>.</td>
</tr>
<tr>
<td>17.5 days, 8 eyes</td>
<td>.</td>
<td>8</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>19.5 days, 12 eyes</td>
<td>.</td>
<td>12</td>
<td>.</td>
<td>12</td>
</tr>
</tbody>
</table>

PLATE 2

Figs. A–G. Cross-sections of eyes irradiated in the female body at different stages of pregnancy and fixed before birth (22nd day of pregnancy). Fig. A. After irradiation at 14.5 days of pregnancy; the whole inner eye cup layer transformed into pigmented layer. The pigment epithelium of the retina is vacuolized at the posterior eye pole; the outer iris layer cannot be distinguished from the rest of the pigment epithelium; the lens epithelium covers the lens over the whole of its surface; the inner eye layer is moved a little to one side from the lens by the basal lens substance flowing out through the capsule rupture which is visible on the neighboring sections. Figs. B, C. After irradiation at 15.5 days of pregnancy. Fig. B. On the level of the optic nerve an undifferentiated retina is preserved; the optic nerve does not run outside the eye; the pigmented area of the inner iris layer is strongly increased; the outer iris layer is not preserved along the whole rim. Fig. C. More central section of the same eye as fig. B; the whole internal eye cup layer is built from the pigmented iris layer, pigmentation being weaker in proximity to the posterior lens pole. Figs. D, E. After irradiation at 17.5 days of pregnancy. Fig. D. Case of significant increase of the inner layer of the iris-ciliary body rudiment and its pigmented part; the outer iris layer is well expressed. Fig. E. Case where no increase of the pigmented part of the inner layer occurred though it is straightened and pressed against the lens; the flexure of the ciliary zone appeared in the outer layer. Figs. F, G. After irradiation at 19.5 days of pregnancy; no increase of the pigmented iris zone occurred. Fig. F. Both layers of the iris and ciliary zone are present at the beginning of folding of the latter. Fig. G. Case of greater damage of the ciliary zone similar to the less expressed after irradiation at 17.5 days of pregnancy (fig. E).
To be sure that the increase of the pigmented iris area arose as a result of the combined effects of irradiation and lens influence upon the inner eye cup layer, the following additional experiment was performed (series BII). Female rats at 15-5 days of pregnancy were exposed to 400 r of X-rays and the eye rudiments were implanted into the anterior chamber 3 h after irradiation. Some rudiments (12 cases) were implanted together with the lens rudiment and surrounding tissues (experimental control). In the other rudiments (23 cases) the lens rudiment was removed before implantation through a cut in the cornea and without previous trypsinization. The irradiated eye rudiments were cultivated for 5 days (approximately the period between irradiation and fixation in the previous experiments). The results obtained have confirmed the conclusion that the increase of the pigmented iris area is not a consequence of irradiation alone. In eye rudiments with lens almost the whole inner layer of the eye cup became a pigmented layer (Plate 3, figs. A, B). On the posterior pole, either minute retinal regions were preserved, or the iris layer had weaker pigmentation than in its more anterior regions. These implants reproduced accurately the eye structure of irradiated embryos developed in the uterus (Plate 2, figs. A, C). This fact confirmed the conclusion that the development of irradiated eyes depends upon the dose and stage of irradiation and is not much affected by the environment (Rugh & van Dyke, 1962). In those rudiments where the lens had been removed before implantation the inner eye cup layer consisted of undifferentiated retina in which numerous mitoses could be seen (Plate 3, figs. C, D). The iris rudiment was absent and this additionally confirms the conclusion that at 15-5 days of pregnancy it is still undetermined. Thus the increase in the area of pigmented iris observed in the previous experiments (BI) was a consequence of the inductive lens effect upon the reduced mass of competent cells of the inner eye cup layer. The comparison of the eyes implanted with and without lens shows that the lens itself, additionally to irradiation, inhibits division of the eye rudiment cells.

