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Original Articles

The Influence of Hormones and Other Substances on Lens Regeneration in vitro

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Culturing the dorsal iris epithelium of a newt with a pituitary gland in organ culture greatly enhances the ability of the iris epithelium to produce advanced lens regenerates in vitro. In an attempt to elucidate the mechanism by which the pituitary enhances lens regeneration irido-corneal complexes from adult newts were cultured in medium to which various substances had been added either singly or in numerous combinations. Prolactin, insulin, hydrocortisone, and thyroxine failed to enhance the production of advanced lens regenerates in any of the doses or combinations tested. Similarly, addition of 50 μg/ml of sodium or calcium ascorbate had no effect on the progress of lens regeneration in vitro. Addition of dibutyryl cyclic-AMP caused an inhibition of depigmentation and regeneration at high doses. The results of these experiments show that the effects of the pituitary cannot be duplicated by hormones which other authors have asserted to be beneficial to limb or tail regenerates in vitro. The results with cyclic AMP suggest that prolonged exposure to high doses of cyclic AMP inhibit regeneration and indicate that further studies on the fluctations in cyclic AMP levels throughout the process of lens regeneration must be done.

Introduction

There has been considerable interest in the role of hormones in regeneration for some time. Numerous experiments have been carried out in vivo to determine if hormones of pituitary or other origin have effects on limb and lens regeneration [1–4]. Recently, progress has been made in studying the role of hormones in regeneration under the controlled conditions of an organ culture environment [5–7]. Studies on the regenerating lens [6, 7] have shown that the entire process of lens regeneration can occur in a 3-week period of organ culture if iris and pituitary gland are cultured in close proximity for at least 10–15 days. The studies of Vethamany-Globus and Liversage [5] showed that combinations of hormones of various origins (growth hormone, insulin, hydrocortisone, and thyroxine) would enhance cartilage differentiation and other regeneration processes in newt tail blastemas in culture. These studies, however, involved only a very short (98 h) culture period, and utilized blastemas which were already in the process of regeneration. In order to further investigate the role of hormones of various origins and other substances in promoting lens regeneration in vitro, the following experiments were undertaken.

Medium was enriched with different doses and combinations of prolactin, insulin, hydrocortisone, and thyroxine. Prolactin was chosen rather than growth hormone since it has been shown to have a pronounced effect on limb regeneration in vivo [1, 4] and since prolactin is known to be secreted in large amounts by newt pituitaries which have been separated from hypothalamic connections [8]. This would be the case in organ culture where the pituitary is known to markedly enhance production of advanced lens regenerates from the dorsal iris epithelium. Other substances such as dibutyryl cyclic-AMP (But_2cAMP) and theophylline were chosen as a result of reports by Thorpe et al. [9] that cyclic AMP (cAMP) levels increase during the regenerative process. Ascorbic acid was also tested since it has been reported to facilitate lens differentiation from chick neural retina cells in tissue

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culture [10]. This is important since it is also known that in adult newts lens fibers may regenerate from neural retinal cells [11].

