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# Interkinetic nuclear migration during the early stages of lens formation in the chicken embryo

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The optic vesicle in the chicken embryo, after growing out sufficiently to establish contact with the overlying ectoderm, adheres very firmly to the latter for a period of 12–15 h (McKeehan, 1951). Within the area of adhesion the surface ectoderm is transformed into a lens placode under influence of the developing retina. Little is known about the biochemical changes involved in this tissue transformation. Langman (1959) and Langman & Maisel (1962) found a cytotoxic effect of lens protein antisera and specific α-crystallin antibodies on presumptive lens cells shortly after induction had started but before the first morphological changes characteristic for the lens placode were visible. They concluded that the synthesis of α-crystallin might be a prerequisite for lens placode formation. Recent studies with the fluorescent antibody technique, however, indicate that the first crystallins are not produced until the very end of the contact period (Zwaan & Ikeda, 1965, 1968; Ikeda & Zwaan, 1966). α-Crystallin appears even later, at about 3·5 days of incubation when the formation of primary lens fibers has progressed far enough almost to obliterate the lens cavity (Ikeda & Zwaan, 1967).

While the appearance of crystallins can thus be regarded as a relatively late event during lens induction, striking cytological changes have been described for the earlier phases of this process (McKeehan, 1951). The nuclei of the surface ectoderm, which are at first irregularly shaped, become oval and arrange themselves with their long axis perpendicular to the apical end of the cell. First distributed at random, they are now displaced towards the basal parts of the presumptive lens cells which gradually increase in height. A similar nuclear orientation can be observed in the cells of the optic cup. This leads to close approximation of the nuclei of the future retinal and lens cells, as these two tissues remain firmly attached to each other at the base of their cells during this time. Weiss (1950) and McKeehan (1951) suggested that these visible occurrences might be associated with or be the consequence of a regrouping of macromole-

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cules in the cells, relative to their basal surfaces, as a result of direct contact between the two cell layers. This regrouping might conceivably be involved in the inductive process.

The availability of tritiated DNA-precursors, together with the development of high resolution autoradiography, has made it possible to follow cellular proliferation and cellular movements in considerable detail. We decided to study the developing lens rudiment with these techniques because of the importance attached to nuclear rearrangement as an expression of induction. Moreover, knowledge on cell multiplication in the lens placode of the chicken embryo is scarce and yet seems necessary for a better understanding of the mechanisms underlying transformation of this tissue. Finally, we hope to obtain information regarding the relationship between DNA synthesis and cell differentiation, as expressed by the onset of crystallin production. A preliminary report of some of our findings has been given elsewhere (Zwaan & Bryan, 1967).

## MATERIALS AND METHODS

Eggs of a White Leghorn strain of chickens were incubated at 39 °C. Tritiated thymidine with a specific activity of 2·0 Ci/mM was obtained from New England Nuclear Corporation (Boston, Mass., U.S.A.). After an incubation of 40–55 h, most embryos were treated with 0·3 $\mu$ Ci $^3$H-thymidine in 0·1 ml saline through a window in the shell. The windows were sealed and the eggs returned to the incubator. In some cases higher doses (up to 5 $\mu$Ci) were given and other embryos were treated twice with 0·3 $\mu$Ci with an interval of 4–5 h.

After an additional incubation for 20 min to 10 h the embryos were harvested, staged after Hamburger & Hamilton (1951), fixed in Bouin's solution and embedded in paraffin. Serial sections of 4 $\mu$ were stained with hematoxylin and ethyl eosin and prepared for autoradiography, essentially after Kopriwa & Leblond (1962). The slides were coated with Kodak NTB 3 liquid emulsion (Eastman-Kodak, Rochester, N.Y., U.S.A.), air-dried and stored at 4 °C in light-tight boxes containing silica-gel. Exposure varied from 1 to 4 weeks. The autoradiographs were developed, fixed and mounted according to routine methods.

A normal series of eye development in the chicken embryo, correlated with stages according to Hamburger & Hamilton (1951), has been described by O'Rahilly & Meyer (1959). In our experience it was impossible to predict exactly the degree of lens development from the over-all degree of development, although a general correlation did exist. The same degree of lens differentiation could be found in embryos of different developmental stages and most often a difference was noted even between the two lens rudiments of the same embryo. We therefore staged the lens according to its own morphology, using the data of O'Rahilly & Meyer (1959; see also Zwaan, 1963).

