Redifferentiation, cellular elongation and the cell surface during lens regeneration

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SUMMARY

(1) Dorsal irises from normal and lentectomized eyes of the newt Notophthalmus viridescens were cultured in vitro in the presence of colchicine (1.25 × 10⁻⁹ to 2.5 × 10⁻⁶ M), vinblastine sulfate (4.4 × 10⁻⁹) and cytochalasin B (20 μg/ml). Explants were treated for 24 h and implanted into host eyes of previously lentectomized newts.

(2) In regenerates treated with colchicine at stages prior to the onset of lens fiber formation, cellular elongation was inhibited or considerably delayed. Cytochalasin B has no effect on lens fiber differentiation when compared with regenerates treated with its solvent, dimethyl sulfoxide.

(3) Colchicine-treated regenerates showed no detectable fluorescence when stained with anti-newt lens fluorescent antibodies, suggesting that cells in which cellular elongation was inhibited were unable to synthesize lens specific proteins.

(4) Ultrastructural observations of elongating lens regenerate cells showed microtubules present in the cytoplasm parallel to the axis of elongation. Microtubules were not observed in depigmented cells from colchicine-treated regenerates. Characteristic vinblastine-induced microtubule crystals were present in depigmented cells from vinblastine-treated regenerates.

(5) Experiments using cell electrophoresis showed that neuraminidase-sensitive groups were detectable at the ionic double layer of cells from implanted regenerates at stages where lens fiber differentiation occurs. Cells from colchicine-treated regenerates showed no significant reduction of their electrophoretic mobility after neuraminidase treatment. This suggests that the appearance of some cell surface components in the redifferentiated cell may also be colchicine sensitive and probably mediated by microtubules.

INTRODUCTION

Surgical removal of the lens from the eye of the newt Notophthalmus viridescens results in the initiation of a sequence of events that cause the cells of the dorsal iris to increase their RNA synthetic activity, initiate DNA synthesis, depigment, elongate, and become organized into a functional new lens. During lens redifferentiation from the depigmented iris cells, the cuboidal depigmented iris cells elongate into lens fibers. In the lens regenerating system, cellular elongation occurs concurrently with a marked increase in protein synthesis which has been associated with the synthesis of lens crystallins (Yamada & Takata, 1963). Wolffian lens regeneration therefore represents a system useful

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for the study of the relationship between cell elongation and cellular processes involved in lens fiber differentiation.

It has been suggested that cytoplasmic microtubules have a role in the acquisition of an asymmetrical cell shape (Wessels et al. 1971); these organelles have also been associated with processes accompanying cellular elongation. Cytoplasmic microtubules have been observed during the invagination and accompanying cellular elongation of the lens placode in the chick embryo (Byers & Porter, 1964). It has also been suggested (Piatigorsky, Webster & Wolberg, 1972a) that de novo synthesis of microtubular protein is necessary for the continued elongation of cultured chick lens epithelial cells; the initial elongation of differentiating lens fibers involving the use of pools of microtubular protein already available in the cytoplasm. The involvement of microtubules in other morphogenetic processes involving changes in cell shape has also been proposed (Arnold, 1966; Grandholm & Baker, 1970; Handel & Roth, 1971; Burnside, 1973).

In the present study, several compounds which have been reported to interfere with microtubule or microfilament integrity have been tested at various stages of lens regeneration. The effects of these compounds on cell elongation and on other processes of lens fiber differentiation have been studied. The results obtained so far indicate that microtubules may play a role in lens fiber elongation and that this process may be associated with the tissue specific protein synthesis which occurs during lens fiber differentiation. Our results also suggest that microtubules play a role in the reappearance of certain cell surface components during redifferentiation.

**Materials and Methods**

Adult newts, Notophthalmus viridescens, were lentectomized according to the method of Eisenberg & Yamada (1966). Prior to lentectomy the animals were kept at 8 °C; after the operation they were kept at 22 °C and fed weekly.

In vitro treatment. At various intervals after lens removal dorsal irises were obtained as described by Zalik & Scott (1969). Irises were cleaned from adhering material and trimmed with iridectomy scissors so that only the mid-dorsal portion of the iris remained. The irises were then placed in 50 % L-15 medium (Grand Island Biological Company) diluted with sterile distilled water and containing 50 units Penicillin-streptomycin (Grand Island Biological Company). This medium will be referred to as control medium. In other experiments specific drugs were incorporated in the control medium in order to test their effects on irises at various stages of regeneration; colchicine (Sigma) ranging in concentrations from $1.25 \times 10^{-3}$ to $2.5 \times 10^{-6}$ M and vinblastine sulfate (Lilly), used at concentrations ranging from $4.4 \times 10^{-8}$ to $4.4 \times 10^{-6}$ M, were used as antimitotic microtubular disrupting drugs; lumicolchicine was prepared according to the method of Wilson & Friedkin (1966) and used at
a concentration of $3.5 \times 10^{-3} \text{M}$. The microfilament disrupting agent cytochalasin B (I.C.I. Laboratories, Cheshire, England) was dissolved in dimethyl sulphoxide (DMSO) and added to the medium at a concentration of 20 $\mu\text{g/ml}$. Controls for this treatment contained the appropriate amount of DMSO. Usually 10–20 irises were placed in 5 ml of medium in a Petri dish 60 mm in diameter and incubated at 22 $^\circ\text{C}$ for 24 h.

