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This Thesis

THE EFFECTS OF COLCHICINE ON LIMB REGENERATION IN AMPHIBIANS

for "Maxima Cum Laude" Recognition, by

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by

has been approved for the Department of Biology

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CHAPTER I

INTRODUCTION

Self-repair characterizes all protoplasm. Every species of animal is capable, at least to some degree, of replacing lost body parts, and in most cases, do so with unvarying accuracy. Either in part or in whole, the organism assumes the characteristics of the old; both in structure and function. This unique power of the body, called regeneration, offers a splendid opportunity to study the fundamental features of cell division, growth, and differentiation.

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the fact that the regenerative capacity tends to vary inversely as the scale of organization. Generally, the lower animals on the phylogenetic scale can replace lost parts better than the more highly developed organisms which rank above them. Hence, such investigation has been undertaken to determine the prerequisites for regeneration, and although the source of the regenerating cells can be accurately pinpointed in many species, there are different theories as to what curbs the regenerative processes in higher animals. In spite of the agreement regarding the controls

1. Roberts Hugh, Experimental Embryology, (Minneapolis, 1948), p. 317.
 2. Paul Weiss, Principles of Development, (New York, 1937), p. 459.

CHAPTER I

INTRODUCTION

Self-repair characterizes all protoplasm.¹ Every species of animal is capable, at least to some degree, of replacing lost body parts, and in most cases, do so with unvarying accuracy. Either in part or in whole, the new structure assumes the characteristics of the old, both in structure and function. This unique power of the body, called regeneration, offers a splendid opportunity to observe the basic features of cell division, growth, and differentiation.

Very obvious in its outward manifestation yet very involved in its fundamental aspects, regeneration has been the subject matter of much research in the past few years. One of the more notable things about this phenomenon, despite its occurrence in all living things, is the fact that the regenerative capacity tends to vary inversely as the scale of organization.² Generally, the lower animals on the phylogenetic scale can replace lost parts better than the more highly developed organisms which rank above them. Hence, much investigation has been undertaken to determine the prerequisites for regeneration, and although the source of the regenerating cells can be accurately pin-pointed in many species, there are different theories as to what curbs the regenerative processes in higher animals. In spite of the vagueness regarding the controls

1. Roberts Rugh, *Experimental Embryology*, (Minneapolis, 1948), p. 317.

2. Paul Weiss, *Principles of Development*, (New York, 1937), p. 459.

directing regeneration, there still remains this fact - some organisms are good regenerators, others are not.

Among the higher forms that regenerate rather well are the amphibians.¹ During their larval stages, regenerative power is very high, and in urodeles, it persists even after metamorphosis. For example, when the limb of a newt or salamander is cut off, immediately cells migrate and are mobilized in the wound area. Cell division begins and soon a white, cone-shaped blastema appears. The blastema elongates, and presently several tissues of a normal limb become differentiated. After a period varying with the age and state of the individual and with the conditions of the environment, a fairly typical limb is found in the place of what had been an amputation stump. This same situation occurs in the tadpole larva of the frog. Here, on large scale, is cell division, growth, and differentiation, an excellent opportunity to study the controls which operate regeneration. Also, the effects of various chemicals, heat, light, PH, and other factors which influence development can be investigated.

Among those chemicals suggested by Rugh which may be effective in altering the regenerative process is the alkaloid colchicine.² The recent evidence obtained concerning this drug definitely show it to influence the mitotic process very significantly. Yet it is not constant in its performance with all organisms. In agriculture, great success has been achieved in producing larger, newer, and more valuable plants with the use of colchicine, because it induces polyploidy (an increase in the number of chromosomes in the plant

1. Ibid.

2. Rugh, op. cit., p. 319

cells).¹ Various investigators report similar reaction in animal cells exposed to the drug, but they are in disagreement as to whether it is a mitotic stimulant or inhibitor, since many of them report a hindrance to the growth process caused by administration of colchicine.

The purpose of this work is to interpret the effects of colchicine as observed on regeneration of amphibian limbs, and in particular, those limbs of the tadpole larva of the frog. But before this is done, regeneration itself will be considered, followed by a more detailed discussion of colchicine and its effects.

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1. "Chemical Allows Mice to Survive Lethal X-Ray Dose", Science News Letter, Vol. 73 (March 1, 1958), p. 141.

1. Weiss, *op. cit.*, p. 458

2. C. H. Child, Patterns and Problems of Development, (Chicago, 1949) p. 11.

3. Weiss, *op. cit.*, p. 459

CHAPTER II

REGENERATION

Weiss defines regeneration as "the repair by growth and differentiation of damage suffered by an organism past the phase of primordial development,"¹ Another concept is offered by Child - "regeneration is reconstitution by outgrowth of new tissue from the cut surface and its differentiation into a part of an individual, often, but by no means always, more or less similar to the part removed."² These are simply two ways of looking at the regenerative process, but regardless of how it is defined, the important thing to realize is that there is no fundamental difference between the regeneration of an organ and its first development - both are of the same nature and follow the same principles. In fact it would seem that the capacity for regeneration is simply a residue of the original capacities for growth, organization, and differentiation, and the extent to which an organism can regenerate depends upon the extent of its ontogenetic development.³

Some regeneration is observed in all forms in which growth and the proliferation of new cells continues throughout life. Hydra, planaria, earthworms, amphibians, crustaceans, starfish, and mammals can regenerate, but the extent to which they can do so is not the same for each. The hydra, starfish and earthworms can restore all lost parts; crustaceans and amphibians can regrow

