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Cell replication and terminal differentiation in the embryonic chicken lens: normal and forced initiation of lens fibre formation

By JOHAN ZWAAN[1] AND ROBERT E. KENYON, JR[2].

[1]*Department of Ophthalmology, Massachusetts Eye and Ear Infirmary and Harvard Medical School, 243 Charles Street, Boston, Massachusetts, MA02114, U.S.A.*

[2]*Department of Anatomy, School of Medicine, University of Virginia, Charlottesville, Virginia, U.S.A.*

SUMMARY

The relation between cell replication and lens fibre formation was studied in normal 5-day chicken embryos and in embryos in which the lens was reversed. After this microsurgery the epithelial cells were facing the retina and were forced to start fibre differentiation. Embryos were treated with [^{3}H]thymidine followed by autoradiography or with Colcemid to induce metaphase arrest.

About half of the control epithelial cells were actively replicating (in contrast, in the earliest stages of lens formation all cells are multiplying). Half of the non-dividing population was accounted for by the annual pad cells. The other half, probably in a resting phase (GO) of the cell cycle, was perhaps slightly more common in the central epithelium. This suggests that a germinative zone may be forming in the lens epithelium as early as 5 days of embryonic life.

Normal epithelium had a cell cycle time of about 11 h, with S-phase, M, G1 and G2 lasting 5·5, 0·5, 2, and 3 h. After reversal the labelling index remained at control levels for about 4 h and then dropped to zero at 9 h. Similarly, the mitotic index gradually decreased to zero in 15.

It appears that reversal forces the epithelial cells to cease replication when they reach G1. Cells already in GO may form fibres without first going through DNA synthesis. The very sharp boundary between dividing and non-dividing cells at the border of the optic cup suggests that the retina may regulate the irreversible cessation of cell replication associated with lens fibre differentiation.

INTRODUCTION

In the earliest stages of chicken lens development growth is accomplished by replication of all lens cells (Zwaan & Pearce, 1970, 1971). With the transformation of the posterior lens vesicle wall into primary lens fibres DNA synthesis and mitosis stop in this area (Modak, Morris & Yamada, 1968; Persons & Modak, 1970); cell division becomes restricted to the anterior epithelium. Late in embryonic life replication for the most part becomes restricted even more,

to a narrow peripheral band of the epithelium termed the germinative zone (Reeder & Bell, 1965; Hanna & Keatts, 1966; Persons & Modak, 1970). The most peripheral zone of the anterior epithelium, in between the germinative zone and the equator, shows no cell division. Cells of this region are more columnar than the remainder of the epithelium and collectively are known as the annular pad. Additional cells are formed in the germinative zone throughout life. Some, being displaced towards the anterior pole, may enter the resting population of the central epithelium. Most become part of the annular pad and differentiate into lens fibres, once they reach the equator.

It is not known how cell replication or its cessation are regulated in the lens tissue. Influences from the neural retina and the mesenchyme of the optic cup rim promote lens fibre differentiation (Coulombre & Coulombre, 1963, 1969), one aspect of which is the stopping of cell cycle activity.

Coulombre & Coulombre (1963) have devised an elegant technique to force all epithelial cells to initiate lens fibre formation almost immediately. This is done by surgically rotating the lens of the 5-day embryo, causing the epithelium to face the retina. The original lens fibres, now opposite the cornea, cease elongation and the cells at the equator become the source for a new anterior epithelium, which is almost normal 10 days postoperatively. The neural retina may regulate this intriguing phenomenon.

Coulombre & Coulombre (1963) described the long-term morphological effects of lens reversal. We tried to extend their work by asking if epithelial cells immediately stop replicating after lens reversal or if they are arrested in a particular phase of the cell cycle. To answer these questions it was necessary to obtain information on cell population kinetics of the normal 5-day embryonic chick lens, and to study possible alterations in cell replication following lens reversal.

MATERIALS AND METHODS

Embryos

White Leghorn eggs were incubated at 38 °C for 5 days. A window of about 1·5 cm^2 was cut into the shell and egg membrane. Stage-27 embryos (Hamburger & Hamilton, 1951) were selected and a small opening was made in the amnion above the right eye. With a no.5 watchmaker's forceps the eye was punctured at the limbus opposite the choroid fissure; this site is less vascularized than others. By gently opening and closing the forceps the wound was enlarged circumferentially. The lens capsule was grasped at the posterior pole and the lens was extracted. The same lens, or in a few cases one from a donor embryo, was reintroduced into the eye, with the epithelium facing the retina and the fibres the cornea. [^{3}H]thymidine or Colcemid were injected into the eye through the same incision (see below). Controls were treated as the experimental embryos, but their lenses were not extracted. Afterwards the

windows in the shells were sealed with parafilm and wax. The eggs were returned to the incubator for varying times.

Labelling methods

Embryos were treated with 0·5 μCi[^{3}H]thymidine in 10 μl (specific activity 6·7 or 2·0 Ci/mmole; New England Nuclear Corp., Boston, Massachusetts). In the first series label was injected intraocularly immediately after lens rotation. At hourly intervals up to 15 h after injection the eyes were removed, fixed in Carnoy's liquid and embedded in paraffin. 58 usable reversed lenses were obtained.

In the second series the label was administered 1–24 h after reversal. Eggs were incubated for one additional hour prior to harvesting. 36 reversed and labelled lenses were obtained.

In control embryos the precursor was applied intraocularly and eyes were removed up to 24 h later. In addition we took left, untreated, eyes from embryos that had been operated and injected on the right side. The left eyes had labelling indices comparable to those of injected controls; apparently diffusion or recirculation of precursor was sufficient to insure normal labelling of the uninjected eyes. Because results for the two control groups were the same, the data were pooled. 44 lenses were used, 30 from injected right eyes and 14 left ones.

Autoradiography

Sections of 4 μm were cut parallel to the optical axis of the eye and stained with Harris–Lillie haematoxylin and aqueous eosin. Slides were coated with Kodak NTB-2 liquid emulsion (Eastman–Kodak Organic Chemicals, Rochester, New York), air dried and stored at 4 °C in light-tight boxes containing silica gel. Test slides indicated that an exposure of 14 days was adequate to show a maximum percentage of labelled cells. This time was used consistently. The autoradiographs were developed, fixed and mounted according to routine methods (Kopriwa & Leblond, 1962).

Colcemid treatment

10 μl of a 2×10^{-5}M solution of Colcemid (CIBA, Fairlawn, New Jersey) in buffered saline was injected into the right eye of nine embryos. Four were obtained 1 h, the others 2 h after treatment. The eyes were removed, fixed and embedded. 4 μm sagittal sections were stained with haematoxylin and eosin.