In vivo culture. After in vitro incubation irises were rinsed in control medium and implanted into the eyes of newts lentectomized immediately prior to the insertion of the tissue; one iris was implanted in each lentectomized eye. One to twenty-five days after the iris implantation, host newts were anesthetized, sacrificed and eyes with the implanted irises were fixed and processed for microscopical examination. Light microscopy was carried out on host eyes fixed in Bouin’s fixative, wax embedded and stained with hematoxylin-eosin. For electron microscopic observations, 15-day regenerates cultured in vitro for 24 h in either control or drug-containing media were fixed in 3 % glutaraldehyde in 0.1 M phosphate buffer pH 7.4 followed by 2 % osmium tetroxide in the same buffer. The material was dehydrated in graded ethanol solutions embedded in Epon, sections stained with uranyl acetate and lead citrate and observed with a Philips 200 electron microscope.

For immunofluorescent studies newt lens homogenates (five lenses/ml) were prepared. Rabbits were injected three times at 2-week intervals with 2 ml of lens homogenate mixed 1:1, v/v, with Freund’s adjuvant. Two weeks after the last injection animals were bled and the antisera were obtained. Before use the antiserum was absorbed with newt tissue powder obtained from lentectomized newts. Reaction of the antiserum with newt lens extract after immunoelectrophoresis revealed the presence of $\alpha$, $\beta$ and $\gamma$ crystallins. Tissues were fixed in 95 % alcohol at 4 $^\circ\text{C}$ and processed according to Sainte-Marie (1962). Deparaffinized sections of control and drug-treated regenerates were treated first with lens antiserum, washed and then exposed to fluorescein-labelled goat anti-rabbit immunoglobulin (Microbiological associates). Controls consisted of lens regenerates exposed to serum from a non-injected rabbit. Observations were performed on a Leitz fluorescence microscope with the appropriate filters.

Some experiments were designed to investigate the effects of colchicine on the incorporation of uridine or thymidine on lens regenerates. Fifteen-day regenerates were cultured in vitro in either control medium or in medium containing colchicine at a concentration of $2.5 \times 10^{-5} \text{M}$. Irises were then implanted into host newt eyes and animals were injected with either 10 $\mu$Ci of uridine ($5-[\text{H}^3] 29.5 \text{Ci/mm}$) or 30 $\mu$Ci thymidine (methyl-$[\text{H}^3]$ 16.9 Ci/mm, New England Nuclear Corp.). Animals were sacrificed 12 h later and eyes were fixed. In experiments designed to measure amino acid incorporation into protein, 10- and 15-day regenerates were cultured in either control or colchicine containing medium as described previously. Irises were then implanted into host lentectomized newts and were allowed to develop in the host eyes for 12 h, 5, 10,
15 and 20 days after implantation. Three hours before sacrifice, animals were injected with 5 μl of D,L-leucine (4,5-[H\(^3\)] 35-4 Ci/mM, New England Nuclear). Tissues were fixed in Bouin's fixative, embedded and sectioned. Sections were treated with cold 5% TCA for 10 min, washed, and slides were covered with Kodak NTB-2 liquid autoradiographic emulsion. Slides were exposed for 3 weeks, developed with D-11, fixed for 5 min in Kodak fixer and stained with hematoxylin-eosin. Slides with sections from \([H^3]\)thymidine- and \([H^3]\)uridine-treated regenerates were placed in 10% \(H_2O_2\) overnight and washed several times in distilled water before being exposed to the autoradiographic emulsion.

In some experiments cell electrophoresis was performed on cell suspensions from regenerate irises. Fourteen-day regenerates were treated for 24 h \textit{in vitro} either in control medium or in media containing 2.5 × 10\(^{-5}\) M colchicine. Regenerates were implanted into lentectomized host newt eyes and cultured \textit{in vivo} for 1–8 days. At the desired time intervals the implanted irises were removed from the host newt eyes and cell dissociation was accomplished by the method of Zalik & Scott (1972). In some experiments aliquots of the cell suspension were incubated in 0.118 M-NaCl containing 25 μl/ml of neuraminidase (from \textit{Vibrio cholerae}, Behringswerke) and 0.005 M-CaCl\(_2\) pH 7.2. Controls consisted of cells placed in saline without enzyme. Cell suspensions were incubated at 37°C for 30 min, washed and suspended in 0.118 M-NaCl pH 7.2; cell electrophoresis was determined in a cylindrical cell electrophoresis apparatus (Bangham, Flemans, Heard & Seaman, 1958), equipped with the micro cell attachment.