Methods

Adult newts, *Notophthalmus viridescens*, were obtained from Bill Lee's Newt Frams, Oak Ridge, Tennessee. Animals were soaked for 30 min in a weak permanganate solution [12]. Following this treatment the heads and eyes of each newt were rinsed in a strong stream of 70% ethanol followed by a rinse with sterile distilled water. Irido-corneal complexes (ICC's) [6] were dissected from eyes and rinsed three times in phosphate buffered saline (Dulbecco) diluted to 80% mammalian tonicity and containing twice the concentration of antibiotic and antimycotic agents used in the normal culture medium (see below). The lens and adherent retinal tissue was dissected from the ICC's in the phosphate buffered saline prior to final explantation. ICC's were then placed upon sterile pieces of Gelman "Metricel" membrane filters (0.45 μ pore size) which had been rinsed several times overnight in culture medium. These were in turn placed upon sterile pieces of lens paper. The entire combination was then supported by a stainless steel grid in the central well of an organ culture dish. Culture medium was added to the well to a level such that the lens paper acted as a wick to draw a sufficient amount of fluid over the cultured tissue. The medium was formulated as follows: L-15 (Leibovitz) – 50%; sterile distilled water – 40%; fetal calf serum – 10%; kanamycin – 200 μg/ml; polymixin E (Colymicin, colistin sulfate) – 200 IU/ml; fungizone – 5 μg/ml [13]. Medium was changed every third day. All operations, medium preparation, and medium changes were performed in a laminar airflow hood to minimize contamination. Prolactin (Ovine, NIH) was dissolved in 0.9% NaCl at pH 9.0. It was added to the medium in concentrations of 5, 10, and 50 μg/ml. Insulin was dissolved in a small volume of 0.5 N HCL and diluted to the desired stock concentration. Hydrocortisone was first dissolved in ethanol and diluted to a desirable stock concentration with water. It was then added to the culture medium in concentrations of 5, 10, and 50 μg/ml. Thyroxine was dissolved initially in weak base and diluted to a desirable stock concentration. It was added to the medium in concentrations of 10^{-8}, 2×10^{-8}, and 10^{-7} mg/ml. Sodium or calcium ascorbate was added to medium at a concentration of 50 μg/ml. As a control for the possible effects of added calcium ions another experiment was performed in which calcium chloride was added to the medium to yield an equivalent increase in the calcium ion concentration of the medium. Theophylline (10^{-5} M) and dibutyryl cyclic 3',5'-adenosine monophosphate (But_2cAMP; 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} M) were also added to several groups of cultures. All of the above reagents were purchased from Sigma Chemical Co. Twenty-one days after explantation, cultures were fixed and prepared for histologic evaluation. Responses were characterized as described previously [6, 7]. In all experiments controls consisted of ICC's cultured in modified L-15 medium [6].

Since it appeared that treatment with a low dose of insulin caused an enhancement of depigmentation, measurement of the volume of depigmented masses in several of these cultures was undertaken and compared to that in control cultures. To this end three of the most advanced regenerates obtained in the treated and control groups were evaluated. The depigmented area in each serial section through the regenerate was traced with the aid of a camera lucida. The area of each tracing was measured with a polar planimeter and the volume of the depigmented mass computed from these measurements [14].

Results

The results of the hormone enrichment experiments are summarized in Table 1. It is clear that none of the combinations or doses tested caused any significant increase in the production of advanced lens regenerates in culture. The primary response in the majority of cases was only depigmentation. It appeared that there was an increase in the number of cases which showed some lens fiber differentiation following treatment with the lowest doses of insulin and thyroxine. However, treatment simultaneously with these two hormones resulted only in the formation of depigmented cell masses. This percentage of cases

Table 1. Influence of hormone enrichment of the culture medium on lens regeneration in vitro

Treatment	Response of the iris		
	Class I	Class II	Class III
No treatment	17/47	28/47	2/47
Plus pituitary	3/20	2/20	15/20
All hormones			
Dose I	4/10	5/10	1/10
Dose II	4/10	6/10	
Prolactin			
Dose I		9/10	1/10
Dose II	2/8	6/8	
Dose III	5/8	3/8	
Thyroxine			
Dose I	1/10	6/10	3/10
Dose II	4/10	6/10	
Dose III	2/10	8/10	
Insulin			
Dose I		6/9	3/9
Dose II	3/8	4/8	1/8
Dose III		10/10	
Cortisol			
Dose I	4/10	5/10	1/10
Dose II	6/10	4/10	
Dose III	7/10	3/10	
Paired combinations (all dose I)			
Prolactin + insulin	5/8	3/8	
Prolactin + thyroxine	3/10	7/10	
Thyroxine + insulin	2/10	8/10	
Prolactin + thyroxine + insulin	2/16	13/16	1/16

Class I = no depigmentation (stages I–II)
Class II = depigmentation only (stages III–V)
Class III = depigmentation and lens fiber formation (stages VI–X)
See text for dose levels (I corresponds to lowest dose)