Having demonstrated the possibility of an experimental increase of the pigmented iris area and having shown the specific roles of the lens and of irradiation in this phenomenon, we analysed the following stages of the eye development in order to find the stage of determination of the iris and ciliary body areas. Females were irradiated at 17-5 and 19-5 days and the embryos

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**Plate 3**

Figs. A–D. Eye rudiments X-irradiated with 400 r in the female body at 15-5 days of pregnancy and cultivated subsequently in the anterior chamber for a period of 5 days. Fig. A. Eye rudiment cultivated with lens; the inner eye layer is represented by the thin pigmented layer; in proximity to the posterior lens pole two small retinal thickenings are seen. Fig. B. Sector of the same eye at greater magnification; the difference in morphology of the inner iris layer from the pigment epithelium of the retina is noticeable. Figs. C, D. Eye rudiments cultivated without lens; in both cases the inner eye cup layer is built of the undifferentiated retina with mitoses.
were then sacrificed before birth and studied histologically (series BIII). The results are given in Tables 1 and 3. After irradiation at 17-5 days of pregnancy the length of the whole iris–ciliary rudiment and its pigmented part was much increased in half the cases, the increase of the length of the latter having been much greater ($K = 0.49$). The pigmented iris area consisted of a very fine cell layer much less intensely pigmented than the inner iris layer in the series BI (Plate 2, fig. D). In the other cases the inner layer of the iris–ciliary body rudiment was straightened and pressed against the lens (Plate 2, fig. E), but there was no increase of the pigmented area ($K = 0.29$). The disintegration of retinal layers and rosette formation were observed. Thus at this stage the increase of pigmented iris area at the expense of the ciliary body zone was realized with greater difficulties than at the previous stage and in some cases was not realized at all. The comparison of these results with the data of series A, where in some cases the inner iris layer was preserved after lens removal at 17-5 days of pregnancy, but in other cases transformed into the retina rudiment, allows us to conclude that at 17-5 days of pregnancy the internal iris layer and the boundary between it and the ciliary zone are in a state of labile determination. Finally, after irradiation at 19-5 days the rudiments of iris and ciliary body were both present (Plate 2, fig. F), but were delayed in their development when compared to the eyes of intact newborn rats. In some cases of greater radiation damage the ciliary zone was straightened and pressed against the lens epithelium as in the case of the previous stage (17-5 days of pregnancy), but the pigmented area of the inner iris layer was not increased in them also (Plate 2, fig. G). In all the cases after irradiation at 19-5 days of pregnancy, $K = 0.28$, i.e. did not change in comparison to the control. To exclude an objection that the increase of the pigmented area of the inner iris layer was absent in this series because of the small time interval between irradiation and fixation a pregnant female was irradiated at 19-5 days and the litter were sacrificed when they were 3 days old (5 days after irradiation). In these animals iris and ciliary body had a

Table 3. The time of determination of the neural iris–ciliary body rudiment in rat embryos according to the data on lens removal (series A) and irradiation (series B)

<table>
<thead>
<tr>
<th>Stage in days of pregnancy</th>
<th>Inner layer of the iris</th>
<th>Inner layer of the ciliary body</th>
<th>Outer layer of the iris</th>
<th>Outer layer of the ciliary body</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-5</td>
<td>–</td>
<td>–</td>
<td>±</td>
<td>–</td>
</tr>
<tr>
<td>15-5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>17-5</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>19-5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

−, Undetermined state; ±, state of labile determination; +, state of stable determination.
structure typical of this stage. Thus the segregation of the inner layer of the common iris–ciliary body rudiment into the iris and ciliary body areas in rats was beginning to be realized during the 18th day of pregnancy and was correlated with the appearance of heterogeneity by the duration of $G_1$-phase of the mitotic cycle in the proliferating cells of this rudiment. At 19-5 days of pregnancy the areas of iris and ciliary body were already determined.

Proliferative activity progressively decreased in the inner iris layer as development proceeded. The ciliary folds region includes the zone of high proliferative activity at the boundary between the iris–ciliary body and retinal rudiments (Fig. 3). The differentiation of the iris and ciliary body continued after birth. After irradiation of the head region of mice between the 1st and 8th post-natal day with 1000 r, the straightening of ciliary folds as well as the inhibition of pigment accumulation in the inner iris layer were still possible. The disturbance caused by irradiation decreased with age and completely disappeared after the 12th day (Pierro & Chase, 1962).

In spite of the fact that after irradiation at 15-5 days of pregnancy no cell death was observed in the outer neural layer of the iris–ciliary body rudiment, the rudiment did not always keep its individuality (Table 2). In some cases it did not differ from the pigment epithelium of retina in its thickness and cell form though it did possess the stromal mesenchyme of the iris rudiment (Plate 2, fig. A). In other cases the external layer as a thickened structure was not preserved along the whole rim, that is why on the cross-sections it was present only at the one side of pupil margin (Plate 2, figs. B, C). Ciliary folds were always absent. After irradiation at 17-5 days of pregnancy the external iris layer was present in all cases though its dimensions were somewhat reduced when compared to control eyes. The formation of the ciliary body flexure in the external layer (Plate 2, figs. D, E) could be realized or inhibited. And, finally, after irradiation at 19-5 days of pregnancy the ciliary flexure in the external layer was always present. Thus, according to the results of experiments on irradiation at 14-5 and 15-5 days of pregnancy the external iris layer was in a state of labile determination and the outer ciliary folds layer was still undetermined. At 17-5 days of pregnancy the outer iris layer was determined and the zone of ciliary folds was in a state of labile determination. At 19-5 days of pregnancy both parts of the eye margin were determined.