RESULTS

The extent of development undergone by the cultured regenerates was determined by comparison with the stages proposed by Yamada (1967) according to the following criteria: (a) ratio of pigmented to depigmented cells in the area making up the regenerate; (b) degree of elongation and extent of nuclear degeneration in primary lens fibers; (c) presence and ratio of secondary lens fibers. In all of the experiments performed the staging was done blind.

Retardation due to experimental manipulation. Intrinsic retardation. The methods chosen for \textit{in vitro} drug treatment and \textit{in vitro} culture offer certain advantages. They allow for a better control of the time and drug concentration to which the regenerate is exposed, and they eliminate drug effects on the experimental animals which in turn may influence the process under investigation. However, these conditions do have several drawbacks: the iris tissue is subject to a certain degree of injury imposed by the manipulation of the tissue, and the iris, although implanted into the optic chamber of a lentectomized newt, may not be surrounded by the same conditions existing in the original lentectomized eye at the developmental stage of the iris implant. A question arising in these experiments is the extent to which our experimental conditions
Table 1. Intrinsic retardation experienced by control iris regenerates implanted into lentectomized eyes

<table>
<thead>
<tr>
<th>Days post-lentectomy</th>
<th>Actual† age</th>
<th>Stages</th>
<th>Apparent age—Average apparent age‡</th>
<th>Intrinsic retardation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16</td>
<td>IV–VI</td>
<td>9–16—14</td>
<td>2</td>
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<tr>
<td>2</td>
<td>18</td>
<td>IV–VI</td>
<td>9–16—14</td>
<td>4</td>
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<td>5</td>
<td>21</td>
<td>V–VI</td>
<td>12–16—15</td>
<td>6</td>
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<td>6</td>
<td>22</td>
<td>VI–VIII</td>
<td>12–19—16</td>
<td>6</td>
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<tr>
<td>7</td>
<td>23</td>
<td>VII–VIII</td>
<td>15–19—17</td>
<td>6</td>
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<tr>
<td>10</td>
<td>26</td>
<td>VII–IX</td>
<td>15–20—18</td>
<td>8</td>
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<tr>
<td>15</td>
<td>31</td>
<td>VIII–IX</td>
<td>15–20—20</td>
<td>11</td>
</tr>
<tr>
<td>20</td>
<td>36</td>
<td>X</td>
<td>18–25—22</td>
<td>14</td>
</tr>
</tbody>
</table>

† Actual age represents the sum of the days post-lentectomy, one day for the in vitro culture and 15 days for the in vivo culture.

‡ Apparent ages were obtained by comparing the observed stages with those described by Yamada (1967). The average age in days for each regenerate was obtained and these values were used to calculate the average apparent age for each of the groups studied. Therefore, the average apparent age is not necessarily the numerical mean of the apparent age. These values as well as those for intrinsic retardation represent only estimates and no further quantitative assessment of the data is intended.

Influence the progress of lens regeneration. To obtain an estimate of this parameter, sections of each implanted regenerate were examined under the light microscope and assigned a stage (Yamada, 1967) according to the differentiation it had attained. Although a considerable variation in developmental stages occurs within time intervals after lentectomy, an approximate estimate of the average time at which a certain regeneration stage may occur can be obtained. Each implanted iris was assigned an ‘apparent age’ in days, by this procedure. The apparent age of the iris was then compared to its ‘actual age’. The actual age of the regenerate was calculated as the sum of the numbers of days after lentectomy, plus one day for in vitro culture and the number of days it was cultured in vivo. The intrinsic retardation was obtained by subtracting the apparent age from the actual age of the implanted irises. Table 1 summarizes the intrinsic retardation experienced by irises at succeeding stages of regeneration cultured for 24 h in vitro and subsequently implanted into host newt eyes for 15 days. For normal irises as well as for regenerates at 2, 5, 6, and 7 days after lentectomy, ten explants were used while, for 10-, 15-, and 20-day regenerates, 20 explants were examined, in these studies. It can be seen, from the data in Table 1, that the intrinsic retardation shown by the implants increases with the age of the regenerate. This would seem to indicate that irises at later stages of regeneration are more sensitive to experimental manipulation and surrounding environmental conditions in the ocular environment.
Table 2. Effect of colchicine concentration on lens regeneration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Stage</th>
<th>Apparent age—Average apparent age</th>
<th>Retardation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>VIII-X</td>
<td>15—25—20</td>
<td>11</td>
</tr>
<tr>
<td>Colchicine</td>
<td>2.5 x 10^-6 M</td>
<td>VI-VII</td>
<td>12—18—16</td>
<td>15</td>
</tr>
<tr>
<td>Colchicine</td>
<td>2.5 x 10^-5 M</td>
<td>V</td>
<td>12—15—13</td>
<td>18</td>
</tr>
<tr>
<td>Colchicine</td>
<td>2.5 x 10^-4 M</td>
<td>V</td>
<td>12—15—13</td>
<td>18</td>
</tr>
<tr>
<td>Colchicine</td>
<td>1.25 x 10^-3 M</td>
<td>III</td>
<td>8—11—10</td>
<td>21</td>
</tr>
</tbody>
</table>

Fifteen-day regenerate irises were treated in vitro for 24 h then incubated in vivo for 15 days; the actual age of regenerates was 31 days. Average apparent age was calculated as in Table 1.