Counting methods

For maximum accuracy all counting was done with the 100 × oil immersion objective at a magnification of 1250 ×. Nuclei with at least four superimposed silver grains were considered labelled. Background labelling was 0–4 grains

per 100 μm^2. Total number of epithelial cells in each section, labelled and unlabelled nuclei and mitotic figures, and in the case of the Colcemid-treated embryos, nuclei arrested in metaphase were scored. For the labelling indices a minimum of four alternating central sections (or about 1500 cells) was counted for each lens. For the mitotic scoring at least ten sections (3000–4000 cells) were used. To obtain the labelling patterns in control lenses labelled and unlabelled nuclei were counted in groups of 10 cells from the last annular pad cell on one side to the one on the other side. The last annular pad cells were considered to be the last cells bordering the anterior aspect of the obliterated lumen of the previous lens vesicle stage. Since the number of cells per section varied from about 300 to 400 the average labelling index in groups of 10 was determined for the first 140 cells from each equator. Any cells remaining in the middle were included in a separate group (M), containing about 10–100 cells. Its average percent labelled cells was plotted in the middle of the curve.

Calculations of cell population kinetics

The definitions of various parameters of cell population kinetics and their abbreviations are listed in Table 1. At this stage of development the lens is enclosed entirely within its capsule and cell loss or addition need not be taken into account.

Table 1. *Definitions of the symbols used*

Term	Symbol
Total cell generation time (cell cycle duration)	G
Potential population doubling time (assuming no cell loss)	T
DNA-synthetic phase	S
Mitosis	M
Postmitotic gap phase	G1
Post-DNA-synthetic gap phase	G2
Time spent in phase indicated by subscript	t . . .
Total number of cells in the population	N
Number of cells in phase indicated by subscript	N . . .
Fraction of cells in division (mitotic index)	M.I.
Fraction of cells arrested in metaphase by drug treatment	A.I.
Time after drug treatment	t_a
Fraction of cells labelled by [^{3}H]thymidine (labelling index)	L.I.
Growth fraction or proliferation index	f
Resting phase (reversible)	G0

Cell cycle times were measured by the method of Quastler & Sherman (1959). Even though some cells are dropping out of the cell cycle in the 5-day embryonic lens, this organ resembles an exponentially growing population. Correction factors, λ and ln 2, need to be introduced to compensate for the resulting exponential age distribution of cells within one cycle. According to Steel (1968):

$$\lambda = \frac{T}{t_S}\left\{ \exp\left[\frac{\ln 2}{T}(t_{G2} + t_S) \right] - \exp\left[\frac{\ln 2}{T}(t_{G2}) \right] \right\} \quad (1)$$

where t_{G2} includes the mitotic time. In practice ($t_{G2} + \frac{1}{2}t_M$) was used, the error thus introduced being very small. T, the population doubling time can be calculated independently from the percentage of Colcemid-arrested metaphases:

$$T = \frac{\ln 2 \times t_A}{A.I.} \quad (2)$$

Mitotic activity can also be used:

$$T = \frac{\ln 2 \times t_M}{M.I.} \quad (3)$$

The mitotic duration, which needs to be known to use Equation (3) can be obtained from the Colcemid data:

$$t_M = \frac{t_A \times M.I.}{A.I.} \quad (4)$$

or from the [^{3}H]thymidine labelling experiments:

$$\frac{\lambda \times t_S}{L.I.} = \frac{\ln 2 \times t_M}{M.I.} \quad (5)$$

We determined the growth fraction based on the work of Mendelsohn (1962):

$$f = \frac{G \times L.I.}{\lambda \times t_S} \quad (6)$$

As shown below, the epithelium of the 5-day embryonic chicken lens does not constitute a pure exponentially growing homogeneous population. Therefore, the calculated values for the population kinetics paramaters should be considered as qualitative rather than as quantitatively exact.

RESULTS

The 5-day embryonic lens (Fig. 1) has a pseudostratified anterior epithelium with three to five layers of nuclei. The epithelial thickness increases from the centre to the periphery and the annular pad, 30–40 cells wide beginning from