## RESULTS

*Histological observations.* An adequate description of the structure of the presumptive lens cells, as seen by light microscopy (McKeehan, 1951), has appeared before and will not be repeated here. One point, not mentioned by the above author, needs emphasis. Most nuclei were observed in the basal halves of the cells. This was particularly clear after elongation of the lens cells became prominent from stage 12 on. A small number of the nuclei however were located in the outer zone of the lens placode. Those immediately adjacent to the apical end were dividing and in fact mitoses were always confined to this area (Plate 1, fig. A).

*Distribution of label.* The results were essentially identical in all phases of lens placode differentiation, but were particularly clear in the later stages, after the lens cells had doubled or even tripled their height.

We will therefore restrict our description to this period (stage 13 or later). Visual observations and grain counts over labeled nuclei were carried out. For the latter the lens placode was divided in three equal zones: a basal zone, adjacent to the retina, a middle zone and an apical zone bordering the lumen. Because of variations in the utilization of $^{3}$H-thymidine and varying exposure times for individual embryos no significance can be attached to absolute grain-count values in different animals. Comparison of these values can only be made among the three zones of individual placodes. A minimum of 5 grains was required to score a nucleus as labeled.

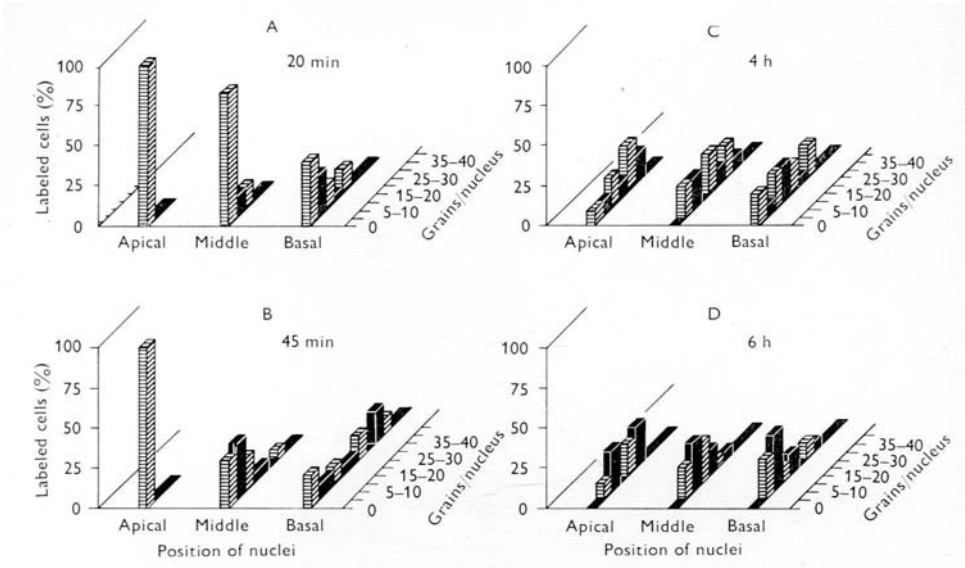
Twenty minutes after application of the label 20–30% of the nuclei in the basal zone were labeled, very few in the middle zone and none in the apical zone (Text-fig. 1 A).

It is clear that nuclei in the S-phase (DNA-synthesis phase) of the cell cycle are restricted to the basal zone, while the non-labeled nuclei in both this and the other two zones must be in the postsynthetic phase (G-2), in mitosis (M) or in the presynthetic phase (G-1).

After 45 min the radioactivity in the basal zone had greatly increased, although a number of nuclei was still negative. There was also an increase of labeled nuclei in the middle zone while the apical zone showed no radioactivity (Text-fig. 1 B).

At 90 min all mitotic figures and other nuclei in the apical zone were still unlabeled (Plate 1, fig. A). After 3–3·5 h (Plate 1, figs. B, C) an occasional grain could be observed over some metaphases but essentially they were negative. Several lightly labeled prophases were seen however. These nuclei must therefore have been in the later part of the S-phase at most about 3 h earlier. Taking into account the time needed for the penetration of $^{3}$H-thymidine into the intracellular pool, for incorporation of the label into DNA and for reaching of the prophase stage, these particular cells must have gone through a postsynthesis phase of at most 3 h.