1. Weiss, op. cit., p. 458

2. C. M. Child, Patterns and Problems of Development, (Chicago, 1949) p. 31.

3. Weiss, op. cit., p. 459

severed limbs or tails; mammals can repair a moderate loss of skin, connective tissue, muscle and bone.¹ This agrees with what has been said above - the more developed an organism is, the more depleted is its regenerative capacity. But there are some lowly forms, such as the ctenophores and rotifers, with practically no regenerative power at all.² There are closely related forms, like the earthworms and the leeches, where the capacity for regeneration varies tremendously.³ There are even some forms that can regenerate better as they increase in age (i.e., Ascidians). In other words, there is not a single group of animals whose regenerative capacity could be safely predicted from its position on the evolutionary scale alone.

From these facts it is evident that the regenerative process must be a complex one. Many factors are involved behind the superficial descriptions seen in the preceding definitions. These include such questions as: What cells are involved - specific regenerative cells, or tissues cells that in normal uninjured animals perform quite different functions (hence, implying dedifferentiation)? What are the influences that induce differentiation of several new tissues in the regenerant? By what means is the new and restored part limited in growth and organization to replacement of the lost part? These questions must all be considered in order that a compact understanding of the regenerative process be attained.

There are still two main concepts regarding the source of

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1. "New Parts for Plants and Animals", Science Digest, Vol. 39 (June, 1956), p. 36.
 2. Weiss, op. cit.
 3. J. W. Buchanan, "Regeneration", The American Naturalist, Vol. 74 (1940), p. 483.

the regenerating cells.¹ Some hold that there are reserve, mesenchyme-type cells in all organs, to be used specifically in regeneration, if the need arises. Others believe that following an injury there is a period of dedifferentiation where the injured cells at the cut surface and those cells nearby lose their specificity, and "withdraw" to an indifferent state, followed by a period of multiplication, and then a redifferentiation into tissues assuming the structure and function of the original tissues.

The adherents to the "reserve-cell theory" base their convictions on histological studies in lower animals, such as planaria and annelids. In planaria, the "formative" or "regenerative" cells of the mesodermal region are the primary source from which new parts arise during regeneration.² Curtis proved this by exposing the planaria to X-rays and radium. He found that this inhibited regeneration, and that the formative cells were destroyed in numbers related to the degree of irradiation and of failure in regeneration. It was impossible to find any such cells in individuals whose ability to regenerate had been completely inhibited. Therefore, the failure to regenerate is due, at least in part, if not wholly, to elimination of these cells.³ The "neoblast" cells in annelids are effected in the same way by X-rays, so they are also considered as reserve regenerative cells.⁴

Most investigators grant that formative cells are responsible for regeneration in planaria. Also, they agree that these forma-

1. Rugh, op. cit., p. 317

2. W. C. Curtis, "The Histological Basis for Regeneration and Reassociation in Lower Vertebrates", The American Naturalist, Vol. 74 (1940), p. 491.

3. Ibid., pp. 491 - 494

4. Ibid., pp. 497 - 498

tive cells are much like the late blastomeres of the embryo.¹ Yet, there cannot be found such a stock of embryonic cells in higher animals. In fact, Curtis admits the possibility for dedifferentiation in these animals, mainly because of Thornton's work on the regeneration of salamander limbs. Yet, Curtis' X-rays have the same retarding effect on regeneration in salamander as in planaria and annelid.² Therefore it is assumed that where there is the same kind of effect by the same agency in so many widely separated cases, the action of similar factors is taking place. Regardless of the source of the regenerating cells, they have the same characteristics.

Thornton's observations on the regeneration of muscle in urodele amphibians is very convincing evidence for dedifferentiation.³ He amputated the forelimbs of larval salamanders (*Amblystoma punctatum*), and watched a new limb regenerate by the formation and differentiation of a regenerating blastema. This blastema was composed of a mass of morphologically undifferentiated cells, which took their origin, at least to a certain extent, from a progressive dedifferentiation of tissues of the limb stump which were cut at the time of amputation. These tissues were chiefly muscle and cartilage, and also included perichondrium, nerve connective tissue sheath, muscle connective tissue, and possibly subcutaneous connective tissue. The dedifferentiated cells of these various tissues became indistinguishable one from another

1. Ibid., p. 497

2. Ibid., p. 498

3. Charles S. Thornton, "The Histogenesis of Muscle in the Regenerating Forelimb of Larval *Amblystoma Punctatum*", Journal of Morphology, Vol. 62 (1938), pp. 17 - 36.

in the blastema.

The formation of the blastema in salamanders can therefore be regarded as a gradual process which is correlated with the rate of dedifferentiation of the injured cells and the mitotic activity of these dedifferentiated cells in the blastema. Following this mitotic increase in blastema cells is the redifferentiation into tissues composing the new forelimbs. Whether these redifferentiated tissues are direct descendents of their corresponding dedifferentiated tissues was impossible to determine, as all the cells in the blastema become identical. However, Weiss maintains that once a cell has assumed a definite course of differentiation, it is never able to switch into a different course.¹ There is dedifferentiation of structure, but not of character; therefore, the regenerating muscle, for example, is derived from dedifferentiated cells of former muscle tissue in the amputated limb.