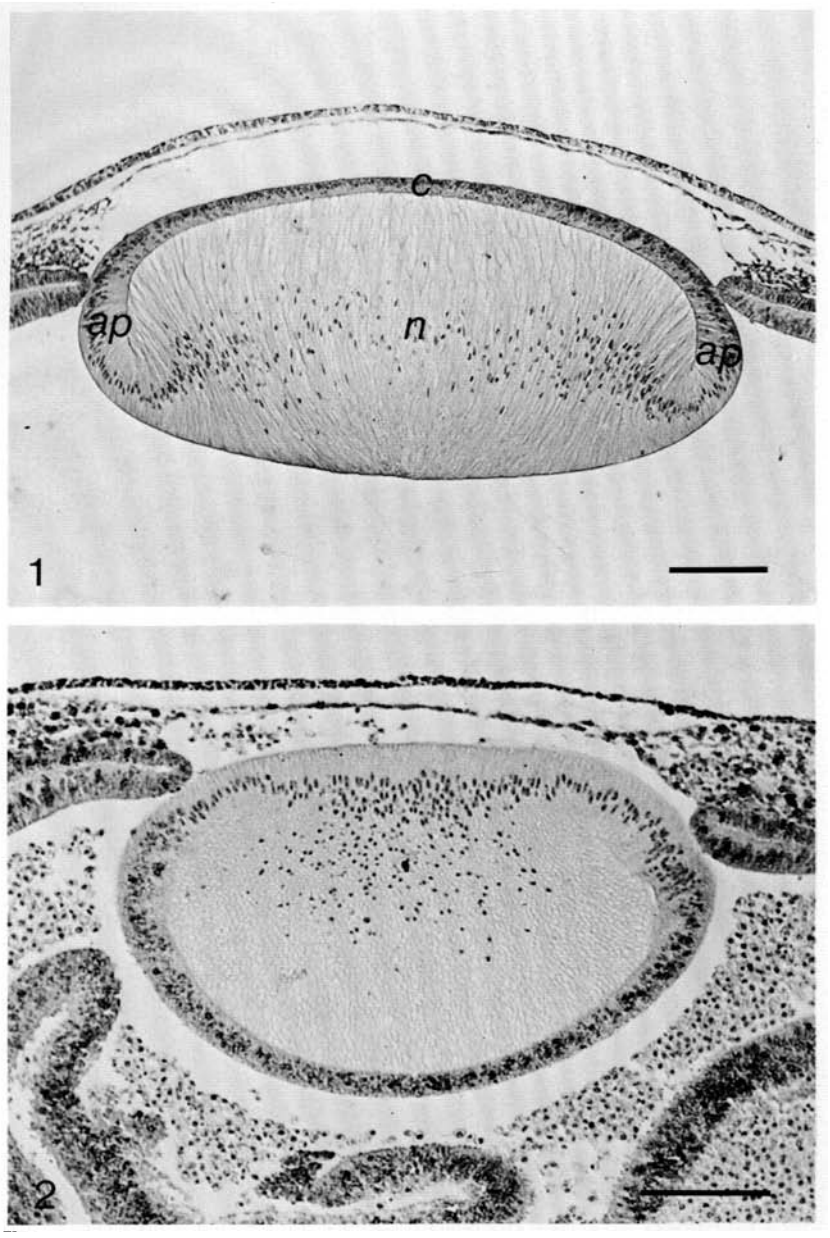


Fig. 1. Cross section through the centre of a 5-day embryonic chicken lens (stage 27). c, central part of the epithelium; *a.p.*, annular pad; *n*, nucleus or core of the lens body (lens fibre mass). × 125. Scale bar = 100 μm.

Fig. 2. Stage-27 lens fixed 3 h after reversal with the epithelium now facing the neural retina. Due to partial collapse of the eye the neural retina is detached and thrown into folds. There is some blood in the vitreous. These artefacts, often seen in the first hours postoperatively, had no discernible effect on the differentiation of the lens epithelium. × 170. Scale bar = 100 μm.

the equator, consists of distinctly columnar cells. The structure of this zone is different from that of the rest of the epithelium. The cells are arranged in a very regular fashion and the nuclei are found in a single layer at the basal pole of the cells, adjacent to the lens capsule.

After the initial trials, reversals of the lens were successful in about 80% of the cases. The morphology of the organ underwent no apparent change during the first postoperative 24 h (Fig. 2). Due to the incision at the corneal limbus and the extraction of the lens a loss of some vitreous often occurred with consequently a partial collapse of the eye. This sometimes led to detachment of the neural retina from the pigment layer and folding of the first. Frequently some bleeding within the eye could be seen as well (Fig. 2). These traumata had no discernible effect on the changes in epithelial cell division that followed lens reversal.

Labelling of the normal lens

To see if regional differences in labelling existed, labelling indices were determined for groups of 10 cells (Fig. 3) at various times after the injection of [^{3}H]thymidine. Variations in the index appeared to be mostly random, but there was some indication that in the central zone of the epithelium perhaps slightly less labelling took place. In the first few hours no reaction was seen in the most peripheral 30–40 cells, but with the passage of time increasing numbers of cells in the annular pad showed radioactivity. Because the overall labelling index was almost maximal after 1 h (see below), indicating that very little free [^{3}H]thymidine was available for incorporation from this time on, the increasing activity in the annular pad was interpreted to be the result of migration of labelled cells into the area and not of late DNA synthesis. It took 14–15 h before cells with radioactive nuclei reached the very end of the epithelium, having been displaced a distance of up to 40 cell widths. This indicates a migration rate of roughly two cell diameters per hour. A summary of the data for all the annular pads counted (Fig. 4) agrees with these interpretations.

Labelling index

One hour after the injection of tracer the control epithelia had an average L.I. of approximately 20. For the next 3 h this increased slightly to 25 (Fig. 5). Apparently, the bulk of the incorporation of the radioactive precursor into DNA takes place within the first hour, after which the label has been diluted enough to contribute little or nothing to DNA being replicated. Diffusion away from the injection site with redistribution through the contents of the egg, catabolism, and dilution by cold precursor may be responsible. In any case, it indicates that a short-term labelling was accomplished and that cell cycle analysis by the labelled mitoses method was feasible. From 6 h after treatment on the L.I. was higher, up to about 30. This may reflect the entry of labelled