After 4, 5 and 6 h virtually all mitoses showed label (Text-fig. 1 C, D; Plate 2, fig. D) and it was not until 8–10 h after application of $^3$H-thymidine that unlabeled mitotic figures could again be observed at the lumen.




Text-fig. 1. Representative examples of grain counts over the lens placode nuclei of individual chicken embryos in stages 13 and 14 at various times after exposure to $^3$H-thymidine. Twenty minutes after labeling grains are almost exclusively found over nuclei in the basal parts of the cells (A) indicating that DNA synthesis is restricted to this zone. After 45 min there is a shift of label to the middle zone (B) and after 4 (C) and 6 (D) h nuclei in the outer zone are radioactive. Thus the nuclei, after DNA synthesis is completed, migrate from the basal zone to the apical end of the cell.

We conclude that the nuclei of the lens placode cells replicate their DNA while in the basal zone close to the optic cup. The cells contract to the lumen for division. When this is complete the daughter cells elongate again and their nuclei return to the basal zone.

Because it takes at least 8 h after treatment with tritiated thymidine for un-

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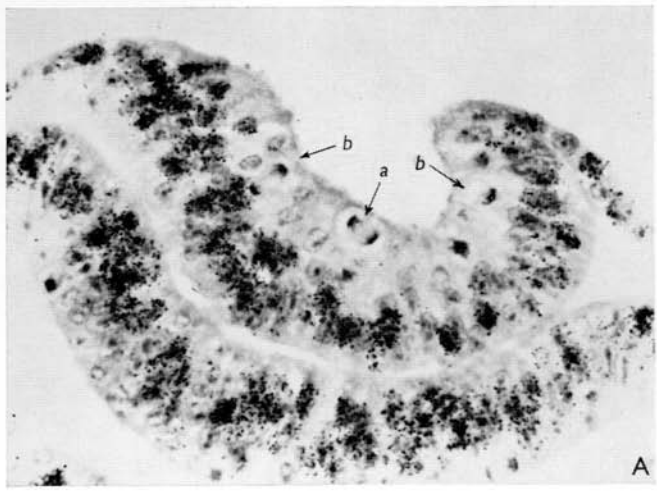
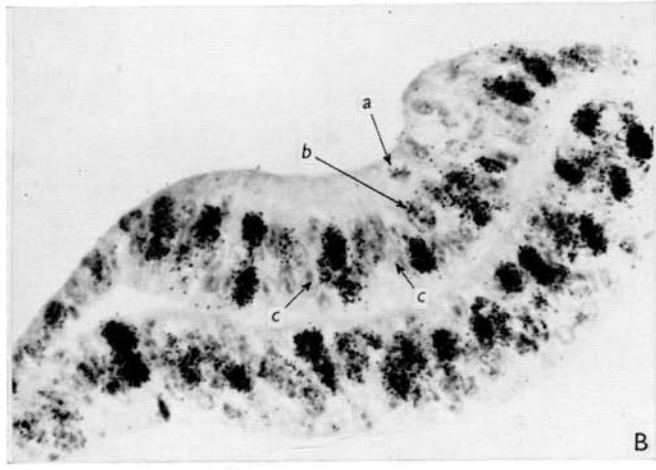
PLATE 1

Fig. A. Autoradiograph of a stage 14·5 lens rudiment 90 min after treatment with $^3$H-thymidine. Labeled nuclei are exclusively located in the basal zone of the cells, while mitoses are restricted to the apical zone. Mitotic figures may be oriented parallel (*a*) or perpendicular (*b*) to the lumen.

Fig. B. Autoradiograph of a stage 14 lens placode 3 h after application of $^3$H-thymidine. A metaphase figure at the lumen (*a*) shows no label but a prophase (*b*) does. Several nuclei in the basal zone (*c*) are unlabeled, while the others have many grains.

Fig. C. Autoradiograph of a stage 14 lens placode 3·5 h after exposure to $^3$H-thymidine. Metaphases (*a*) still show no grains but many may be seen over prophases (*b*).