It is interesting to note Thornton's detailed description of dedifferentiating muscle cells.² First there is degeneration of sarcoplasm which disappears by fragmentation and sarcolysis. Then several nuclei degenerate, becoming either pycnotic or fragment into scattered chromatin granules and probably removed by phagocytosis. However, there are several nuclei that remain unaltered and, surrounded by a bit of cytoplasm, assume a more oval shape and migrate into the blastema. Here they join similar oval-shaped nuclei from other dedifferentiated tissues. Mitotic increase of blastema cells follows, and then redifferentiation of tissue

1. Weiss, op. cit., p. 468.

2. Thornton, op. cit., pp. 23 - 29.

which is no different from the primordial development.

So it is seen that both theories concerning the origin of regenerating cells have substantial evidence supporting them, but dedifferentiation appears to be the only possible source in higher animals (i.e., amphibians). Now the influences that induce dedifferentiation of new tissue in the regenerant must be considered, and by what means regeneration may be inhibited.

To understand what may promote redifferentiation, it is probably better to first consider what retards the process. Two separate investigations on this subject were conducted on frogs, and the conclusions are in agreement - there is a decrease in regenerative ability as the skin increases in thickness.¹ In other words, a skin change at metamorphoses is responsible for loss of regenerative power in frogs.

Rose, one of the experimenters, observed on frog tadpoles that are losing their ability to regenerate that the skin is slowly increasing in thickness.² Also, it was noted that the skin is being freed from underlying tissue with the production of lymph spaces, and this freeness of the skin allows it to pinch down and seal a wound within a few days. So a thickening and loosening of the skin appears as a decrease in regenerative ability occurs. Rose further observed that an epithelium quickly grows over the exposed surface, closely followed by epidermis and dermis. In animals that do regenerate, an epithelium grows over quickly, but usually that is all. From these observations, Rose

1. S. Meryl Rose, "Methods of Initiating Limb Regeneration in Adult Anura", Journal of Experimental Zoology, Vol. 95 (1944) p. 149.

2. Ibid. Journal of Experimental Zoology, Vol. 97 (1944), pp. 72 - 83.

surmised that the skin covering is responsible for the regenerative failure.

To prove this, he amputated forelimbs on frogs, aging from recent metamorphosis to fully grown. By bathing these freshly amputated limbs in saturated Sodium Chloride solutions for a few days, the epithelium covering was retarded and the skin covering was held back. In most cases, this retardation of skin covering allowed regeneration to occur. In some cases it was almost complete regeneration, while in others, where regeneration was imperfect, a dermis had quickly regenerated, and this layer of skin was probably responsible for inhibiting limb regeneration.¹

Rose emphasized that the skin did not act mechanically in preventing regeneration. Since it was not an inelastic obstruction in the path of the blastema, how did the dermis inhibit regeneration? Another experiment, performed by Gidge and Rose, was more enlightening as to the role of the dermis in regeneration.²

They amputated the forelimbs of recently metamorphosed frogs above the wrist. The amputated limbs were then covered with (1) adult skin, (2) tadpole skin, and (3) only an epithelium (adult skin stripped off). The results showed that all stumps underwent dedifferentiation and redifferentiation, but there was outgrowth only from stumps covered with tadpole skin or with epithelium. In the case of the adult skin covering, the blastema only formed cartilage and collagenous scar tissue with very short outgrowth if any.

1. Ibid., pp. 150 - 166.

2. Natalie M. Gidge and S. Meryl Rose, "The Role of Larval Skin in Promoting Limb Regeneration in Adult Anura", Journal of Experimental Zoology, Vol. 97 (1944), pp. 71 - 85.

Here again it was concluded that the dermis is the skin layer responsible for regenerative failure.¹ Although the tadpole skin had a dermis, it underwent very rapid dedifferentiation. However, the presence of this dermis apparently is necessary to stop dedifferentiation, for in the case of epithelial covering, where the dermis is absent, resorption does not cease and outgrowth begin until a dermis is regenerated. Outgrowth, then, must await the time when dermis is present at the base of the blastema, and internal dedifferentiation has ceased. Therefore, those limbs covered with tadpole skin more nearly approach normal limbs than do stripped limbs.²

How the dermis of adult skin prevents the outgrowth of new tissue was then explained.³ A blastema was formed, but the dermis induces these blastema cells to form fibrous scar before they have an opportunity to grow and differentiate into several new tissues. Because the blastema becomes scar under dermal influence, limbs do not regenerate. A dermis free wound allows blastema cells to grow while still undifferentiated. Later they differentiate, and still are not under the influence of a single established tissue but in a field which is a complex of influences from several tissues. The kind of tissues that regenerate are correlated with the kinds of tissue in the immediate vicinity of the blastema cells. A predominance of old dermis destroys the natural balance and leads to induction of too much fibrous tissue and cartilage - therefore, no outgrowth.