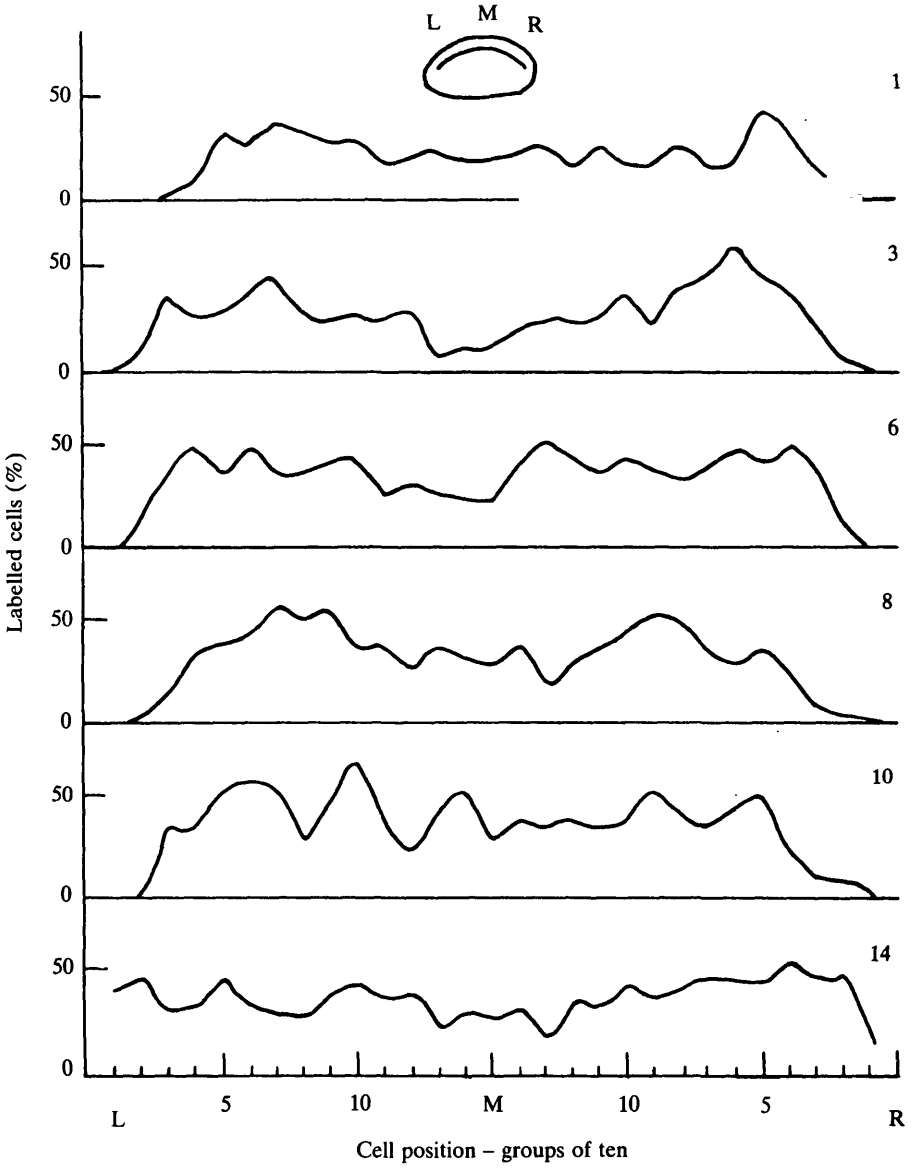


Fig. 3. Labelling indices of epithelial cells counted in groups of 10 from the last cell on the side (L) to the last one on the other side (R), as indicated in the small diagram on the top. Hours after the injection of [^{3}H]thymidine are given at the right margin. The number of cells per cross section varied from about 300 to 400. All central cells, more than 140 cells away from the equator, were therefore grouped together (M) to allow direct comparison of different lenses.

cells into mitosis, causing an increase in radioactive cells. In addition, unlabelled cells are lost for the first 15 h, when they are displaced around the equator to become lens fibres. Both factors may contribute to the small increase in L.I.,

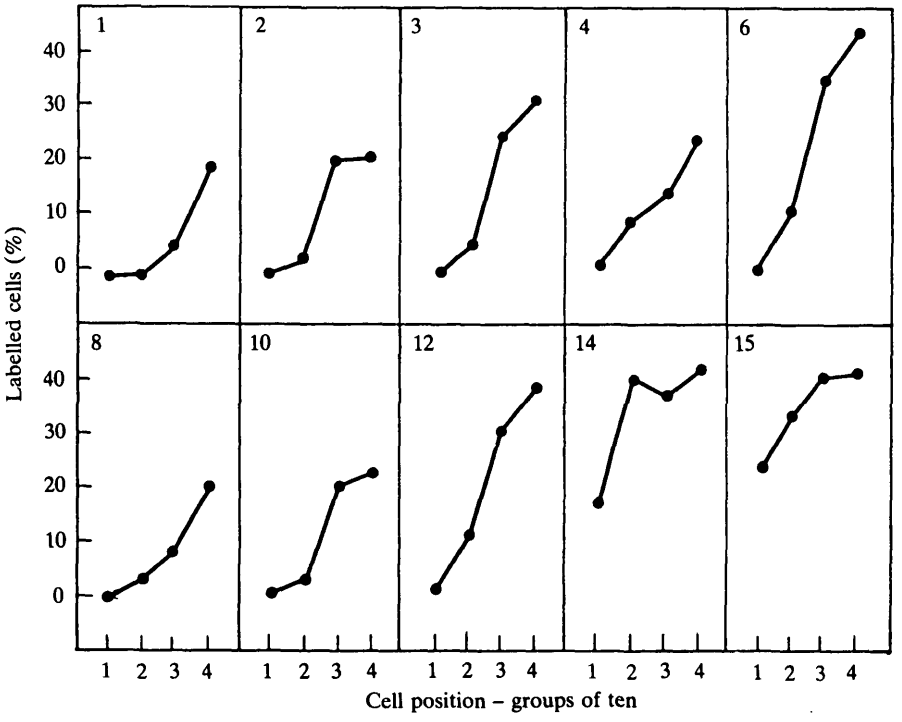


Fig. 4. Average labelling patterns found in the first four groups of 10 cells each in the annular pad, at hours (indicated on the left) after administration of label. The most peripheral cell groups are at the left. With time the curves shift from the lower right to the upper left, showing that labelled cells are migrating into the annular pad from adjacent regions.

which therefore probably does not indicate renewed synthesis of labelled DNA.

The reversed lenses, injected at the time of surgery, showed the same incorporation as the controls (Fig. 5). At the end of the period studied the L.I. of normal lenses were somewhat higher than those of the experimental ones. This is perhaps a sign that cell division and lens fibre formation at the equator, while continuing in the normal lenses, are going down in the reversed organs.

A very different picture was offered by reversed lenses exposed to label at intervals following the operation (Fig. 6). For the first 3–4 h the L.I. were the same as for the controls, but later they started to drop to become 0 at 9 h postoperatively and afterwards. This cessation of DNA synthesis was sharply limited to those parts of the epithelium that had surgically been relocated in the posterior chamber of the eye. In the anterior chamber replicative activity continued. This was particularly well demonstrated when the reversal had not been entirely successful, for instance when the rotation was only 90° rather than 180° (Figs. 7–9). Thus, epithelial cells were facing anteriorly as well as posteriorly. In these cases there was a sharp boundary at the rim of the optic cup between unlabelled posterior cells and labelled anterior ones.