b
a
b
A


a
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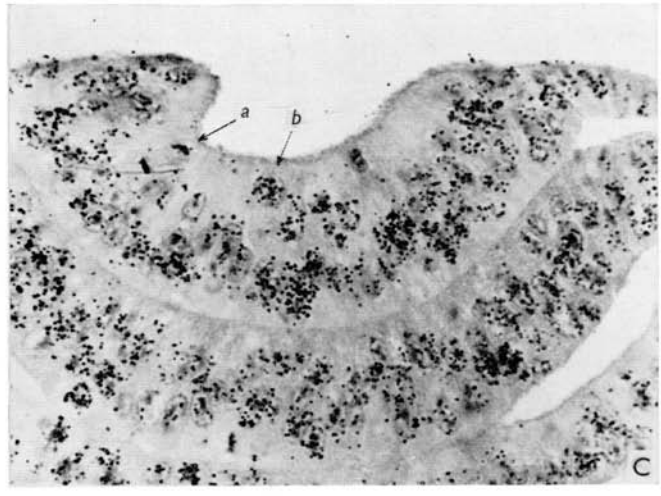
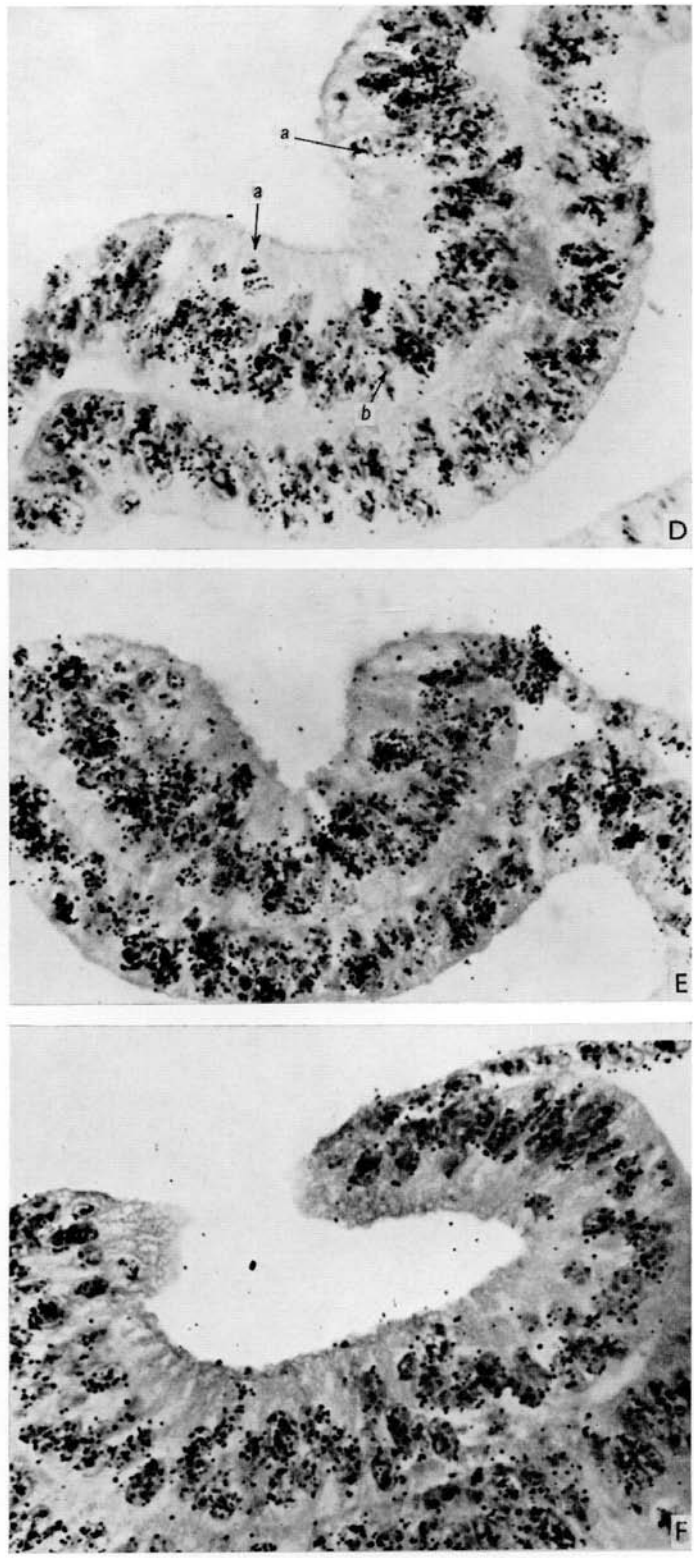
B


a
b
C


a
a
b
D
E
F

labeled mitotic figures to reappear in the apical zone the total cell cycle must last a minimum of 8 h.