Effects of colchicine on lens regeneration

Colchicine-treated regenerates were evaluated and staged in the same manner as the controls. The 'actual age' and the 'apparent age' were calculated and a retardation time, in days, was estimated. The intrinsic retardation time of the controls was subtracted from that experienced by the drug-treated regenerates to obtain a retardation time which is presumed to be due to drug effects.

A series of experiments were first performed in order to investigate the colchicine concentrations that would affect regeneration without being lethal to the cells. To determine this parameter 15-day regenerates were used and the effects of several colchicine concentrations on subsequent cellular elongation was determined. For these experiments regenerates were cultured in the presence of colchicine (2.5 x 10^-6 to 1.25 x 10^-3) for 24 h and incubated in vivo for 15 days. The results of these experiments are presented in Table 2. From the data in this table it can be observed that colchicine at all of the doses used retarded regeneration. At drug concentrations of 2.5 x 10^-6 the regeneration process was retarded, but some cellular elongation occurred as evidenced by the fact that regenerates attained stage VI and VII, in which some cells have elongated into primary lens fibers. Concentrations of colchicine of 2.5 x 10^-5 and 1.25 x 10^-3 completely inhibited elongation; regenerates of this series were composed of masses of...
Cell elongation during lens regeneration
Table 3. Retardation experienced by colchicine-treated 15-day regenerates at different time intervals after implantation in host lentectomized eyes

<table>
<thead>
<tr>
<th>In vivo incubation time (days)</th>
<th>Control</th>
<th>Actual age</th>
<th>R1†</th>
<th>Stages</th>
<th>Age–Average</th>
<th>R2‡</th>
<th>R2 – R1§</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>V–VII</td>
<td>12–18–15</td>
<td>21</td>
<td>V</td>
<td>12–15–12</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>20</td>
<td>X</td>
<td>18–25–22</td>
<td>36</td>
<td>V–VII</td>
<td>12–18–17</td>
<td>19</td>
<td>5</td>
</tr>
</tbody>
</table>

Regenerates were cultured in vitro in the presence of 2\(\times10^{-5}\) M colchicine for 24 h and subsequently implanted into host lentectomized newts. Age and Average refer to apparent age and average apparent age respectively. For further information about constraints of these values see Table 1.

† Retardation in days experienced by the control due to experimental procedures – intrinsic retardation.

‡ R2, Retardation in days experienced by the colchicine-treated irises due to experimental conditions and colchicine treatment.

§ R2 – R1, Retardation due to the effect of colchicine as compared to controls.

Table 4. Effect of colchicine on various stages of lens regeneration

<table>
<thead>
<tr>
<th>Age of iris after lentectomy</th>
<th>Total age</th>
<th>Stage of operated control/av. apparent age</th>
<th>Stage of colchicine-treated av. apparent age</th>
<th>Colchicine retardation (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16</td>
<td>IV–VI/14</td>
<td>IV–VI/14</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>IV–VI/14</td>
<td>IV–VI/14</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>V/14</td>
<td>V–VI/14</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>22</td>
<td>VI–VIII/17</td>
<td>V–VI/14</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>23</td>
<td>VII–VIII/17</td>
<td>IV–V/13</td>
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<tr>
<td>20</td>
<td>36</td>
<td>X/22</td>
<td>VII–VIII/18</td>
<td>4</td>
</tr>
</tbody>
</table>

Irises were cultured in vitro in the presence of 2\(\times10^{-5}\) M colchicine and subsequently implanted in host lentectomized eyes for 15 days. See Table 1 for calculation of average apparent age.

depigmented cells showing no evidence of elongation (Fig. 1). Under histological examination no morphological evidence of cytoplasmic or nuclear damage was observed. Moreover, when regenerates treated with these colchicine concentrations were cultured for 25 days in vivo, they were able to recover and form lens fibers (Table 3). The histological appearance of control and colchicine-treated regenerates is presented in Fig. 1. For subsequent work a colchicine
Cell elongation during lens regeneration

Fig. 2. Effects of colchicine on 7- and 10-day regenerates cultured for one day in vitro and 15 days in vivo. (A) Seven-day regenerate control late stage VI. (B) Colchicine-treated 7-day regenerate stage V cells at the onset of elongation are pointed at (arrow). (C) Ten-day regenerate control; primary lens fibers are evident, stage VII. (D) Colchicine-treated 10-day regenerate in which many depigmented cells are evident. ×250.