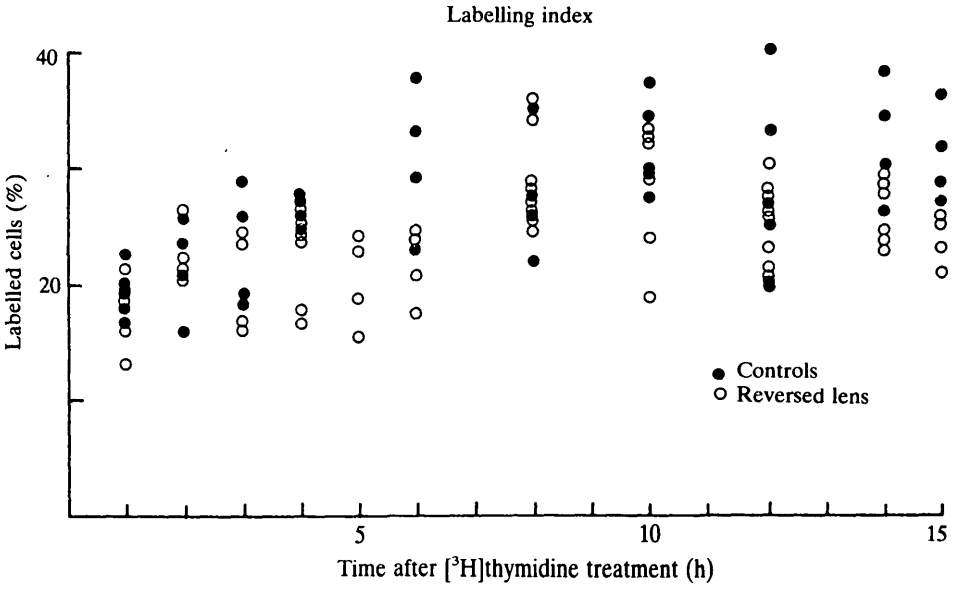
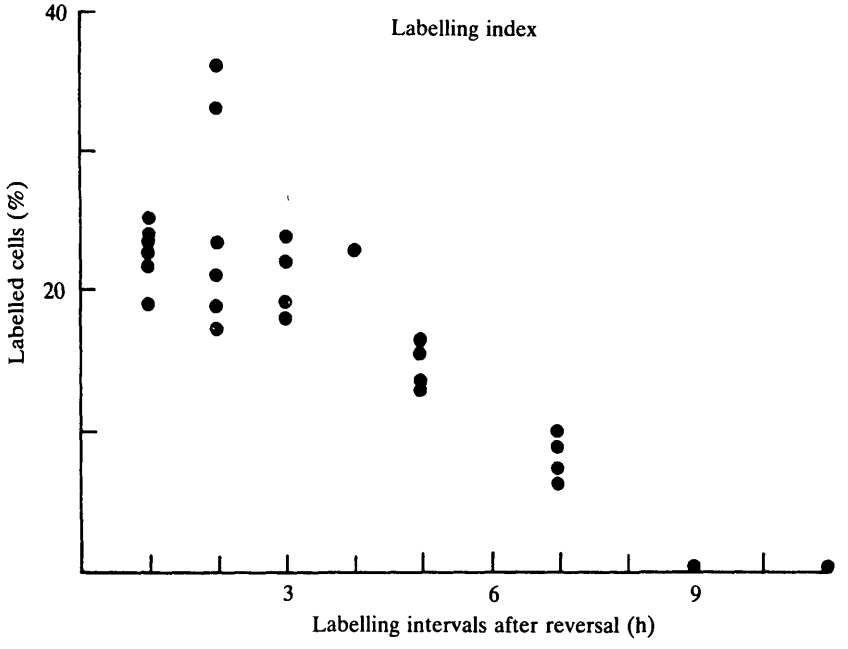


Fig. 5. Labelling indices of the entire epithelia of normal and reversed 5-day embryonic chick lenses. Label was applied at time 0 (in case of reversed lenses immediately after the operation). The index is 0·20–0·25 for the first 4 h and increases to about 0·30 from 6 h on, probably partially due to division of labelled cells. From 14 h onwards control values are somewhat higher than experimental ones, but otherwise the two sets of data are comparable.



Cell cycle studies

In the normal lens the first labelled mitoses were not seen until 3 h after the administration of the short-term label. At 4 h virtually all metaphases were labelled and their percentage did not decrease until after 6 h. It then fell to a low of 20 and rose again to beyond 50% 14 h after the injection (Fig. 10). The approximate lengths of the cell cycle phases are found in Table 2. For comparison the values reported earlier for the very young lens rudiment (Zwaan & Pearce, 1971) are given. The curve showed asymmetry, indicating some heterogeneity in cell cycle times within the dividing population or a slightly less than ideal pulse labelling. The S-phase may be somewhat shorter than calculated because of this asymmetry.

The cell cycle curve of the experimental lenses showed no differences compared with the control curve at first, but from 8 h after labelling onwards the percentage of labelled mitoses remained very high, while values for the control series fell to a minimum. Then, at 15 h, when control values were climbing up again, the reversed lenses abruptly had no (labelled) mitoses at all.

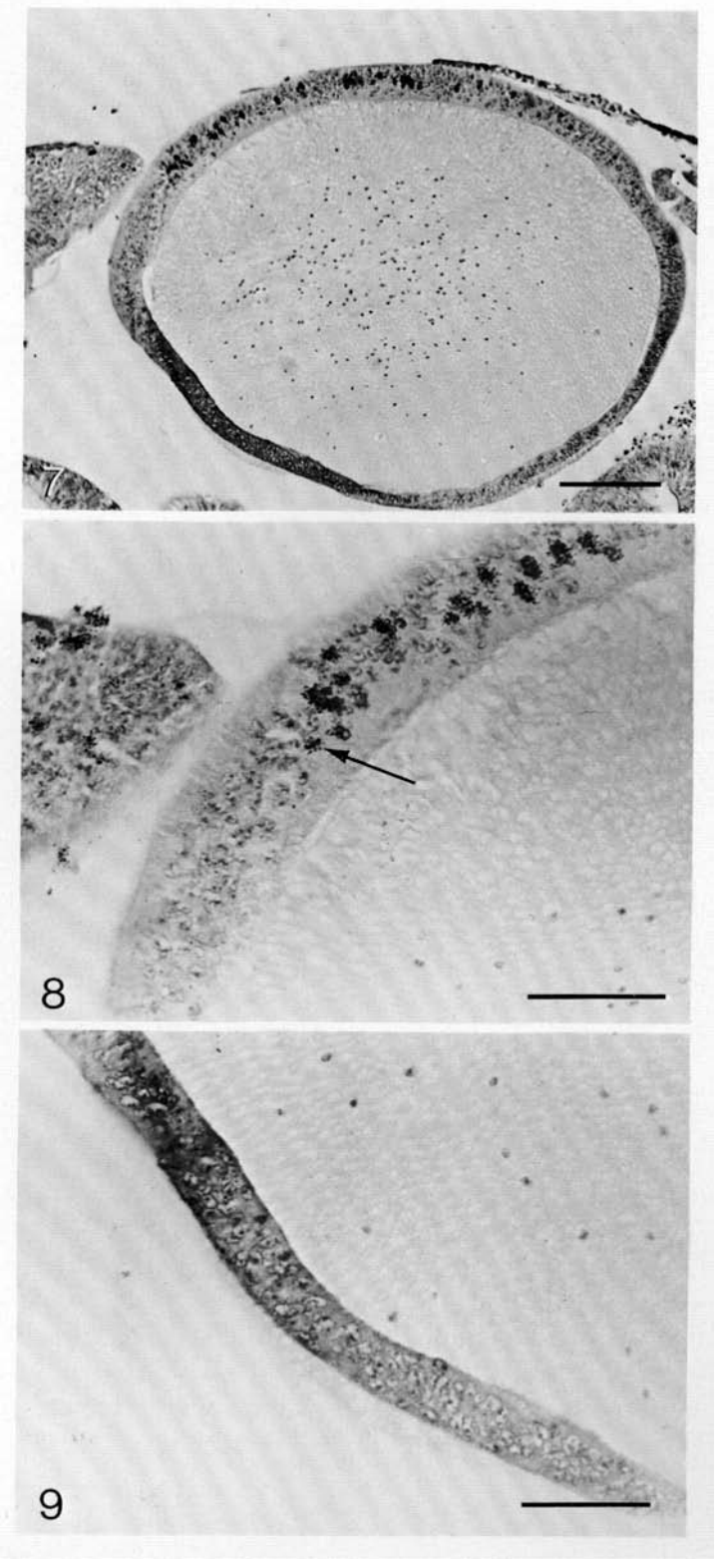
Mitotic activity

Control lenses showed considerable variability in mitotic index, but the average value was about 0·009 (Fig. 11). The epithelia of rotated lenses had the same M.I. for 2 h after surgery. This was followed by a slow decrease over the next 5–6 h. The M.I. then levelled off at about 0·002 until, from 15 h postoperatively onwards, it became 0.