*Double labeling.* After 4–5 h a number of the nuclei in the basal zone still showed no grains (Text-fig. 1C; Plate 2, fig. D). These cells may not have been in the S-phase during the time that $^3$H-thymidine was available or they may have lost their capacity to synthesize DNA. To choose between these a large dose (5 $\mu$Ci) of thymidine was given to 50–52 h embryos with the assumption that this would result in a prolonged availability of label for incorporation into DNA. This stage of development was selected because earlier work has demonstrated the first appearance of crystallins at this time (Ikeda & Zwaan, 1966). Other embryos were treated for a second time with 0·3 $\mu$Ci $^3$H-thymidine after 4 or 5 h. It seemed probable that cells not in the S-phase during the first treatment would have reached this period of the cell cycle during the second exposure to label.

Essentially all cells were labeled 4–5 h after treatment with the high dose (Plate 2, fig. E) or 1 h after a second low dose (Plate 2, fig. F), indicating that all lens placode cells are still capable of DNA synthesis in the stages investigated.

*Thymidine index.* The chicken egg and embryo form a closed system, possibly resulting in a prolonged presence of $^3$H-thymidine. This in turn might lead to erroneous conclusions regarding the generation time of the lens placode cells. We therefore determined the percentage of labeled cells in the population (thymidine index) at various times after treatment. At the low dose of $^3$H-thymidine normally given the index increased over the first hour and then remained constant at about 60–70 %. The tritiated thymidine must therefore have been available for incorporation into DNA for about 1 h. That free label disappears relatively fast is also indicated by the presence of unlabeled nuclei in the basal zone 3–4 h after treatment (Plate 1, fig. B). The experiment where two doses of label were given showed this not to be due to a loss of ability to synthesize DNA.

There is no evidence for synchronous cell division in the lens placode, and we may therefore assume that the cells are randomly divided over the phases of the 8 h cycle at any moment. After 1 h 60 to 70 % of the cells are or have been in the S-phase, which therefore must last 5–6 h.

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PLATE 2

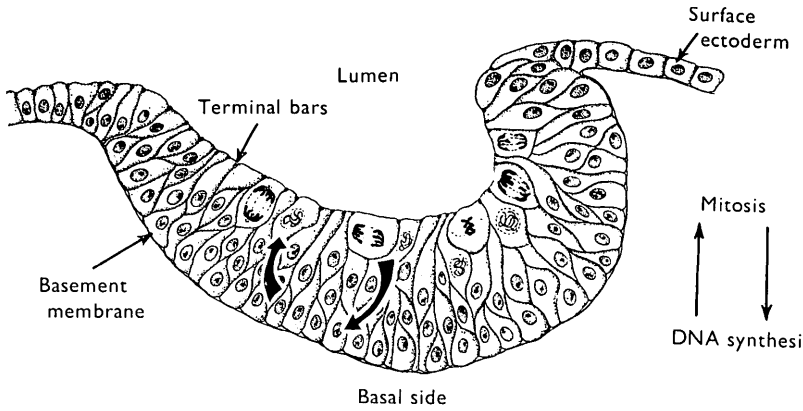
Fig. D. Autoradiograph of a stage 15 lens cup. Label is clearly present over mitotic figures at the lumen (*a*). Some nuclei in the basal zone are negative (*b*). These cells possibly were in G-2 or mitosis when label was applied 5 h earlier and did not return to the basal zone until radioactive thymidine was no longer available.

Fig. E. Autoradiograph of a stage 15 lens cup treated twice with 0·3 $\mu$Ci $^3$H-thymidine, respectively 5 and 1 h earlier. All cells have incorporated the label.

Fig. F. Autoradiograph of a stage 15·5 lens rudiment, treated 5 h earlier with 5 $\mu$Ci $^3$H-thymidine. All cells have grains over their nuclei.

## DISCUSSION

No previous data are available on the cell cycle in the early lens rudiment of the chicken embryo, but our values of 8–10 h for the total generation time, 5–6 h for the S-phase and 2·5–3 h for the G-2 phase agree very well with those of Langman, Guerrant & Freeman (1966) for the neuro-epithelial cells of the neural tube during closure. Cameron (1964) found a DNA-synthesis time of 5–6 h for several organs of the young chicken, and Fujita (1962) reported a total generation time of 5 h for neuro-epithelium in the 1-day-old chicken embryo, which lengthened to 10 h during the sixth day of incubation. As we found a mitotic duration of 30 min by the Colcemid method (J. Zwaan & T. L. Pearce,




Text-fig. 2. Drawing of a stage 14·5 lens rudiment. See text for details.

in preparation), the presynthetic phase must be very short. Studies on the length of the cell cycle by the method of Quastler & Sherman (1959) confirm the present values, at least for lens placode cells during the contact period (in preparation). It should be stressed that our data, primarily based on observations of nuclear migration in single cells, may lack the accuracy of direct counting methods. It is entirely possible therefore that part of the cell population has longer cell cycles than proposed above, particularly in the later stages of lens induction.