1. Gidge, op. cit., p. 85.

2. Ibid.

3. Ibid., pp. 82 - 85.

This leads to a discussion of field characters. It is evident that the pattern of development in a limb regenerant is directed and controlled by the limb field of stumps. Weiss states that the body as a whole exerts no influence upon the character and orientation of a regenerate; only the field of the injured organ itself is responsible for the qualitative determination of the regenerative process.¹ According to Child, this field is a "gradient system", and the disappearance of a potency field is evidently associated with the progressive differentiation of its different tissues.² The subsidence of regenerative capacity in an organ could, therefore, be due to the disappearance of the field.³

A final point to consider about limb regeneration is the nerve influence. Schotte and Butler concluded that no blastema is ever established on a completely nerveless amputated limb.⁴ Weiss states that nervous influences are prerequisite for the regeneration of many organs (e.g., amphibians limbs) whose ontogenetic formation has been entirely free from such influence.⁵ Child concludes that the absence of motor innervation does not prevent leg regeneration, but may retard or prevent growth to full size through absence of function. However, sympathetic innervation is apparently a necessary factor in determining and maintaining the physiological state of the cells composing the blastema.⁶

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1. Weiss, op. cit., p. 471.
 2. Child, op. cit., pp. 285 - 288.
 3. Ibid.
 4. Rugh, op. cit., p. 318.
 5. Weiss, op. cit., p. 446.
 6. Child, op. cit., p. 341.

From these facts about regeneration it can now be determined what will happen when the limbs of frog tadpoles are amputated. There will be a period of dedifferentiation and establishment of a blastema, followed by a mitotic increase of cells from the blastema and redifferentiation into new tissues. This proliferation of cells affords an excellent opportunity to study the effects of colchicine.

and used in medicine. The alkaloid content of the seed is from 0.2 to 0.4 percent.¹

In recent years this chemical has proved to be an effective agent for the induction of polyploidy in plants and animals. Polyploidy is "a condition found in some species in which the number of chromosomes in the somatic cells is some multiple (greater than two) of the haploid number."² This "evolutionary chemical" has been used to originate new species of plants by multiplying the hereditary-carrying chromosomes of old plants, and also has the power of greatly speeding up plant growth when used in weaker concentrations.³ However, these same beneficial results have not been obtained with any consistency in the animal kingdom.

The discovery of the action of colchicine on mitosis was first reported by Ferrius in 1889.⁴ He described in much detail the gastrointestinal mitosis found in two dogs given 10 to 15

1. Ralph Strong, Editor, Kingzett's Chemical Encyclopedia, Sixth Edition, (New York, 1940), p. 238.

2. The American Illustrated Dictionary, 20th Edition, (Philadelphia, 1944), p. 1151.

3. "Colchicine in Small Doses Speeds Plant Growth", Science News Letter, Vol. 48 (July 7, 1945), p. 8.

4. "On the Discovery of the Action of Colchicine on Mitosis in 1889", Science, Vol. 120 (Dec. 23, 1949), p. 692

COLCHICINE

Colchicine is "a yellow, crystalline, alkaloid body . . . extracted from the seeds of the meadow saffron (Colchicum autumnale) . . . It is of poisonous character and bitter taste, and used in medicine. The alkaloid content of the seed is from 0.2 to 0.8 percent."¹

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1. Ralph Strong, Editor, Kingzett's Chemical Encyclopedia, Sixth Edition, (New York, 1940), p. 238.
 2. The American Illustrated Dictionary, 20th Edition, (Philadelphia, 1944), p. 1163.
 3. "Colchicine in Small Doses Speeds Plant Growth", Science News Letter, Vol. 48 (July 7, 1945), p. 8.
 4. "On the Discovery of the Action of Colchicine on Mitosis in 1889", Science, Vol. 110 (Dec. 23, 1949), p. 692

gram tincture of bulbs of colchicum, but dying within 48 hours. In the stomach he found an extraordinary great number of dividing cells. In the Lieberkuhn glands of the intestine nearly all the cells were engaged in mitotic division. Endothelial mitosis could be seen in nearly all vessels. It was rare to see the latest stages of division. He concluded that cellular elements may have been directly excited and stimulated by tincture of colchicum.¹

It was not until the last twenty years that real study on colchicine activity was undertaken. In this time much has been added to Pernice's original findings. It appears that colchicine is a mitotic stimulant, but in not all cases does it cause an acceleration in growth or increase in size. Its primary effect seems to be a doubling of the chromosome number, but in many cases, particularly in the animals tested, there was an inhibition in growth due to a disturbance in the metaphase of the mitotic division. This resulted in a halting of cell division in those cells affected, and in many cases stopping growth completely.

Various species of the plant kingdom have profited much with the administration of colchicine. For instance, the Department of Agriculture was able to produce loretto grapes three times the normal size and resistant to attack from parasites.² Colchicine increased the chromosome number from 38 to 76 in these grapes. In addition, two strains of muscadine

1. Ibid.

2. "Chemical Yields Grapes Three Times Normal Size, Science News Letter, Vol. 68 (Dec. 17, 1955), p. 392.

grapes treated with the alkaloid became twice the normal size, and increased from 40 to 80 in chromosome number.¹

Other examples of success with colchicine in plants were reported. Two Chinese researchers, Lou and Tang, observed a speeding-up of seed germination in corn, rice, wheat, cabbage and mungo bean.² Newcomer, using .4 of one per cent of colchicine added one drop per day on growing points of young oak and chestnut trees, and found that growth went on at double the usual rate.³ An attempt to stabilize the characteristics of a new strain of sorghum seedlings, using colchicine, succeeded in a single year, where in the past it would take many years for such an accomplishment. After only four generations of seedlings the desired changes in the plant were permanently established in the new varieties.⁴

A typical pattern of induced polyploidy with colchicine was observed in the algae Chlamydomonas.⁵ It was found that in a 1.0 per cent solution, cell division was completely inhibited, yet cell enlargement proceeded, producing cells up to ten times the normal volume. Special staining methods made it possible to observe clearly colchicine mitosis (called c-mitosis, where the chromatin clumps in the metaphase), polyploid nuclei, and multinucleate cells.