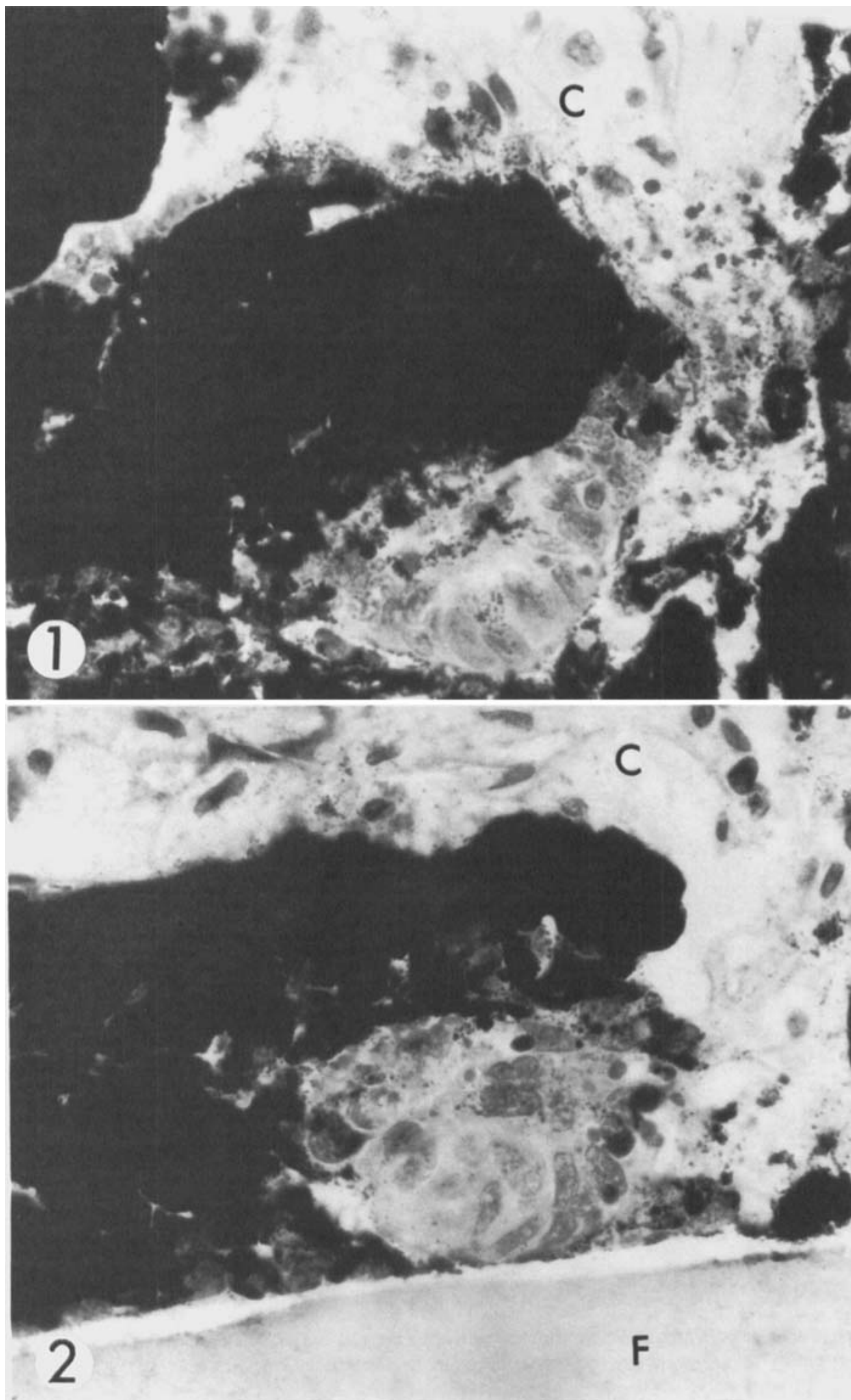
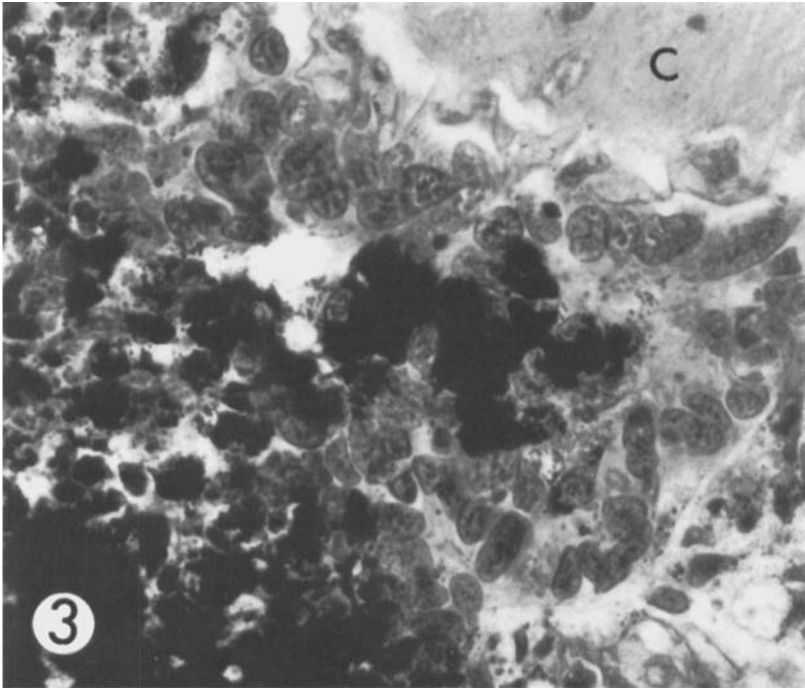