Colcemid arrested cell divisions in metaphase. The number of arrested mitoses varied somewhat but varied less than the number of mitoses. The average value for the arrested metaphase index (A.I.) was 0·021 after 1 and about twice as much after 2 h (Table 2). Interestingly, both normal and arrested mitoses were always located near the border line between epithelium and fibres, which indicates the position of the obliterated lumen of the lens vesicle.

Growth fraction

The population doubling time (T) was calculated from the Colcemid data, and an average value of 34·75 h was obtained. Comparison of T (about 35 h) and cell cycle time G (about 11 h) indicated that a significant percentage of the epithelium was not multiplying or doing so very slowly. Calculation of the growth fraction (f) confirmed this. For the control lens epithelium f was 0·47. There was no marked change after reversal until 5 h had passed, when f began to drop to reach zero at 9 h (Table 3). Thus, after this time interval cell replication has effectively shut down in the reversed lens epithelium.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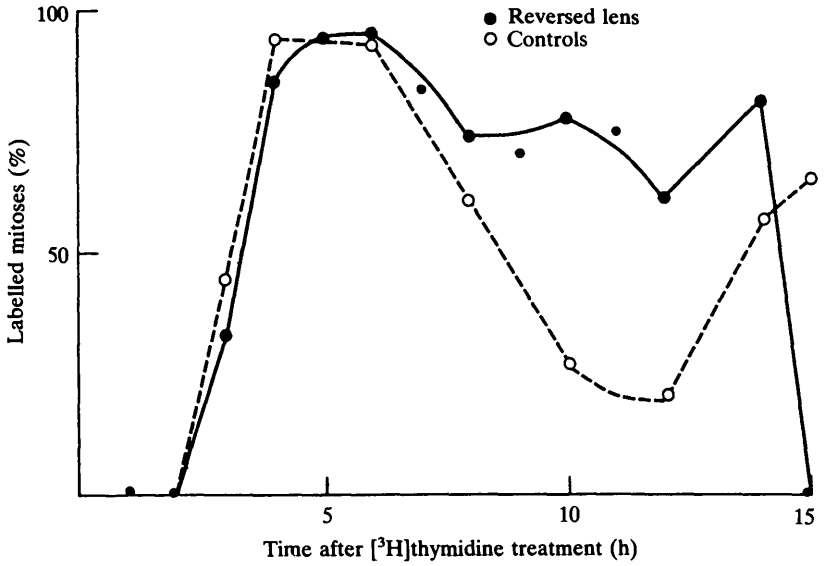

Fig. 10. Labelled mitoses curves of normal and reversed epithelial cells of the 5-day chicken embryo. The values remain high for reversed lenses, even when the control curve declines, but then they drop abruptly to 0 by 15 h after the operation.

DISCUSSION

Cell replication

The replication of lens cells changes significantly between the onset of lens formation at about 2 days of incubation and the period studied here, which is 3 days later. In the earliest stages all cells are dividing (Zwaan, Bryan & Pearce, 1969; Zwaan & Pearce, 1970; 1971). The cell cycle lasts about 8 h and the G1 phase is extremely short. In later stages fibre formation is associated with cessation of cell replication (Reeder & Bell, 1965; Hanna & Keatts, 1966; Modak, Morris & Yamada, 1968; Zwaan & Pearce, 1970) and, in agreement with these authors, we did not find [^{3}H]thymidine incorporation or mitoses in lens fibres.

Fig. 7. Lens rotated 90° instead of 180°. As a result lens epithelial cells are present both anteriorly and posteriorly in this section, outside and inside the rim of the optic cup. The lens was given [^{3}H]thymidine 9 h after reversal and fixed 1 h later. While there are many labelled cells in the anterior part of the epithelium, cells facing posteriorly lack label. × 165. Scale bar = 100 μm.

Fig. 8. Higher magnification of the lens of Fig. 7, near the rim of the optic cup. There is a sharp borderline between anterior labelled cells and posterior unlabelled ones. The arrow indicates the last labelled cell. × 540. Scale bar = 40 μm.

Fig. 9. Posterior epithelium of the same lens. Labelled nuclei are entirely absent. × 490. Scale bar = 40 μm.

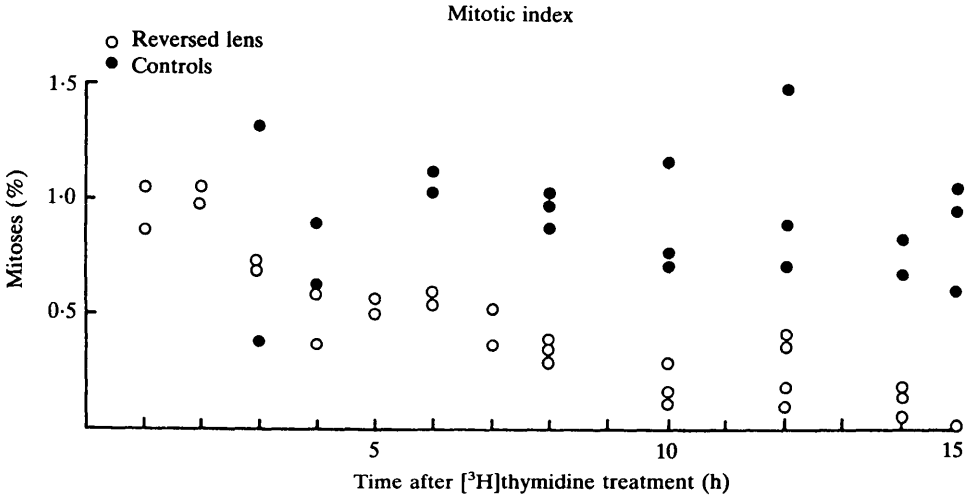

Fig. 11. Mitotic indices of normal and reversed stage-27 lenses. Control values overall do not change; the average index is 0·009. The mitotic index in operated lenses gradually diminishes with a noticeable drop evident 8 h after surgery. No mitoses are seen by 15 h and later.