The development of the outer layer of the iris–ciliary body rudiment differed from that of the inner one in the dynamics of proliferative activity. In spite of the fact that at 15 days of pregnancy the proliferative activity in the outer neural layer of the iris–ciliary body rudiment was already much lower than in the inner one (Text-fig. 3), this level was maintained until the 18th day of pregnancy and had fallen insignificantly by birth. At this time the index of labelled nuclei in the pigment epithelium and inner iris layer sharply decreased and by birth equalled approximately 6 % in both structures (Text-fig. 3). Thus, contrary to previous ideas (Mann, 1949; Dejean, Leplat & Hervouët, 1958;
Duke-Elder & Cook, 1963), it is the external layer of the iris and not the inner one which is more active in its growth (Zavarzin & Stroeva, 1964). Relatively high mitotic activity in the marginal zone of the outer eye cup layer was observed in chick embryos as well (Coulombre, Steinberg & Coulombre, 1963). A high proliferative activity in both layers of the ciliary body area was maintained at the same level between the 20th day of pregnancy and birth (Text-fig. 3).

**DISCUSSION**

The study of the determination time in the development of the iris–ciliary body rudiment in rat embryos has shown the following. The first sign of the transition of the inner layer of the iris–ciliary body rudiment to differentiation when compared to the peripheral retina was a slight decrease of proliferative activity as some cells ceased to reproduce. At this stage the inner layer of the iris–ciliary body rudiment was undetermined and had the same potencies as the peripheral retina in which all the cells at this stage proliferated. After lens removal the iris–ciliary body rudiment differentiated into retina and under certain experimental conditions the peripheral retinal zone might give rise to the pigmented iris layer. The period of labile determination of the iris–ciliary body rudiment correlated not only with the progressive loss of cells from reproductive activity, but also with the increase in the duration of G1-phase in proliferating cells and the appearance of heterogeneity in the cell population in respect of this phase. By this criterion it is the outer iris layer which is the first to enter the state of labile and of stable determination; this layer, however, keeps for a long time a low but stable proliferative activity which contributes to the general growth of the iris rudiment (Zavarzin & Stroeva, 1964). At 17–5 days of pregnancy the inner iris layer, and inner and outer layers of the ciliary area, were in a state of labile determination. Shortly before birth (19–5 days of pregnancy) all these areas of the iris–ciliary body rudiment were already determined. The data obtained concern only the tissue properties of the marginal zone of the developing eye, as it is known that the morphogenesis of the definitive ciliary body folds is achieved later and requires additional factors for its realization. The most important of these factors is the developing intraocular pressure (Coulombre & Coulombre, 1957). The differentiative factors in the development of the outer neural layer of the iris and ciliary body are practically unknown and were not studied. The data on the time of determination of the outer layer of the iris and ciliary body detected by its resistance to X-rays obtained in the present work may facilitate further studies in this direction. A more detailed study of the factors upon which the differentiation of the outer layer of the iris ciliary body rudiment depends will allow us to understand how the correlation between the time of determination and growth of both outer and inner layers is realized during the creation of the definitive structure of iris and ciliary body.
As is shown in the present study the process of determination of the inner layer of the iris-ciliary body rudiment requires a prolonged contact with the lens epithelium. Both structures—iris and ciliary body—arise in the inner layer of the eye cup under the influence of the lens. The final differences in their structures and properties are probably connected not only with possible differences in the influences from the anterior lens epithelium and from its equatorial zone, but also with duration of contact with the lens epithelium, which is more prolonged in the case of the pigmented iris layer. The close contact of the iris rudiment with the lens prevents the folding of the iris, to which the iris-ciliary body rudiment as a whole has a tendency (Stroeva, 1963). The separation of the ciliary zone from the lens took place as soon as the formation of the vitreous body cavity began. This separation caused the preservation of high proliferative activity in the ciliary zone, as well as realization of its ability to fold. The further differentiation of the ciliary zone had properties intermediate between retina and iris kept for a long time. In newts under certain conditions (Wachs, 1920; Hasegawa, 1958) and in anuran tadpoles (Lopashov, 1955; Dabagian & Sheresheva, 1966) the ciliary zone is a source of retinal regeneration and, at the same time, having been in contact with the lens epithelium is able to differentiate into pigmented iris tissue (Dabagian et al. 1966).