There is a time lapse of at least 17 h between the establishment of contact between the optic vesicle and the overlying ectoderm in stage 10 and the first appearance of crystallins in stage 14 (Ikeda & Zwaan, 1966). If the generation time of the lens placode cells is indeed about 8 h, the cells go through at least two full cell cycles before specific lens antigens can be detected. It may be more than coincidental that similar observations were made for the transformation of iris cells into lens cells during Wolffian regeneration in the newt (Yamada, 1966; Eisenberg & Yamada, 1966). Many tissues go through a period of rapid cell division prior to specialization and indeed it has been postulated that release of new information (transcription) may depend upon an immediately preceding replication (Ebert & Kaighn, 1966).

The double-treatment procedure indicates that all cells are still capable of DNA replication in late lens placode and early lens cup stages. Earlier investigations have shown that at least part of the cell population engages in crystallin synthesis at this time (Ikeda & Zwaan, 1966, 1967; Zwaan & Ikeda, 1968). Thus, in contrast to the situation in the regenerating newt lens (Yamada, 1966) the prospective lens cells of the chicken embryo may not have entered the terminal cell cycle when specific proteins are first produced. We hope to confirm this impression by a technique (Yamada, 1966) which combines autoradiography after exposure to $^{3}$H-thymidine with immunofluorescence of individual cells.

It is clear that the position of the presumptive lens cell nuclei in the placode is related to the phase of the cell cycle in which they are found. During DNA synthesis the nucleus is located in the basal zone close to the optic cup and for mitosis it migrates to the apical end of the cell (Text-fig. 2).

Since the first phase occupies a major portion of the cycle and the latter probably a very minor part, it is not surprising that the majority of the nuclei are always seen in the basal zone (McKeehan, 1951).

This nuclear migratory behavior is interesting enough to warrant a further discussion. Schaper (1897) first reported on the exclusive localization at the lumen of mitotic figures in the early embryonic lens, as well as in a variety of other embryonic epithelial organs. It was several decades later that Sauer, on the basis of very careful descriptive studies, explained these findings in his theory on interkinetic nuclear migration. He proposed (Sauer, 1935*a*, *b*) that the early embryonic neural tube formed a pseudostratified epithelium, its cells attached to each other at the lumen by terminal bars. During the interkinetic part of the cell cycle the nuclei were thought to be located in the deeper layers of the neural wall, while for mitosis the cells contracted to the lumen. Sauer suggested that after completion of their division the cells elongated again, their nuclei returning to the basal zone. He later expanded his theory (Sauer, 1936, 1937) to include a variety of embryonic epithelia. In recent years a wealth of experiments on the neural tube, involving the use of radioactive thymidine to label DNA or of colchicine and similar mitosis-blocking agents, have fully confirmed Sauer's views on the replicative behavior of the neuro-epithelial cells (see review by Watterson, 1965). The present work focused on the lens rudiment, but a number of incidental observations were also made on other tissues. Not only the lens placode (and later vesicle) but also the nasal pit, the otic placode and vesicle, the thicker parts of the coelomic walls, the gut and several of its outpouchings and the mesonephric tubules among others show interkinetic nuclear migration. Fujita (1960) also described the localization of mitotic figures next to the lumen in several columnar epithelia.

We conclude that this phenomenon of nuclear migration is widespread among embryonic tissues, associated with a certain cellular formation. Such a formation involves a columnar or pseudostratified epithelium in which all the cells are firmly attached to each other at their apical ends by terminal bars. In view of

the generality of the phenomenon it is difficult to attach *specific* inductive significance to the basal arrangement of nuclei in the lens placode and retina.