A concentration of 2.5 x 10^-5 M was selected since this was the lowest dose that would inhibit cellular elongation under these conditions.

Another parameter to be investigated was the time of in vivo culture at which the effects of colchicine on the treated regenerate would become evident. To
answer this question experiments were performed in which 15-day regenerates were cultured for 24 h in the presence of $2.5 \times 10^{-5}$ M colchicine and then implanted in the eyes of host newts for 5, 10, 15, and 25 days. The results of these experiments are presented in Table 3. Five to ten regenerates were studied for each time interval. It can be observed that a retardation of the regeneration process is already evident in regenerates cultured in vivo for 5 days after colchicine treatment. The most pronounced retardation was observed in regenerates cultured in vivo for 15 days after treatment; no evidence for lens fiber differentiation was observed in these implants. That regenerates were able to recover from the inhibition of regeneration was evident in irises maintained for 20 and 25 days in host eyes where the presence of primary and secondary fibers was observed in implanted irises that had attained stages VI–IX (Fig. 1).

To investigate whether other regeneration stages were also affected by colchicine treatment, experiments were performed in which normal irises and regenerates at 2, 5, 6, 7, 10, 15, and 20 days after lentectomy were used. Tissues were cultured for 24 h in the presence of $2.5 \times 10^{-5}$ M colchicine and implanted in host newt eyes for 15 days. Five irises were examined for each period after lentectomy and drug-induced retardation was estimated as before. The results of these experiments are shown in Table 4. It can be observed that colchicine at this concentration has no noticeable effect on the further development of implants of normal dorsal irises and regenerates at 2 days after lentectomy. Colchicine treatment of regenerates at 6–15 days after lens removal inhibits the further morphogenesis of the implanted irises. The development of the regener-
ates is arrested at stages V or VI where onset of cellular elongation occurs. Regenerates treated at 20 days after lentectomy, when elongated primary lens fibers are already present, also undergo a delay in development; however, all of the regenerates in these series attained the morphogenesis of stages VII–VIII characterized by presence of elongated acidophilic lens fiber cells. The scatter for each group of regenerates varied in a random fashion; this was also the case for the experiments mentioned in the previous section. The effects of colchicine on 7-, 10-, and 15-day regenerates cultured for 15 days in vivo can be observed in Figs. 1 and 2. Lumicolchicine treatment had no appreciable effect on the development of implanted regenerates when compared to the controls.

**Effects of colchicine on the presence of lens specific protein**

In order to determine whether lens specific protein synthesis could occur in the absence of cell elongation, fluorescent antibody studies were performed on 15-day regenerates cultured 1 day in vitro in the presence or absence of colchicine, and then cultured 15 days in vivo. Figure 3 shows the results obtained when regenerates treated with absorbed anti-lens serum were stained with fluorescein-conjugated antirabbit immunoglobulin. In control irises a bright fluorescence was present in all of the lens fiber cells, while a slight fluorescence occurred in the lens epithelium; although not as bright as that in the fiber cells (Fig. 3A). No fluorescence was present in other tissues of the host eye. Under these conditions colchicine-treated irises showed no detectable fluorescence when compared to controls (Fig. 3B). One or two minute spots of fluorescence were present in a few of the internal cells of one of the colchicine-treated irises studied. Four regenerates were examined for the control and three for the colchicine treatments. Newt liver tissue, treated with lens antiserum and stained with fluorescein-conjugated anti-rabbit immunoglobulin, showed no detectable fluorescence. Tests with serum from uninjected rabbits showed no fluorescence in control lens regenerates in situ.

**Effects of colchicine on the incorporation of [H³]uridine, [H³]thymidine and [H³]leucine**

Colchicine has been reported to inhibit nucleoside incorporation in mammalian systems (Hell & Cox, 1963; Creasey & Markiw, 1965). Experiments were conducted to investigate whether incorporation of uridine and thymidine was affected in colchicine-treated regenerates. When autoradiographs of [H³]uridine-treated implants were examined, no significant differences in grain counts per area were detected in control and colchicine-treated regenerates. Similarly the synthetic index did not vary appreciably in control and colchicine-treated implants as measured by [H³]thymidine incorporation. In implanted regenerates labelled with [H³]leucine differences were observed in autoradiographs of control and drug-treated tissues. Since increased protein synthesis occurs first in a localized number of cells at the onset of lens fiber
differentiation (Yamada & Takata, 1963), to obtain an estimate of the incorporation of $[^{3}H]$leucine into protein, autoradiographs of control and colchicine-treated implants were investigated in the following manner. Grain counts were performed in cells which were heavily labelled; similarly grain counts were performed in cells which appeared weakly labelled. Counts were performed for the same number of cells in each class, in randomly chosen sections. The sum of averages of these counts was obtained for several control and colchicine-treated implants. In Fig. 4 differences in incorporation of $[^{3}H]$leucine between control and colchicine-treated regenerate implants is presented. It can be seen that colchicine-treated regenerates exhibit a lower incorporation of this amino acid into protein as evidenced by the lower grain counts present in their autoradiographs. Similarly, the increase in average grain counts per area observed in regenerates cultured for 5 days in vivo is delayed slightly in colchicine-treated regenerates. This result suggests that although colchicine treatment affects amino acid incorporation, it does not completely inhibit protein synthesis in implanted regenerates.