Fig. 1. A photomicrograph of one of the most advanced regenerates obtained by culturing an iridocorneal complex in medium containing insulin (0.14 IU/ml), thyroxine (10^{-8} mg/ml), prolactin (5 μg/ml), and cortisol (5 μg/ml). Compare the morphology of this regenerate with that obtained by culturing the iris in contact with a pituitary gland (Fig. 4). Although some cells appear to be starting to elongate the fiber hillock has not yet begun to appear. *C*, Cornea. × 400

Fig. 2. A photomicrograph of one of the most advanced regenerates obtained by culturing an iridocorneal complex in the presence of 5 μg/ml of prolactin. Again, although some cell elongation has begun, this is not as advanced as the type of regenerates obtained with pituitary glands. *C*, Cornea, *F* Gelman "Metricel" Filter substrate. × 400



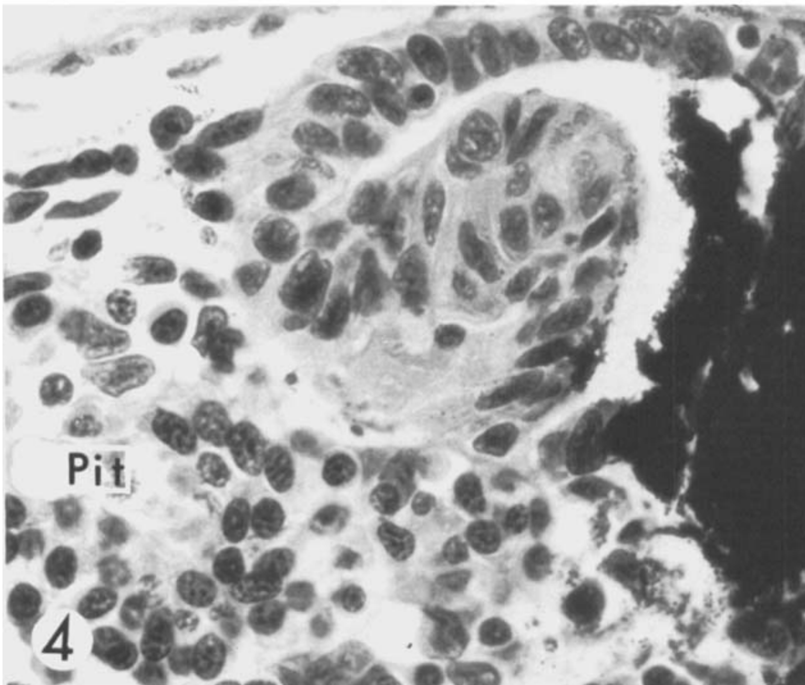


Fig. 3. A photomicrograph of a regenerate typical of those obtained in cultures enriched with thyroxine (10^{-8} mg/ml). This regenerate consists of a disorganized mass of depigmented and depigmenting cells. Few, if any, cells have begun to redifferentiate along lens fiber lines. *C*, Cornea. × 400

Fig. 4. A photomicrograph of an advanced regenerate typical of those obtained in large numbers of irises cultured in contact with a pituitary gland. Note the formation of the fiber hillock (*F*) and the elongating lens fiber cells within it. *Pit*, Pituitary. × 400

depigmenting is not outside the range obtained in control untreated cultures. Although some advanced regenerates do appear following treatment with hormones, the extent of differentiation and the morphology of the regenerates obtained is considerably less advanced than that obtained in cultures of iris plus pituitary gland (see Table 1 and compare Figs. 1–4), and usually not greater than that which may occur in irises cultured without treatment.

The mean volume of the three most advanced regenerates obtained in the irises treated with 5 μg/ml insulin was $2.31 \pm 1.21(SD) \times 10^8$ μm³ that of three regenerates obtained in untreated cultures was $1.6 \pm 0.3(SD) \times 10^8$ μm³. Statistical comparison of these values revealed no significant difference between the two groups.