Table 2. *Cell proliferation parameters of the 5-day embryonic chick lens epithelium, compared with those of the invaginating lens rudiment*

Parameter	5-day lens	Lens labelled at 50 h*
L.I. (after 1 hour)	0·195	0·62
M.I.	0·0091	0·027
Median G	10·6 h	8·4 h
Median t_s	5·5 h	4·9 h
Median $t_{G2} + \frac{1}{2}t_M$	3·2 h	4 h
Median t_{G2}†	3·0 h	3·8 h
Median $t_{G1} + \frac{1}{2}t_M$	2·0 h	0 h
Median t_{G1}†	1·8 h	0 h
t_M (Equation 4)	27 min	22 min
A.I. (t_a = 1 hour)	0·021	0·077
A.I. (t_a = 2 hours)	0·038	
T (Equation 2)	34·75 h	9·0 h
λ (Equation 1)	0·78	1·16
f (Equation 6)	0·47	1·00

*After Zwaan & Pearce (1971)
†Assuming that mitosis takes 27 min

Table 3. *Labelling index and growth fractions of epithelial cells of reversed lenses*

Series	No. of lenses	Time after surgery (in hours)	Time after labelling (in hours)	Average labelling index	Average* growth fraction
Controls	6	–	1	0·19	0·47
Reversals 1	4	1	1	0·17	0·42
2	6	1	1	0·22	0·54
3	6	2	1	0·25	0·62
4	4	3	1	0·21	0·52
5	1	4	1	0·24	0·59
6	4	5	1	0·15	0·37
7	4	7	1	0·08	0·20
8	2	9	1	0	0
9	1	11	1	0	0
10	2	15	1	0	0
11	1	19	1	0	0
12	1	24	1	0	0

*Calculated based on $\lambda = 0{\cdot}78$

Although cell replication is restricted to the epithelium, the distribution of the activity is not homogeneous. DNA synthesis and mitoses do not occur in the peripheral parts of the annular pad. This observation is in conflict with the conclusions of Hanna & Keatts (1966), who found labelled nuclei in the annular pad and deduced that DNA synthesis continued after final mitosis, leading to terminal differentiation in G2. In their study embryos were harvested at least 2 h after labelling, which means that cells had time to migrate into the annular pad from more centrally located regions. Indeed, our results clearly demonstrate the progressively higher L.I. of annular pad cells with time after labelling. Shortly after [^{3}H]thymidine injection the last labelled nuclei are about 40 cells away from the equator, the last mitoses about 25. During the following hours only the label gradually shifts more peripherally until at 15 h labelled nuclei reach the equator. We interpret this to mean, in agreement with Modak & Perdue (1970) and Persons & Modak (1970), that the most anterior annular pad cells duplicate their DNA, divide and gradually are displaced towards the equator, while in G1, to become lens fibres. Yet, this drop-out from the cell cycle can be reversed. As Coulombre & Coulombre (1963) have shown, cells at the equator are capable of regenerating a new anterior epithelium, when the lens is turned around microsurgically.

With a generation time of close to 11 h, half of it devoted to DNA synthesis, half of the nuclei should have incorporated [^{3}H]thymidine after 1 h. Yet, the L.I. was less than 0·2, as also found by Persons & Modak (1970), and the calculated growth fraction was only 0·47. Thus, roughly 53% of the epithelial cells have dropped out of the cell cycle, or have extremely slow cycles. The cells in the annular pad account for at best about 25%. The remaining 28% must be distributed throughout the other regions of the epithelium, although the labelling pattern curves (Fig. 3) suggest that these non-dividing cells are perhaps slightly more concentrated in the central portion.

The most plausible explanation for these non-replicating cells is that they are in a resting phase or G0 (Harding, Reddan, Unakar & Bagchi, 1971). Cells may enter G0 from either the G1 or the G2 phase and apparently can remain there indefinitely until triggered to re-enter the cell cycle. In view of the cell cycle results it is unlikely, that the low L.I. can be explained by the presence of cells with a very long cycle time.

Hanna & Keatts (1966) reported that division is generally confined to a germinative zone just anterior to the annular pad at the time of hatching in the chick. Our data suggest that as early as 5 days there may be a tendency towards a concentration of labelled cells in the region just anterior to the annular pad. Modak, Morris & Yamada (1968) reported labelling to be slightly higher peripherally than centrally in stages 21–25, while at 5 days there was definitely a difference (Persons & Modak, 1970). Together, these results indicate that a germinative zone already may begin to form by 5 days of incubation.

The lengths of the cell cycle phases are not greatly different between 2 and 5

days of embryonic life, except for that of G1. This phase increases from almost non-existent to about 2 h, accounting for most of the lengthening of the cell generation time.

Lens reversal

The cell cycle of reversed lenses closely follows that of the controls for the first 8 h. This is the time necessary for the labelled block of cells to pass through a normal S, G2 and M phase. However, by 10 h the two curves are no longer comparable and by 15 h the labelled mitoses curve falls to zero in the experimentals, indicating that all reversed cells have ceased replication. The other aberrant points (between 10–15 h) may represent cells that were in late S at the time of reversal and labelling, and instead of stopping in G1, continued through the cycle. With a normal cell cycle length they would be seen as labelled mitoses 14 h or so later, but with only half as many grains. Alternatively, a few cells may have had a longer than normal cell cycle duration. These cells would normally be masked statistically, but may show up when the cells of average cycle length drop out following reversal. Finally, for a few cells we may not have attained a pulse label. At any rate, the drastic decrease in M.I. after 10 h indicates that the number of cells involved is quite small.

The L.I. after lens reversal agree with the cell cycle data and stay about the same for the first few hours. Then, as early as 5 h after the surgery, cells are dropping out and by 9 h the L.I. has decreased to zero. That there is no increase in [^{3}H]thymidine label following reversal makes it likely that G0 cells do not have to re-enter the cell cycle before terminal differentiation. The M.I. steadily declines to zero at 15 h after surgery. Together these data indicate that forced lens fibre formation, as in the normal situation, takes place in G1.