Research done with colchicine on animal cells indicates that

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1. Ibid.
 2. "Colchicine in Small Doses Speeds Plant Growth", op. cit.
 3. Ibid.
 4. "Breeding Short-cut for Plants Successful", Science News Letter, Vol. 64 (Nov. 28, 1953), p. 345.
 5. "Colchicine Induced Polyploidy in Chlamydomonas", Science, Vol. 124 (July 26, 1956), p. 25.

the chemical does induce polyploidy, yet opinion is divided on the question as to whether it is a mitotic stimulant or inhibitor. However, most experimenters acknowledge the stathmokinetic (c-mitosis) property of colchicine on animal cells, irregardless of whether it stimulates or inhibits mitosis.

Experiments performed on the fruit fly larvae by Braungart and Ott produced the first successful results using colchicine on animals.¹ Using a fine hypodermic needle they injected the alkaloid into regions of the brain of three day old larva, where later, cells were found to have undergone doublings in the numbers of chromosomes.

Testing the rate of regeneration of a lower animal (Pelmato-hydra oligactis) in a colchicine medium, Sturdevant showed that the degree of inhibition of regeneration is a direct function of the concentration of colchicine.² Hydra were examined in colchicine concentrations ranging from 0.0033% to 0.00000033%, compared to pond water controls, and although colchicine inhibited growth, cytological examination showed there was a greater proportion of mitotic figures in the colchicine treated specimens. This indicated that the latter had not completed their period of growth in regeneration, while the controls had. Metaphase figures were most abundant in the colchicine treated specimens, proof of c-mitosis.

Other cases where colchicine proved to be effective on animal

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1. Frank J. Sturdevant and others, "Effects of Colchicine on Regeneration in Pelmatohydra Oligactis", Science, Vol. 114 (Aug. 31, 1951), pp. 241 - 2.
 2. "Colchicine Injection Changes Chromosomes in Fruit Fly", Science News Letter, Vol. 42 (Aug. 1, 1942), p. 67.

cells were reported. Experiments performed to discover the lethal dosage of colchicine for the hamster revealed that there was a marked increase in the number of metaphase figures and an absence of spindle fibers in many cells of the small intestine.¹ In testing the lethal X-ray dose for mice, it was discovered that those given the colchicine compound survived longer.² Colchicine, in concentrations from 10^{-3} to 10^{-6} accelerated the effect of pituitary in causing ovulation in isolated frog's ovaries, although the chemical alone failed to incite egg release, and eggs from colchicine injected frogs failed to divide when fertilized with normal sperm.³

One investigation of noteworthy attention was conducted by Samartino and Rugh.⁴ Using *Rana Pipiens* frogs, they studied the effect of colchicine on amphibian ovulation, fertilization, and early development. Concentration ranging from 10^{-3} to 10^{-6} were used, and colchicine was found to act as a capillary poison, to prevent ovulation when injected into sexually mature female frogs prior to anterior-pituitary stimulation, to prevent cleavage to normal eggs exposed to spermatozoa previously treated with colchicine, to effect gastrulation to such an extent that exo-gastrula were

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1. "Sensitivity of Hamster to Colchicine", Science, Vol. 126 (Oct. 18, 1957), p. 749.
 2. "Chemical Allows Mice to Survive Lethal X-Ray Dose", op. cit.
 3. M. K. McPhail and K. M. Wilbur, "The Stimulating Action of Colchicine on Pituitary - induced Ovulation of the Frog", The Journal of Pharmacology and Experimental Therapeutics, Vol. 78 (1943), p. 313.
 4. G. T. Samartino and R. Rugh, "Effects of Colchicine on the Frog in Relation to Ovulation and Early Development", Proceedings of the Society for Experimental Biology and Medicine, Vol. 63 (Oct. - Dec., 1946), pp. 424 - 7.

the rule, to inhibit the appearance of organ anlage when applied just below the normal appearance time, to retard general growth of the larva to such an extent that cephalic structures were markedly stunted and hatching prevented. The cytological picture was one of many pycnotic nuclei (former metaphase cells which has been agglutinated), occasional multi-nucleated cells, few metaphase figures and apparent resting stages of mitosis.¹

They conclude that colchicine acts as a depressant in mitosis in the frog, as the drug prolongs the mitotic cycle, and in certain concentrations stops it completely. The lack of the prerequisite increases in viscosity, due to the influence of colchicine, may account for the lack of spindle formation and for the bizarre figures seen in colchicine-treated cells.²

Experiments show that the predominant effect of colchicine is to arrest mitosis in metaphase. Bucher stated that the arrest appears apparently early in metaphase and has been attributed to an inhibition of the mechanism of spindle formation.³ The cells in the arrested state usually show the chromatin accumulated in a dense irregular clump surrounded by a halo of pure protoplasm. Larger doses of the drug and prolonged administration cause many of these arrested cells to degenerate and thin clumps of chromatin to fragment. However, with smaller doses the effect of the drug has shown to be reversible; after the drug has been removed the

1. Ibid., p. 426.

2. Ibid.

3. W. Buschke, J. Friedenwald, and W. Fleishman, "Effects of Colchicine", Bulletin of Johns Hopkins Hospital, Vol. 73 (1943), p. 149.

arrested cells escape its influence and complete their mitotic cycles yielding two normal daughter cells.¹

Concerning the question as to whether colchicine stimulates cells to enter into mitosis or not, there is varied opinion. For example, Bucher showed that the rate of entrance of cells into mitosis is not increased in colchicine poisoning, and with large doses the mitotic rate is in fact somewhat decreased.² On the other hand, the work of Paff gives results which are not in harmony with Bucher's conclusions.³ Using varying amounts of colchicine for different lengths of time on 48 hour chick embryos, Paff advanced evidence which indicates that colchicine may produce so many colchicine figures (inhibited mitosis) as to be explained only on the basis that cells had been stimulated to pass into mitosis. For in addition to this increase in colchicine figures, there is an increase in the total number of cells. Therefore, not all the cells are arrested in mitosis in rapidly dividing cells, colchicine may stimulate as well as inhibit.