Table 2 summarizes the results obtained when culture medium was enriched with But_2cAMP, theophylline, sodium or calcium ascorbate, or Ca^{2+}. Again none of the treatments caused an increase in the number of advanced lens regenerates formed. The results obtained with sodium ascorbate are data pooled from two experiments. The first time the experiment was performed 90% of the cultures depigmented. When the experiment was repeated only 40% of the irises underwent depigmentation. The differences between the effects of sodium and calcium ascorbate necessitated the study of the effects of equivalent doses of calcium ion alone. It is clear that calcium alone has neither a positive nor a negative effect on the progress of lens regeneration in vitro.

Table 2. Response of iridocorneal complexes to longterm culture in medium enriched with ascorbic acid, calcium, theophylline or cyclic AMP

Treatment	Response of the iris		
	Class I	Class II	Class III
Controls (no treatment)	23/64	37/54	4/54
Na-Ascorbate (50 μg/ml)	12/30	17/30	1/30
Ca-Ascorbate (50 μg/ml)	9/10	1/10	
Calcium (1.28×10^{-4} M)	4/9	5/9	
Theophylline (10^{-5} M)	2/10	6/10	2/10
cAMP			
10^{-8} M	1/8	7/8	
10^{-6} M	3/10	7/10	
10^{-4} M	3/8	5/8	
10^{-2} M	16/16		

Class I = no depigmentation (stages I–II)
Class II = depigmentation only (stages III–IV)
Class III = depigmentation and lens fiber differentiation (stages VI–X)

Only the highest dose of But_2cAMP utilized (10^{-2} M) had any significant effect on the progress of lens regeneration in vitro. It is interesting to note that cAMP appears to inhibit rather than promote the process of lens regeneration. It is not possible, at least at this point, to determine if this effect is a real inhibition or simply a result of toxicity of the high dose of cAMP.

Discussion

The results presented above indicate that the process of cell-type conversion during lens regeneration in organ culture is markedly enhanced by only a limited number of conditions. The term "enhancement" is used here since it is known that even in organ culture and especially in tissue culture [15] cell-type conversion from iris epithelium to lens will occur in many cases. Although the iris has an inherent tendency to produce lens cells this tendency is greatly enhanced in organ culture by the presence of neural retina [16] or pituitary gland [6, 7]. The experiments described in this paper were undertaken to determine if the enhancing effects of the pituitary gland could be duplicated by treating the irido-corneal complexes with hormonal substances, or with substances known to be involved in the stimulation of differentiation in other systems. Although the treatments described in this paper are not extensive enough to unequivocally rule out a role of hormones or the adenyl cyclase-cyclic AMP system in the enhancement of lens regeneration in organ culture by the pituitary the results suggest that such a link is unlikely.

The results of the hormone treatment experiments are interpreted as being essentially negative since even though some treatments may have increased the number of cases depigmenting (e.g., the low dose of insulin) none of the treatments resulted in the formation of well-developed advanced lens regenerates in a frequency comparable to that found in iris-pituitary combinations. Likewise, since no differences could be found in the volume of depigmented masses in insulin treated and control cultures this finding is also interpreted as a negative result. Since mammalian hormones were used one might argue that such substances would not be expected to have any effect on amphibian tissues. Experiments utilizing hormones of amphibian origin have not yet been performed. However, it is known that the hormones used in the present study are biologically active in amphibians in vivo [1, 4] and in vitro [5]. It is known that antisera to mammalian pituitary hormones will cross react with substances in amphibian pituitary glands [17–21]. One cannot equate immuno-

logic cross reactivity with biological activity, but in light of the other evidence available it would appear safe to assert that the negative results are probably not due to a preference of the iris tissues for amphibian rather than mammalian or synthetic hormones. It is possible that the results are due to destruction of the added hormone by lysosomal enzymes in the iris itself. The difference between the hormone enrichment studies and those in which the iris is cultured in contact with the pituitary gland could be explained by the hypothesis that the pituitary may produce a sufficient excess of material(s) so that the necessary amounts reach the iris cells despite the presence of lytic enzymes.