It is known that the influence of retina on lens epithelium leads to the transformation of epithelial cells into lens fibres (Mikami, 1941; Velikanova, 1963; Coulombre & Coulombre, 1963; Coulombre, 1965a, b; Reyer, 1966a, b) and the influence of the lens epithelium in its turn inhibits proliferative activity and neuroblastic potencies in the cells of the inner layer of the eye cup which are in contact with it (present communication). It is easy to understand how both transitional zones, the ciliary zone in the inner eye cup layer and the equatorial lens zone, are interrelated in their localization as was mentioned by Coulombre & Coulombre (1963). In the zone of separation of the inner layer of the eye cup from the lens epithelium the cells of the latter begin to be influenced by retina and transformed into lens fibres while all the cells lying anterior to the lens epithelium are preserved from this influence by the close tightening to the internal iris layer. The ciliary folds rudiment, in its turn, having suffered some transformations under lens influence is preserved in the zone of separation from further lens influence and transformation into iris. It is not excluded that the influence of the equatorial zone of the lens upon the ciliary area though weakened by distance is still realized after separation. Thus the properties different from those of the peripheral retina may be determined in the ciliary area.

Though the molecular mechanisms of the lens influence upon the inner layer of the iris-ciliary body rudiment are unknown and require special methods for their study the present investigation shows that one of its results is an inhibition of cell reproduction in the inner layer of the eye rudiment and another is the
switching of the eye rudiment cell to pigment synthesis. Now it is known that cells of the whole primary eye rudiment acquire not only neuroblastic potencies, but are also melanoblasts as well, i.e. according to the terminology of Gordon (1959) unpigmented cells potentially capable of melanin synthesis. The eye cells are switched from the neuroblastic path on to the melanocyte one by means of the mesenchyme which surrounds the eye cup (Stroeva, 1960; Lopashov, 1963) and the lens (Stroeva, 1963, present communication). Though the cells of both structures, the pigment epithelium of the retina and the pigmented iris layers, belong to the class of epithelial melanocytes they differ from each other in morphological and physiological properties. In the eye of adult animals they definitely differ in their ultrastructure, but obviously represent modifications of a common cell type characterized by smooth endoplasmic reticulum (Porter & Yamada, 1960; Toussimis & Fine, 1961; Bernstein, 1961). The outer iris layer shows, beside melanocyte differentiation, muscle differentiation as well. In the ciliary zone a fourth potential type of cell differentiation in the eye rudiment is shown (after neuroblastic, melanoblastic and myoblastic), secreting cells which form the aqueous humor and pass it into the inner eye medium (Kinsey, 1950; Kinsey, Jackson & Terry, 1945; Kinsey & Palm, 1955; Pease, 1956; Kuhlman & Kaufman, 1960). When keeping in mind that in newts (Stone, 1950a, b) and some other animals the differentiation of the pigmented eye parts does not exclude the possibility of their later revealing initially suppressed neuroblastic potencies, the eye rudiment cells of vertebrates seem to represent an interesting system for studying ontogenetic control mechanisms by genome function.

**Summary**

1. In experiments combining irradiation and cultivation of rat eye rudiments in the anterior chamber with and without lens at successive developmental stages (14-5–19-5 days of pregnancy), the time of determination of the neural part of the iris–ciliary body rudiment was studied.

2. At 14-5 and 15-5 days of pregnancy the inner layer of the iris–ciliary body rudiment is still undetermined. After lens removal and subsequent eye cultivation in the anterior chamber the pupil margin thickens and transforms into the retina rudiment. After X-irradiation at 400 r which causes destruction of more than half of cells in the retina rudiment a significant increase of the pigmented iris occurs at the expense of the areas of the ciliary body and peripheral retina in the developing eye in situ, as well as in the eyes cultivated in the anterior chamber. In some cases the whole inner layer of the eye cup transforms into pigmented iris. During the cultivation of irradiated eyes without lens the inner eye cup layer keeps the structure of the retina rudiment. At these stages proliferating cells of the inner layer of the iris–ciliary body rudiment and of the peripheral area of the retina have the same total duration of the mitotic cycle and of its separate phases, but these zones differ a little in the number of cells which take part in proliferation.