Thus it is seen that the properties of colchicine are by no means settled. Much work is still to be done concerning its influence on mitosis in animals. Yet it is fairly certain that on limb regeneration in frogs, colchicine will inhibit growth. The work done in this matter will now be examined.

1. Ibid.

2. Ibid.

3. G. H. Paff, "The Action of Colchicine upon the 48-Hour Chick Embryo", American Journal of Anatomy, Vol. 64 (1939), pp. 331 - 339.

CHAPTER IV

EXPERIMENTS PERFORMED

The original plan was as follows: Rana clamitans tadpoles would be the experimental animals. The breeding period and delayed metamorphosis of this species are such that induced methods would be required in view of the time limit on this work. These animals usually breed in the summer months, and the tadpoles hibernate for a winter, followed by a quick metamorphosis in the spring. Work did not begin until after hibernation had begun, so it was necessary to obtain frogs from a Biological Supply House. Pituitary-induced ovulation and artificial fertilization would be the means used for obtaining the tadpoles. Their lengthy metamorphosis would necessitate the use of thyroid hormone for the premature appearance of limbs. Amputations would be made at various stages in the development of the limbs. Once regeneration started, some tadpoles would be placed in weak colchicine solutions; others would be used as controls. Close microscopic observation could determine any gross morphological differences in the colchicine tadpoles, and some conclusion could be made as to whether colchicine stimulates or inhibits growth.

Before the actual procedure and results are presented, various difficulties that arose during the course of the work and altered these original plans must be explained. A high mortality rate among both the frogs and tadpoles throughout the year caused many repeated experiments and much valuable time was lost, particu-

larly in ordering new shipments of animals from the Supply House. First, a red-leg infection brought a decrease in the number of frogs available for the induced breeding. Not until the third attempt at this method for securing tadpoles were successful results obtained, and realizing that additional time would be needed for growth of these tadpoles to a size suitable for application of the thyroid hormone, it became necessary to order large tadpoles from the Supply House, and these became the experimental animals. However, directions for the application of the thyroid powder to the tadpoles were somewhat vague, and only after two attempts to induce metamorphosis was the powerful influence of this hormone realized. Those tadpoles that survived the excessive thyroid dosage proved to be unfit for amputations and colchicine treatment. Therefore, three orders were sent to the Supply House before healthy tadpoles with well-developed limbs were available for amputations.

The laboratory conditions set up for the culture of the frogs and tadpoles were suggested by Rugh¹. Frogs were kept in aquaria containing sand and a small volume of water. A culture of worms provided the food source. Tadpoles were raised in aquaria, battery jars, or an aluminum pan, and those chosen for special observation or treatment were separated into finger bowls. Distilled water was the culture medium. Occasionally, salts of Sodium Chloride, Potassium Chloride, Calcium Chloride, and Sodium Bicarbonate were added to the water, but never did the total salt content exceed 0.385%. Sprigs of Elodea were placed in the medium to add oxygen, but Rana clamitans tadpoles can tolerate a low oxygen environment,

1. Rugh, op. cit., pp. 48 - 52.

so these were not absolutely required. Space conditions were never so "crowded" that the rate of development would be considerably decreased. Laboratory temperatures ranged from 20-25 degrees Centigrade. Slightly boiled lettuce was the food source, which proved to be an adequate diet. Water was changed daily, and fresh boiled lettuce added every other day.

In spite of these precautions, many of the animals died. The red-leg infection among the frogs was practically impossible to stop, so several frogs were eliminated in this manner. Many of the tadpoles of the induced breeding experiment died, but their death rate never seriously affected their availability for thyroid treatment. It was this treatment itself which caused the high mortality rate that hampered experiments with colchicine.

With these preliminary difficulties explained, a detailed discussion of the actual experiments will now be related. For the sake of convenience, these will be divided into three categories: (1) induced breeding; (2) thyroid treatment, and (3) amputations and consequent colchicine treatment on regenerating limbs. However, the same group of tadpoles was not used for all three phases; often, two of these experiments were being conducted simultaneously.

Rugh's procedure was followed for the induced breeding experiment¹. Pituitary glands dissected from sexually-mature frogs, both male and female and in numbers corresponding to the season, were to be injected into a large female, preferably one just received from hibernation conditions. Within three days, if the pituitary hormone had been absorbed in sufficient concentration by the female's cir-

1. Ibid., pp. 102 - 107.

culatory system, many eggs in the metaphase of the second maturation division could be stripped from the uteri of the frog. These eggs could be artificially fertilized if stripped into a small volume of water containing viable sperm from fresh, macerated frog testes. By two and one-half hours, the eggs would be in the two-cell stage, and development would proceed according to conditions set up in the laboratory.