**Effects of vinblastine in implanted regenerates**

Although considerable retardation was induced by vinblastine sulfate, we regard studies on the effect of this alkaloid as inconclusive because of evidence
Cell elongation during lens regeneration

Table 5. The effect of cytochalasin B on various stages of lens regeneration

<table>
<thead>
<tr>
<th>Age of post-lentectomy (days)</th>
<th>Total age (days)</th>
<th>Stages of control/average apparent age</th>
<th>Stages of treated/average apparent age</th>
<th>Retardation</th>
</tr>
</thead>
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<tr>
<td></td>
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<td></td>
<td>Cytochalasin B</td>
<td>DMSO</td>
</tr>
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<td>0</td>
<td>16</td>
<td>IV-VI/14</td>
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<td>31</td>
<td>VII-X/20</td>
<td>IX-X/20</td>
<td>11</td>
</tr>
</tbody>
</table>

Irises were cultured in vitro in the presence of cytochalasin B (20 μg/ml) for 24 h and subsequently implanted into host lentectomized eyes for 15 days. See Table 1 for estimates of average apparent age.

Effects of cytochalasin B on lens regeneration

A series of experiments was conducted in order to investigate the effect of this chemical on irises at increasing time intervals after lentectomy. Regenerates were treated in vitro with cytochalasin B and maintained as implants for 15 days. The results of these studies are presented in Table 5. The data show that under the experimental conditions of this study there is no observable effect of cytochalasin B on lens regeneration.

Ultrastructural observations were performed in 15-day regenerates and were confined to regions of the regenerates where cells involved in the process of depigmentation or depigmenting cells at the onset of elongation were present. In control regenerates, depigmented cells were associated rather loosely; they appeared to send cellular processes which intertwined and came close to each other. In some regions, cells were present in close apposition to one another and desmosomes occurred. In agreement with earlier studies (Dumont & Yamada, 1972) the cytoplasm was rich in mitochondria, ribosomes and profiles of rough endoplasmic reticulum; microfilaments were observed located immediately below the cell surface. Microtubules were present in all areas of the thin layer of cytoplasm surrounding the nucleus of the elongating cell. They were located just below the cell surface, in the intermediate areas of the cytoplasm and close to the nuclear membrane. Microtubules were oriented for the most part parallel...
Table 6. Effect of neuraminidase on electrophoretic mobilities (μm/sec/√cm) of 14-day regenerates cultured for different time intervals

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Saline (S)</th>
<th>Neuraminidase (N)</th>
<th>Difference (S – N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0a</td>
<td>1.875</td>
<td>1.775</td>
<td>0.100</td>
</tr>
<tr>
<td>2b</td>
<td>1.904</td>
<td>1.73</td>
<td>0.174</td>
</tr>
<tr>
<td>3</td>
<td>1.951</td>
<td>1.919</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>1.821</td>
<td>1.40</td>
<td>0.421**</td>
</tr>
<tr>
<td>4</td>
<td>1.94</td>
<td>1.48</td>
<td>0.46**</td>
</tr>
<tr>
<td>5</td>
<td>1.992</td>
<td>1.636</td>
<td>0.356**</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.384</td>
<td>0.616**</td>
</tr>
<tr>
<td></td>
<td>1.77</td>
<td>1.38</td>
<td>0.39**</td>
</tr>
</tbody>
</table>

Fourteen-day regenerates were cultured for 24 h in vitro and subsequently implanted into host lentectomized eyes. Cell suspensions were obtained by trypsinization of irises and electrophoretic mobilities were determined: a, determined in cell suspension prior to culture; b, EPM determined in cell suspensions 24 h after in vivo culture.

** When compared by a t test the differences were significant by a probability < 0.5%. Where probability values are missing the differences are not significant.

to the axis of elongation (Fig. 5), although in many sections some appeared to run perpendicular to this axis. Sections of several colchicine-treated regenerates were observed; although the cellular organization of regenerates was similar to that of controls, no microtubules were observed in the cytoplasm of depigmented cells while microfilaments were still visible in regions of the cytoplasm. Lattices presumably composed of microtubule crystals were regularly observed in the cytoplasm of depigmented cells of vinblastine-treated regenerates (Fig. 5). These lattices were similar to those described by Wilson, Bryan, Ruby & Mazia (1970) and identified as precipitated lattices of microtubular protein (Bensch & Malawista, 1968; Bensch, Marantz, Wisniewski & Shelanski, 1969).