The causes of cell elongation and nuclear migration are not clear. Sauer (1937) has shown that crowding, in combination with a limitation of the surface area of the epithelial cell through the terminal bar system, cannot account for elongation of the cells after mitosis in all cases. He rather thinks that specific forces within the cell itself are of influence and attaches importance to cytoplasmic fibers, which were seen in the elongated stage of the cells but disappeared during mitosis, and which were very similar to spindle fibers. In the case of the chicken lens placode Byers & Porter (1964) have described the appearance of large numbers of long, straight microtubules oriented from base to apex in the presumptive lens cells from stage 11 on. Similar observations have been made on the embryonic neural tube of the mouse (Herman & Kauffman, 1966). According to Porter (1966) microtubules may be associated with intracellular transport of cytoplasmic components or may form a cytoskeletal system, sometimes involved in the development of marked assymmetries of cell shapes. In our view it is highly probable that the elongation of the lens placode cells is an active process related to the assemblage of microtubules within the cytoplasm. Whether rounding up of the cells for mitosis involves the breakdown of these structures remains to be seen. Once the forces causing elongation of the cell body have been removed the cell may round up passively because maintenance of a sphere requires the least free surface energy. Alternatively, the process may be a more active one. The terminal bars, which have been demonstrated in the placode cells by electron microscopy (Cohen, 1961; Hunt, 1961; Byers & Porter, 1964) and light microscopy (McKeehan, 1951) will in either case cause the cells to round up towards the lumen.

Why the cells exhibit this complex dynamic behavior is a matter of speculation. Langman *et al.* (1966) feel that it may be related to invagination and later closure in case of the neural groove. The wedge shape of the proliferating neuroepithelial cells anchored at the lumen by terminal bars might mechanically lead to formation of the neural tube. This theory may not be universally valid. When the presumptive retina invaginates to form the optic cup the free cell surface is convex rather than concave, yet mitotic figures are still found exclusively at the lumen, while DNA synthesis is restricted to the outer zone of the tissue (Sidman, 1961). Large parts of the coelomic cavity wall are virtually flat but do exhibit interkinetic nuclear migration. Invagination of the lens placode is independent of the underlying optic vesicle and probably due to forces operating within the lens ectoderm itself (Coulombre, 1965). We feel that in addition to cell elongation a variety of factors may be involved: contraction of the terminal bar network (McKeehan, 1951), obliteration of intercellular spaces (Hunt, 1961), and possibly others such as changes in cell adhesiveness.

Inductive processes have been shown to play a role in the differentiation of many tissues undergoing interkinetic nuclear migration. We propose that there

may be a relation between these two phenomena, though not a specific one. If we assume that differentiation is dependent on the release of specific information by the nuclei of the induced tissue, it might be advantageous if these nuclei are in close approximation to the source of inductive influences during a major part, or possibly a specific phase, of the cell cycle. This is precisely what occurs during interkinetic nuclear migration. At the same time the system allows for an orderly growth of the organ with retention of the architecture of the tissue, due to the anchoring of its cells at the lumen before and after division.

SUMMARY

1. A study was made of the replicative behavior of lens placode cells of the chicken embryo by autoradiography after $^{3}$H-thymidine application.

2. Approximate values for the total generation time, S-phase and G-2 phase were found to be a minimum of 8, 5–6 and 3 h respectively.

3. All placode cells were still capable of DNA synthesis, despite the fact that at least some of them are known to produce specific proteins at this stage.

4. The cells underwent interkinetic nuclear migration: during DNA synthesis the nuclei were located in the basal part of the cell and for mitosis they moved towards the apex.

5. The possible significance of this is discussed, particularly in relation to lens induction.

RÉSUMÉ

*Migration nucléaire intercinétique pendant les phases précoces de la formation du cristallin chez l'embryon de poulet*

1. Une étude autoradiographique du comportement replicatif des cellules du cristallin de l'embryon de poulet a été effectuée par l'incorporation de thymidine-$^{3}$H.

2. La durée totale du cycle cellulaire, la durée de la phase S et celle de la phase G2, correspondent approximativement à 8, 5–6 et 3 h respectivement.

3. Toutes les cellules du placode sont capables de synthétiser du DNA, bien que certaines, tout au moins, synthétisent déjà des protéines spécifiques.

4. Les cellules subissent une migration nucléaire intercinétique: pendant la synthèse du DNA, les noyaux se localisent dans la partie basale de la cellule, tandis qu'au moment de la mitose, ils se trouvent autour du lumen.

5. La signification éventuelle de ces résultats est discutée par rapport à l'induction du cristallin.

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