The first attempt was unsuccessful. Only four male and two female frogs were available for supplying pituitary glands at a time when at least ten male or five female donors are needed for sufficient hormone concentration. Also, this attempt was one month earlier than the suggested period for ovulation induction (November to July)¹. These two factors were largely responsible for the unsuccessful stripping of the female's body cavity.

To overcome these defects, a frog pituitary set was obtained from the Biological Supply House, and the next attempt to induce ovulation began well within the suggested breeding time. The pituitary set included the glands of one female grass-frog, two males, and a unit of frog pituitary suspension. Following directions, the suspension was injected into a large female, and seventy-two hours later, the frog was stripped. Many eggs were obtained, but only after lengthy and vigorous pressure on the frog's abdomen. These eggs were stripped directly into water containing viable sperm from two pairs of macerated testes (viable sperm observed under microscope immediately after stripping). However, after a few days, it was obvious the eggs had not been fertilized. The jelly membrane that

1. Ibid., p. 107.

surrounds Anuran eggs in water did not appear. The eggs appeared no different from a cluster of eggs that could be observed in the ovary of a female's body cavity before the breeding season. In addition, the donor of the eggs died four days after stripping. Therefore, it was concluded that the pituitary suspension was not sufficient to induce maturation of the eggs at that particular time. Consequently, the eggs obtained were merely bundles of ovarian eggs stripped directly from the body cavity by extreme pressure, and this accounts for no jelly membranes around the eggs (the jelly secreted by the oviduct). Naturally, these eggs could not be inseminated by the sperm.

For the third attempt, a large supply of frogs was obtained to insure a plentiful amount of pituitary hormone, and this was successful. Glands from six females were injected into a large female that had been recently received from a thirty-five-day refrigeration period to simulate hibernating conditions. Within seventy-two hours, approximately six hundred eggs were easily stripped from the frog into 10 ccs. of distilled water (containing sperm from two pairs of testes). Although cleavage could not be detected, four days after insemination many elongate forms were observed moving in the swollen jelly membranes, and about one hundred embryos were separated from the mass. These embryos went through the normal larval development, and responded fairly well to laboratory conditions already described. After eighty days, forty of these tadpoles were alive and growing, the biggest measuring four centimeters in length. However, the late arrival of these tadpoles made them ineligible for work with colchicine, but thyroid

experiments were performed using these small tadpoles.

The powder used for the thyroid experiments was obtained from the same Supply House. Directions called to sprinkle a "pinch" of the thyroid powder on the water in which the animals were kept. This was done daily over a seven-day period on a group of thirteen tadpoles, varying in length from forty-five to sixty millimeters. Within four weeks, eight of these tadpoles had died. The survivors had well-developed hind-limbs, and at least one fore-limb on each had appeared, thereby exposing the gills. These animals showed much difficulty in respiring. Their bodies had tapered immensely, and some had resorbed their tail to within a few centimeters of their body. None of these survived limb amputation.

Similarly, a group of thirty large tadpoles were treated with the thyroid, but reduced amounts of the powder were added. The daily dosage varied from 2×10^{-4} to 5×10^{-5} , and treatment lasted six days. However, the results were similar to those seen above. At the end of two weeks, twenty-six of the tadpoles had died. They responded to the thyroid in the same manner as the previous group (stop eating, appear sluggish and bloated, with fluid-filled cavities, tapered bodies, and reduced tails, but usually well-developed hind-limbs). Four additional tadpoles from this same group had been set aside for closer observation, two of these receiving the same thyroid dosage, and two serving as controls. Within one week, legs had appeared on the thyroid tadpoles, and their bodies had begun to taper. By ten days, both had died. When compared with the controls, both appeared to have "shrunk", their total body length decreasing about four centimeters.

A careful investigation revealed the reasons for these rapid deaths. The dosage of thyroid given the tadpoles was in some cases as much as ten times in excess of the suggested dosage by Rugh (1×10^{-5})¹. The overdose of thyroid caused an acceleration of development which carried beyond the tolerable limit and the tadpoles were literally "burned up". A total of nine tadpoles survived the overdose, and their deaths came with the amputations.

With the lethal dosage of the thyroid powder roughly established, an additional group of large tadpoles was ordered. However difficulties in shipping reduced their number to eight, but this was enough for experimental purposes. These eight tadpoles were treated with the thyroid for a nine-day period, the daily dosage being kept below the 1×10^{-5} concentration. They responded perfectly to the treatment. Hind-limb buds appeared within one week, and by three weeks they were developed enough for amputations. The tadpoles never appeared sluggish, and they fed on boiled lettuce as normal. Their bodies did taper slightly, but their total length remained the same. Seven of these survived amputations.

A final determination of the thyroid influence on the small tadpoles from the induced-breeding experiment brought interesting results. Six larger tadpoles (three for thyroid and three for control purposes) were used. All were fifty-six days old and three centimeters in length. The thyroid tadpoles, in a finger bowl containing 200 ccs. of water, received a minute trace of thyroid daily for six days. At the end of this time, tiny limb buds had appeared on all three tadpoles. Their bodies had tapered considerably,

1. Ibid., p. 324.

although remaining the same length. They had stopped eating. After twelve days, two had died. The third, after twenty-eight days, was surprisingly still alive, with proportionately well-developed hind-limbs and tiny fore-limb buds.