The possibility also exists that the premise of hormonal involvement in regeneration is a false one. Little direct evidence exists that the newt pituitary actually releases hormonal materials in vitro. This seems unlikely for several reasons. First, it is known that the pituitary survives well during the 3-week culture period and that many acidophils are surviving at the end of the culture period (Connelly, personal observation). This is similar to the result seen in ectopic pituitary grafts in amphibians [8, 22]. Such grafts are known to be active in the production of a prolactin-like substance [4, 8]. Finally, it has been shown that newt pituitaries cultivated for 27 days in vitro can support survival and limb regeneration when implanted into the lower jaws of hypophysectomized newts [23]. However, until studies using radioimmunoassay or combined immunocytochemistry and radioautography are performed to follow the synthetic activities of specific cell types in the newt pituitary in organ culture this final possibility cannot be ruled out.

The result obtained in those experiments designed to alter the levels of cAMP in the iris are interesting in light of the results of Ortiz et al. [24] and Ortiz and Yamada [25]. One might expect that increased levels of cAMP would lead to a more rapid depigmentation or to a larger number of depigmented cells. However, the results obtained with a high dose (10^{-2} M) of But_2cAMP are precisely the opposite. It is possible that the present results do not accurately reflect the normal condition since the irido-corneal complexes were cultured continuously in the presence of cAMP. Perhaps one must alter the levels in a periodic, as yet undetermined cycle. The existing data on cyclic AMP levels in lens regenerates during the whole process of regeneration are not complete. Thus, we have no way of knowing how levels of this substance fluctuate with time. Of course it is clearly possible that the results obtained here reflect an overall toxic effect of increasing amounts of But_2cAMP in the medium.

The present results combined with earlier reports from this laboratory and others [6, 15, 26, 27] indicate that cell-type conversion in lens regeneration can be initiated by explantation of the iris to organ or tissue culture conditions in at least a limited number of cases. It is not known why some irises in organ culture fail to undergo depigmentation nor is it known why others which do depigment fail to produce advanced lens regenerates. Thus, it is not possible at this time to determine whether enhancement of lens regeneration by the pituitary gland in vitro is a result of an effect on dedifferentiative or redifferentiative events. Circumstantial evidence suggests that the critical step(s) affected by the pituitary occur sometime around day 10 after explantation or at least after Stage IV–V of regeneration has been attained [6, 26, 27]. It has been hypothesized that iris epithelial cells must undergo a requisite number of cell divisions prior to redifferentiation [28]. It is possible that the pituitary exerts its effect by enhancing the progress of cells through the cell cycle in organ culture. Unpublished observations in my laboratory show that in the presence of the pituitary gland the incorporation of labeled thymidine by the iris epithelial cells is higher than in irises cultured alone for 12 days. These results are complicated by the fact that most cultures with pituitary are producing lens regenerates while most of the irises cultured alone are not.

It also appears that there is a correlation between cellular elongation and lens fiber crystallin protein syntheses [29]. It is possible that the pituitary enhances production of advanced lens regenerates in organ culture by influencing this aspect of redifferentiation. No data are yet available concerning such comparisons of irises in situ and those maintained in organ culture with or without the pituitary gland.

Analysis of experiments of the type presented above is hampered by the lack of an easily applicable quantitative measure of enhancement of lens regeneration. For example, individual variation in the size of the regenerates produced and the lack of a strict morphologic identity between regenerates obtained in situ and in vitro complicate statistical evaluation of results. It is not possible to measure subtle responses of the iris to treatments which might be important in identifying the mechanism by which the pituitary gland enhances lens regeneration in organ culture. Thus, we are able to recognize only those treatments which produce marked enhancement (80–90% of the cases forming advanced regenerates) and this is a true fault of the assay system. However, when one is interested in duplicating such effects one would like to be able to achieve high levels of enhancement.

The differences in my results and those obtained with tail regenerates in vitro [5] could be a result of several factors. The most obvious is that the two processes have basically different requirements for progress in culture.

Another possibility is that since the tail regenerates tested were already in the process of regeneration while the irises in my experiments were required to initiate the entire sequence of regenerative events in culture, the tails had passed through some critical step(s) prior to explantation which allowed them to show a response to hormones in vitro.

It is also difficult to explain the differences between differentiative capacity of iris epithelial cells in tissue culture and those maintained as an intact organ. Until we know more about the relationship between the cell cycle and regeneration, and about the similarities in cell cycle times of iris cells in tissue and organ culture further speculation about the cause of the greater frequency of lens cell differentiation in tissue culture is unwarranted.

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