Morphology of annular pad

There is a steady increase in cell height from the anterior to the posterior annular pad. At the same time the nuclei take up a basal position in the cells, close to the lens capsule. In contrast both normal and arrested mitoses are at the opposite side of the tissue, where the cell apices are. This is very reminiscent of the process of interkinetic nuclear migration seen in early lens rudiments (Zwaan *et al.*, 1969), where cells elongate in G1, remain columnar in S and part of G2, and then round up again for the next division. Microtubules are not required to maintain the elongated shape, because their disruption with Colcemid has no influence on the cell shape (Pearce & Zwaan, 1970). We have proposed that the driving force for the cell elongation and invagination of the lens placode may be cell crowding, caused by continuous cell division in a restricted area (Zwaan & Hendrix, 1973). It was suggested that the restriction of the lens territory was due to changes in the extracellular matrix between lens rudiment and prospective retina (Hendrix & Zwaan, 1975).

A similar series of events may be responsible for the columnar shape of the annular pad cells. Cell multiplication within the lens epithelium may create a driving force leading to cell crowding, while the lens capsule and the underlying lens fibre mass may restrict the area available to the annular pad. Cells may be packed in this fashion and forced to elongate. The process would be significantly reinforced if cell volume increased at the same time and, indeed, Beebe, Johnson, Feagans & Compart (1981) have recently published a model of fibre morphogenesis based on volume change of individual lens fibres.

Role of the optic cup

Coulombre & Coulombre (1963, 1969) demonstrated that lens reversal, which causes the epithelium to face the retina, leads to differentiation of the epithelium into lens fibres. Similarly, in mouse embryos fibre formation is dependent on the presence of presumptive retina (Muthakkaruppan, 1965). Beebe, Feagans & Jebens (1980) have partially purified a factor, lentropin, from chicken vitreous, which stimulates lens epithelial cells to elongate and to increase crystallin synthesis. It is unclear if lentropin also causes cells to stop dividing. At any rate, operationally the neural retina appears to be the source of influence(s) causing lens fibre differentiation. This was dramatically demonstrated in experiments, in which the lens reversal was only partially successful. With epithelial cells both inside and outside the optic cup a very sharp boundary was observed between replicating and non-replicating cells exactly at the rim of the optic cup. Whatever the molecular basis for this phenomenon, it is obvious that it allows for a continuous adaptation of the size, position and shape of the lens to the size and shape of the optic cup. This, in turn, tends to adjust the ocular system towards the optically most desirable configuration.

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REFERENCES

BEEBE, D. C., FEAGANS, D. E. & JEBENS, H. A. H. (1980). Lentropin: a factor in vitreous humor which promotes lens fiber differentiation. *Proc. natn. Acad. Sci., U.S.A.* **77**, 490–493.

BEEBE, D. C., JOHNSON, M. C., FEAGANS, D. E. AND COMPART, P. J. (1981). The mechanism of cell elongation during lens fiber differentiation. In *Ocular Size and Shape Regulation during Development* (ed. S. R. Hilfer and J. B. Sheffield), pp. 79–98. New York: Springer-Verlag.

COULOMBRE, J. L. & COULOMBRE, A. J. (1963). Lens development: Fiber elongation and lens orientation. *Science* **142**, 1489–1490.

COULOMBRE, J. L. & COULOMBRE, A. J. (1969). Lens development: IV. Size, shape, and orientation. *Invest. Ophthal.* **8**, 251–257.

HAMBURGER, V. & HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

HANNA, C. & KEATTS, H. C. (1966). Chicken lens development: epithelial cell production and migration. *Expl Eye Res.* **5**, 111–115.

HARDING, C. V., REDDAN, J. R., UNAKAR, N. J. & BAGCHI, M. (1971). The control of cell division in the ocular lens. *Int. Rev. Cytol.* **31**, 215–300.

HENDRIX, R. W. & ZWANN, J. (1975). The matrix of the optic vesicle-presumptive lens interface during induction of the lens in the chicken embryo. *J. Embryol. exp. Morph.* **33**, 1023–1049.

KOPRIWA, B. W. & LEBLOND, C. P. (1962). Improvements in the coating technique of radioautography. *J. Histochem. Cytochem.* **10**, 269–284.

MENDELSON, M. L. (1962). Autoradiographic analysis of cell proliferation in spontaneous breast cancer of C3H mouse. III. The growth fraction. *J. natn. Cancer Inst.* **28**, 1015–1029.

MODAK, S. P., MORRIS, G. & YAMADA, T.(1968). DNA synthesis and mitotic activity during early development of chick lens. *Devl Biol.* **17**, 544–561.

MODAK, S. P. & PERDUE, S. W. (1970). Terminal lens differentiation. I. Histological and microspectrophotometric analysis of nuclear degeneration. *Expl Cell Res.* **59**, 43–56.

MUTHUKKARUPPAN, V. (1965). Inductive tissue interaction in the development of the mouse lens in vitro. *J. exp. Zool.* **159**, 269–288.

PEARCE, T.L. & ZWAAN, J. (1970). A light and electron microscopic study of cell behaviour and microtubules in the embryonic chicken lens during Colcemid. *J. Embryol. exp. Morph.* **23**, 491–507.

PERSONS, B. J. & MODAK, S. P. (1970). The pattern of DNA synthesis in the lens epithelium and the annular pad during development and growth of the chick lens. *Expl Eye Res.* **9**, 144–151.

QUASTLER, H. & SHERMAN, F. G. (1959). Cell population kinetics in the intestinal epithelium of the mouse. *Expl Cell Res.* **17**, 420–438.

REEDER, R. L. & BELL, E. (1965). Short- and long-lived messenger RNA in embryonic chick lens. *Science* **150**, 71–72.

STEEL, G. C. (1968). Cell loss from experimental tumors. *Cell Tissue Kinetics* **1**, 193–207.

ZWAAN, J., BRYAN, P. R. & PEARCE, T. L. (1969). Interkinetic nuclear migration during the early stages of lens formation in the chicken embryo. *J. Embryol. exp. Morph.* **21**, 71–83.

ZWAAN, J. & HENDRIX, R. W. (1973). Changes in cell and organ shape during early development of the ocular lens. *Amer. Zool.* **13**, 1039–1049.

ZWAAN, J. & PEARCE, T. L. (1970). Mitotic activity in the lens rudiment of the chicken embryo before and after the onset of crystallin synthesis. I. Results of treatment with Colcemid. *Wilhelm Roux Arch. Entw. Mech. Org.* **164**, 313–320.

ZWAAN, J. & PEARCE, T. L. (1971). Cell population kinetics in the chicken lens primordium during and shortly after its contact with the optic cup. *Devl Biol.* **25**, 96–118.

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