Evident that the thyroid is effective irregardless of the size or age of the tadpoles, a tiny amount of the hormone was added to the remainder of the induced-breeding tadpoles. Three minute traces, at three-day intervals, were sprinkled on approximately three liters of water containing thirty tadpoles. These ranged from 2-1/2 to 6 cms. in length. The desired effects were obtained within two weeks, for tiny limb buds had appeared on most of the tadpoles, with no toxic effects whatsoever. All tadpoles reacted normally and continued feeding as before. At the termination of this work, which was three weeks after the first application of the thyroid, the larger tadpoles had well-developed limbs, some of these reaching 1/2 cm. in length. However, as the size of the tadpoles decreased, so did the limb development in proportion to body size. The smaller tadpoles did possess distinct limbs, but these were tiny when compared with the body-limb ratio of the large tadpoles. Also, the smaller tadpoles seemed to be sluggish and were not as active as the larger tadpoles. Their bodies had tapered considerably, whereas the larger animals had for the most part retained their oval shape. Therefore, it appears there is a very significant susceptibility range among the tadpoles to the thyroid hormone. Although it can induce limb formation on all sizes, the effects are much more desirable on larger tadpoles, and they react to the thyroid much better than the smaller tadpoles do.

The results of some of the amputations have already been mentioned. The right hind-limb of seven tadpoles that survived the thyroid

overdose was amputated, but obviously none of these animals had recovered from the thyroid, and within one week, all had died. Three of these had been placed in 10^{-4} colchicine solutions after amputations, and these were first to die, indicating that the colchicine solution might be toxic. Autopsy of all the tadpoles showed excessive fluid in the body cavity.

The final amputation series was much more conclusive in that the tadpoles used were in good condition after the thyroid treatment, so death could not be attributed to overdose of the hormone. The right hind-limb of eight tadpoles was amputated in the tibio-fibula area. A sharp razor blade, previously sterilized, was used as the amputating instrument. The tadpoles ranged from 4-1/2 to 6 cms. in total body length, and all had well-developed limbs. The largest segment cut from any of the tadpoles measured 3/4 cms. Only one tadpole failed to survive, and death was definitely due to excessive bleeding following the cut. None of the remaining seven lost blood, and all reacted normally after the amputation.

The survivors were separated into finger bowls. Four were to be subjected to colchicine, and the remainders were to serve as controls. Close measurements taken at various intervals would note any changes in size or growth of the limbs. Colchicine was not used until the first signs of regeneration appeared, which was seven days after amputations. At this time, swellings were observed in the stumps, indicating the blastema had formed, and regeneration would follow shortly. The colchicine tadpoles were placed in a 10^{-4} solution of the chemical for a two-hour period, being closely observed for toxic effects. No severe reactions were detected, so on the following day the tadpoles were placed permanently in the colchicine solution. Concentrations

varied from 10^{-8} to 10^{-4} , and solutions were changed daily. Three days after the first exposure to colchicine, one tadpole died. Only the chemical itself could be determined as the cause of death. However, the remaining tadpoles were retained in the colchicine solutions, and one week after the initial exposure, no changes could be detected in the size of their limb stumps. Three days later, while in a 10^{-4} solution, these tadpoles had died. No symptoms of any kind preceded their death. Since the controls were alive and in good condition, it was definitely determined that colchicine is toxic to tadpoles, at least in 10^{-4} or more concentrated solutions.

It should be noted that although the control tadpoles survived the amputations, never did they resume feeding on the lettuce available to them. Only tiny amounts of feces could be found in the water. More important, however, is the fact that four weeks after the amputations had been made, no regeneration had occurred in the limbs of these controls. Their stumps had swollen, but this was the only change that could be observed in their limbs. If regeneration was to occur, it should certainly have begun within four weeks. The indications are that the thyroid may disturb the metabolism of the tadpoles to such an extent that the natural regenerative powers are lost. It could also indicate that there is only a short period between the beginning of limb formation and the loss of regeneration powers in frog tadpoles. However, the limited evidence from these experiments prevents any definite conclusions on these matters.

not discovered before the time limit on this work expired. There is certainly much more investigation to be done on this subject, and on the effects of colchicine in general. The sparse amount of literature on this subject verifies this. Since colchicine is such a

SUMMARY AND CONCLUSIONS

During the course of this work, the effects of three chemical substances on Rana clamitans frogs and tadpoles have been at least partially determined.

Hormones from frog pituitary glands, if in sufficient concentration and at a proper time of the year, can induce ovulation in the pre-breeding season of female frogs. The eggs obtained can be fertilized by the sperm extract of fresh frog testes.

Thyroid powder, if applied to the tadpoles in a non-toxic dose, will induce metamorphosis. The dosage of the thyroid depends on the size of the tadpoles, and larger tadpoles react more favorably to the hormone. A safe maximum dose would be somewhere under a 10^{-5} concentration, and this dose would decrease as the size of the tadpoles decreased. A non-toxic dose will certainly cause the formation of limbs. However, the metabolism of these tadpoles may be upset enough to prevent regeneration, and more specific work could be done concerning this matter.

Colchicine is toxic to tadpoles in a 10^{-4} concentration, and perhaps even in less concentrated solutions. As to its effect on mitosis in tadpoles, and in particular, its effect on limb regeneration, it could not be determined, since a safe non-toxic dose was not discovered before the time limit on this work expired. There is certainly much more investigation to be done on this subject, and on the effects of colchicine in general. The sparse amount of literature on this subject verifies this. Since colchicine is such a

remarkable chemical, such matters are worthy of investigation.

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