Cell electrophoresis

The purpose of these experiments was to determine whether colchicine treatment would affect the appearance of certain cell surface groups in the regenerating iris. Neuraminidase sensitive groups present in cell suspensions of normal irises cannot be detected at the cell surface when dedifferentiation is completed. These groups reappear as redifferentiation occurs, at 15 days after lentectomy and later. It was of interest to determine whether colchicine treatment

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Fig. 5. Electron micrographs of sections from 15-day regenerates. (A) Section through an elongating cell from the central portion of the regenerates. Note the sub surface microtubules (Smr) and a desmosome (D) between two elongating pigmented cells, ×42000. (B) Section through a depigmented cell from a 15-day regenerate treated with vinblastine sulfate for 24 h. A typical vinblastine-induced crystal (V) is present; microfilaments (F) can also be discerned in the cytoplasm (×80000).
Regenerates at different days after lentectomy were cultured in vitro in the absence (control) or in the presence of colchicine (2 x 10^{-5} M). They were then implanted into host lentectomized eyes for 4 days. After this interval implanted irises were removed and cell suspensions were obtained. For each experiment aliquots were incubated in the presence or absence of neuraminidase (25 u./ml).

* Probability < 1% or less; ** Probability < 0.5% or less; *** Probability < 0.1% or less. Where probability values are missing the differences between saline and enzyme treated cells are not significant.

would affect the appearance of neuraminidase sensitive groups, detectable by cell electrophoresis during redifferentiation.

A first series of experiments were performed to determine the sequence of appearance of neuraminidase sensitive groups in cultured iris implants. Fourteen-day regenerates were cultured in vitro in control medium and the effects of neuraminidase on the electrophoretic mobilities was determined in cell suspension of regenerates implanted into host newts for 1-4 days. From the data presented in Table 6 it can be seen that surface groups sensitive to neuraminidase treatment appear in cells of cultured regenerates 2-4 days after implantation into host newt eyes.

Regenerates 8-21 days after lentectomy were then cultured in vitro in the presence of colchicine and implanted into newt host eyes for 4 days. The results presented in Table 7 indicate that enzyme sensitive groups are present in cells from 14- and 15-day control regenerates; however, the electrophoretic mobilities of cells from colchicine-treated regenerates are not affected by neuraminidase treatment. The presence of enzyme sensitive groups in 8-day colchicine-treated regenerates is to be expected, since these groups are still present in 7- and some 10-day regenerates during the normal course of regeneration (Zalik & Scott,
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1973). The results obtained with 16- and 21-day regenerates suggest that neuraminidase sensitive groups already present at the cell surface (Zalik & Scott, 1972) were not affected by colchicine treatment.

DISCUSSION

Lentectomy stimulates the iris epithelial cells of the newt's eye to undergo DNA synthesis (Eisenberg & Yamada, 1966; Reyer, 1971; Yamada & Roesel, 1969) and proliferate. Concomitantly with these processes, melanosomes disappear from the pigmented iris epithelium either by dilution during cell division or by an active process on which the iris epithelial cells discharge the melanosomes invested in portions of the cell membrane (Eguchi, 1964; Dumont & Yamada, 1972). After completion of this phase of dedifferentiation, some cells retreat from the cell cycle, elongate, proceed to synthesize lens specific proteins and transform into lens fiber cells (Yamada, 1967). One of the questions pertinent to our studies is how many of the above mentioned processes are affected by the colchicine treatment. Cell cycle parameter studies on the dedifferentiating iris epithelium (Yamada, Roesel & Beauchamp, 1975) have indicated the presence of two types of cells in the dorsal iris; those which transform into lens cells after the complete loss of melanosomes and follow the pathway of conversion, and those involved in the pathway of retrieval. The latter are represented by cells who enter mitosis, undergo a partial loss of melanosomes, and subsequently redifferentiate into pigmented iris cells (Yamada, 1976). Colchicine binds to microtubular subunits (Borisy & Taylor, 1967a, b) and evidence suggests that slow morphogenetic movements that involve assembly of these subunits tend to be colchicine sensitive (Margulis, 1973). In our experiments, retardation induced by this alkaloid was first observed in 6-day regenerates, and increased in 7- and 10-day regenerates. Mitotic figures first become evident in the iris epithelium 4–5 days after lentectomy, while in 7- and 10-day regenerates a large number of mitotic cells occur (Eisenberg & Yamada, 1966; Yamada & Roesel, 1971). It is conceivable that some of the colchicine effects observed at these stages were due to its interference with mitosis, since it has been suggested that at least six cell cycles are necessary for the iris melanocyte to transform into a lens fiber (Yamada, 1976). The appearance of depigmented cells in 7- and 10-day colchicine-treated regenerates cultured for 15 days could be explained by extrusion of melanosomes in cells in which cell division is halted. Colchicine effects on 15-day regenerates, however, cannot solely be explained by its interaction with microtubular subunits during formation of the mitotic spindle, since at this stage many cells in the regenerate have left the cell cycle and are beginning to elongate to follow the pathway of conversion. A more likely possibility in this case is that colchicine affects lens cell elongation by its interaction with microtubular subunits. That the effects of colchicine are not due to cell death is indicated by the general healthy appearance of the affected regenerates and their
incorporation of \([H^3]\)uridine and \([H^3]\)thymidine into high molecular weight compounds. The ability of colchicine-treated regenerates to recover from the effects of this alkaloid after a suitable interval, argues against an irreversible non-specific damage induced in this system.