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3. At 17-5 days of pregnancy the rudiments of the inner layer of the iris and ciliary folds are in a state of labile determination. In lens-free eye rudiments of this age cultivated in the anterior eye chamber, the inner layer of the iris–ciliary body rudiment is preserved in some cases though it has no typical structure and is not pigmented. Ciliary folds are absent. After irradiation at 400 r an increase of pigmented area at the expense of the ciliary area takes place in half the cases, but in other eyes no increase of the pigmented area is observed, though the ciliary zone is straightened and pressed against the lens.

4. The preservation of the normal ratio of the pigmented part of the inner iris layer and unpigmented ciliary folds after irradiation shows that both the areas of the eye margins and the boundary between them are already determined at 19-5 days of pregnancy.

5. Conclusions are drawn on the time of determination of the outer layer of the iris–ciliary body rudiment from its resistance to X-irradiation in conditions of separation from the inner layer. At 14-5 and 15-5 days of pregnancy the outer iris layer is in a state of labile determination; at 17-5 days of pregnancy it is already determined. The outer ciliary folds layer is in a state of labile determination at 17-5 days of pregnancy and is determined at 19-5 days of pregnancy.

6. The comparison of data obtained with the results of autoradiographic study (³H-thymidine) shows that the period of labile determination of the iris and ciliary body rudiments is correlated with the progressive removal of cells from mitotic activity, as well as with the increase in the duration of G₁-phase in proliferating cells and the appearance of heterogeneity in the cell population in respect of the duration of this phase.

РЕЗЮМЕ

Соотношение процессов пролиферации и детерминации в морфогенезе падужины и цилиарного тела у крыс

1. В комбинации опытов облучения и культивации зачатков глаз крыс в передней камере с линзой или без неё на последовательных стадиях развития (14,5–19,5 суток беременности) были изучены сроки детерминации нейральной части цилиарно–радужинного зачатка.

2. На стадиях 14,5 и 15,5 суток внутренний листок цилиарно–радужинного зачатка ещё не детерминирован. После удаления линзы и последующей культивации глаза в передней камере зрачковый край утолщается и дифференцируется в зачаток сетчатки. После облучения в дозе 400 р, вызывающей деструкцию более половины клеток зачатка сетчатки, происходит значительное увеличение пигментированной радужины за счет зон цилиарных складок и периферической сетчатки in situ и в культивируемых зачатках глаз. В части случаев весь внутренний листок глазной чаши трансформируется в пигментированный листок радужины. При культивации облученных глаз после удаления линзы внутренний листок глазного
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бокала сохраняет строение зачатка сетчатки. На этих стадиях пролиферирующие клетки внутреннего листка цилиарно—радужинного зачатка и периферической сетчатки имеют одинаковую продолжительность митотического цикла и его отдельных периодов, но слетка различаются по количеству клеток, находящихся в цикле репродукции.

3. На стадии 17,5 суток беременности зачатки внутреннего листка радужины и цилиарных складок находятся в состоянии лабильной детерминации. В беллизированных зачатках этой стадии, культивируемых в передней камере, в части случаев зачаток внутреннего листка радужины сохраняется, хотя и не имеет вполне типичного строения и не пигментируется. Цилиарные складки отсутствуют. После облучения к рождению в половине случаев пигментированная зона увеличивается за счет цилиарных складок, в других глазах такого увеличения не происходит, хотя цилиарная зона была спрятана и прижата к линзе.

4. Сохранение нормального соотношения зон пигментированной части внутреннего листка радужины и неpigmentированных цилиарных складок после облучения свидетельствует о том, что территории этих зачатков уже детерминированы на стадии 19,5 суток беременности.

5. Сделаны заключения о сроках детерминации наружного листка цилиарно—радужинного зачатка по его радиорезистентности в условиях разобъщения с внутренним листком в опытах облучения. На стадиях 14,5 и 15,5 суток наружных листок радужины находится в состоянии лабильной детерминации и уже детерминирован на стадии 17,5 суток; наружный листок цилиарных складок находится в состоянии лабильной детерминации на стадии 17,5 суток и уже детерминирован на стадии 19,5 суток.

6. Сопоставление полученных результатов с данными авторадиографического исследования (³H-тимидин) показывает, что состояние лабильной детерминации изученных зачатков коррелирует как с прогрессирующим выходом клеток из митотического цикла и с увеличением длительности периода G₂, так и с возникновением гетерогенности клеточной популяции по продолжительности этого периода.

REFERENCES


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