A noticeable effect of colchicine on 15-day regenerates was the inhibition of cellular elongation which occurs during lens fiber formation. The notion that the inhibitory effects of this alkaloid are due to its interaction with the microtubular apparatus is supported by ultrastructural observations. Microtubules normally oriented along the axis of elongation, disappeared after colchicine treatment (Hornsby, 1975) and characteristic vinblastine-induced crystals were detected in non-pigmented cells of 15-day regenerates. Our results are in agreement with those of other investigators (Piatigorsky et al. 1972a, b) studying lens fiber formation in the cultured chick lens epithelium. The presence of lens fibers in 20-day colchicine-treated regenerates suggests that once elongation has occurred it cannot be reversed by colchicine, and in this respect our results are in agreement with those of Pearce & Zwaan (1970) in the developing chick lens.

Yamada & Takata (1963) and Takata, Albright & Yamada (1964) have observed that onset of crystallin synthesis in the prospective primary lens fiber of 15-day regenerates coincides with a noticeable increase in incorporation of amino acids into protein observable by autoradiography. Our data with implanted regenerates show that this secondary enhancement of protein synthesis is retarded when compared to regenerates in situ. In colchicine-treated regenerates the increase in amino acid incorporation is more gradual and never reaches the magnitude of the increase for controls. When absorbed anti-lens serum was applied to control and colchicine-treated regenerates, a bright fluorescence was observed in the lens-cell aggregates formed by the implanted regenerates, while no detectable fluorescence was observed in colchicine-treated irises. An interpretation of these results is that in the latter \([H^3]\)leucine was incorporated into proteins that are not lens-specific, although the possibility that the level of lens-specific proteins in colchicine-treated regenerates was not high enough to be detected by the fluorescent antibody method also has to be considered. An attractive explanation of these results is that elongation and synthesis of tissue-specific protein occur in concert and that inhibition of elongation resulted in the inhibition of tissue-specific protein synthesis. A relationship between microtubules and orientation of tissue-specific proteins has been reported during myogenesis (Fishman, 1967; Warren, 1968). However, the possibility that colchicine inhibits tissue specific protein synthesis directly cannot be discarded.

There is recent evidence suggesting that microtubules may play a role in the modulation of receptors at the cell surface (Yahara & Edelman, 1972; de Petris, 1974; Oliver, Ukena & Berlin, 1974; Edelman, 1976). Previous work from this laboratory has shown that surface components present in the pigmented iris cell disappear during dedifferentiation and reappear at the onset of redifferentiation (Zalik & Scott, 1972, 1973; Zalik, Scott & Dimitrov, 1976). From the
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data in Table 7 it can be concluded, that, while the cells in control implanted regenerates possess neuraminidase sensitive groups at their surfaces, these groups are not detectable in cells of colchicine-treated regenerates. Although a variety of factors may affect the detection of specific charged groups at the ionic double layer by cell electrophoresis (see Cook & Stoddart, 1973) one possibility is that the lack of effect of neuraminidase on the cell’s EPM reflects the absence of groups sensitive to this enzyme at the cell surface. A possible explanation for this phenomenon is that a colchicine sensitive event presumably microtubule-mediated may be involved in the appearance of certain groups at the cell surface. An inhibition of cell aggregation by colchicine has been reported by several investigators (Waddell, Robson & Edwards, 1974; Lackie, 1974), and it has been suggested that microtubules may be involved in the insertion of new membrane or in redistribution of surface groups involved in adhesion (Waddell et al. 1974; Edwards, Campbell, Robson & Vicker, 1975). There is also evidence suggesting that neuraminidase sensitive groups may be involved in adhesion (Vicker & Edwards, 1972; Lloyd & Cook, 1974). The possibility exists that, during lens fiber differentiation, cellular elongation and insertion of new adhesive sites at the cell surface occur in synchrony and are microtubule mediated. However, a direct effect of colchicine on the cell membrane (Wünderlich, Miller & Speth, 1973; Furcht & Scott, 1975) cannot be excluded.

Cytochalasin B had no observable effect on elongation or morphogenesis regardless of the developmental stage of the regenerate. However, microfilaments were present in elongating cells of regenerates; and, in other systems, these structures have been shown to reappear rapidly after removal of this drug (Wessells et al. 1971). Short-term experiments are needed before a role of microfilaments in cellular elongation is discarded in this system.

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REFERENCES


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