ON THE ORIGIN OF EPIDERMAL PROTUBERANCES IN THE TAIL OF BULLFROG LARVAE FOLLOWING THE INJECTION OF THE LATHYROGENIC AGENT, SEMICARBAZIDE HYDROCHLORIDE

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CHAPTER I

INTRODUCTION

Alteration of connective tissues, skeletal lesions, aneurysms of arteries, and other skeletal deformities in rats and chick embryos have been demonstrated by numerous experiments where the seeds of Lathyrus odoratus or its extracts, and other compounds having lathyro- genic activity, have been added to the diets of these organisms. The characteristic lesions of lathyrism have been produced in amphibians by adding lathyrogens to their aqueous environments. However, only a few experiments have been designed to observe the changes produced by injecting lathyrogens directly into the organism. The present investigation was undertaken in an effort to observe the changes produced by injecting semicarbazide hydrochloride directly into the ventral fin of bullfrog larvae of Rana species, since semicarbazide was reported to produce lesions similar to beta-aminopropionitrile.

The results of this investigation indicated that semicarbazide caused connective tissue degeneration and epidermal protuberances following its injection into the ventral fin of bullfrog larvae of Rana species. However, the mechanism of the reaction was not determined.
CHAPTER II

REVIEW OF LITERATURE

Lathyism, the name applied to the alteration of connective tissues, skeletal lesions, aneurysms of arteries, distorted epiphyseal plates, scoliosis, and other skeletal deformities produced by lathyrogenic agents, was produced experimentally more than a quarter of a century ago (Geiger, Steenbock, and Parsons, 1933) by feeding rats seeds of the flowering sweet pea—Lathyrus odoratus. However, Stockman (1929) wrote that lathyism has been known since the time of Hippocrates and was common in the past in India and northern Africa; small local outbreaks occurred frequently in Italy, France, and elsewhere in southern Europe. Since then several compounds, including semicarbazide (Gross, Levene, and Orloff, 1960), beta-aminopropionitrile, aminoacetonitrile, methyleneaminoacetonitrile, and mercapto-ethylamine (Karnovsky and Karnovsky, 1961) have been tested to determine their lathyrogenic activity.

According to Karnovsky and Karnovsky (1961), the experimental disease has been studied mostly in the rat, but changes produced in various other species (for example, mice, rabbits, turkey pouls, chick embryos, frogs, and salamanders) were similar, in that essentially mesenchymal tissues were affected. Gross, Levene and Orloff (1960) stated that embryo fragility and the appearance of extractable collagen were always associated following application of lathyrogenic compounds into the chick embryo. Chang, Witschi, and Ponseti (1955) reported that beta-aminopropionitrile caused a bending of the femurs in chick embryos. Belanger (1959) announced that severe osteolathyism was induced in chicks
of different ages by a diet containing fifty per cent seeds of *Lathyrus odoratus*. In these chicks, most of which became paraplegic after seven days, a meningeal tumor, articular and bony deformities, spontaneous fractures and osteoporosis were observed.

Ramamurti and Taylor (1959) fed weanling rats a diet containing 0.3 per cent semicarbazide. The animals developed the characteristic lesions of osteolathyrysm. This condition was the same as that produced by beta-aminopropionitrile, aminoacetanitrile, and mercapto-ethylamine. These lesions consisted of kyphoscoliosis, displacement of epiphyses, and dislocations of joints. A pathological study of the skeletal lesions showed widening, disorganization, and tears of the epiphyseal plates, with the zone of maturing cartilage showing the greatest increase in width. The severe kyphoscoliosis was due to derangement and displacement at and through the epiphyseal plates of the twelfth thoracic or first lumbar vertebra.

Amato, Sliema, Malta, and Bombelli (1959) observed growth interference, lesions of the smaller blood vessels, raising of the periosteum, formation of exostoses, and osteoporosis in rats when fed *Lathyrus odoratus* seeds. Ponseti and Shepard (1954) mentioned that rats given diets containing such seeds developed distortions of the normal arrangements of rows of cartilage cells in the epiphyseal plates and loosening in the attachments of the tendinous and ligamentous insertions. These effects produced many different kinds of deformities such as kyphoscoliosis, subluxation and dislocation of joints, degeneration of intervertebral discs, and distortions of growing epiphyses. Many other authors seemed to be in agreement.
as to the kind and number of effects produced by Lathyrus odoratus in rats (Geiger, Steenbock, and Parsons, 1933; Ponseti and Baird, 1952; Churchill Gelfant, LaLich and Angevine, 1955; Menzies and Mills, 1957; Karnovsky and Karnovsky, 1961).

Lathyris has been demonstrated in other animals by numerous experiments in which the seeds of Lathyrus odoratus or its extracts, and other compounds having lathyrogenic activity, have been added to the diets of these organisms. Chang, Witschi, and Ponseti (1955) indicated that Lathyrus odoratus seeds produced dislocation of joints and loosening of inter-segmental septa in amphibians. However, Levy and Godman (1955), using the crystalline form of the active principle of Lathyrus odoratus in spring water and placing amphibian embryos in it, reported that small tumorous growths appeared on the dorsolateral aspects of the trunks. The suggestion was made that these tumors resulted as a consequence either of a focal degeneration of the notochordal sheath, leading to secondary proliferative ectodermoses or prolapses, or of a direct stimulation to proliferation of the notochordal cells. From descriptions of the skeletal, articular and ligamentous deformities of rats and other animals, it might have been expected that the principal effect of the Lathyrus factor on Amblystoma larvae would have been manifested in the skeletogenous and supporting connective tissue.

It has been known that in mammals sweet pea diets cause scoliosis. The scoliosis and other skeletal deformities in rats appeared to be due to widening and disruption of the epiphyseal plates and to loosening and detachment of tendinous and ligamentous insertions (Ponseti and Shepard,
In Xenopus larvae dislocation of the joints of the hind legs and loosening of the septa of the metameres of the dorsal musculature were produced by feeding seeds of sweet pea (Chang, Witschi, and Ponseti 1954). Similar lesions can be obtained by using aqueous extracts of sweet pea in great dilution. However, animals after metamorphosis under the same diet did not develop any deformities. Cameron (1963) using semicarbazide in aquarium water indicated that no gross dislocations in Xenopus larvae were observed as described by Chang, Witschi, and Ponseti, (1954), when using Lathyrus odoratus seeds. However, histological examination revealed clefts between the connective tissue and the epiphysis of the tibio-tarsal joint when compared with the control animals.

Ponseti and Shepard (1954) suggested that lesions of the skeletal and of other mesodermal tissues in rats fed sweet pea seeds were due to defective formation or excessive destruction of the chondroitin sulphate of the ground substance, since the lesions occurred in areas in which the ground substance contained chondroitin sulphate as the only or the main mucopolysaccharide. Ramamurti and Taylor (1959) set forth the idea that lathyrogenic agents might possibly interfere with the metabolism of the epiphyseal plate. There appeared to be a decreased polymerization of the ground substance of the epiphyseal cartilage. Using other chemicals of similar structure, with the same reactive groups, he also found that they did not show lathyrogenic properties and concluded that it was not a particular chemical structure, configuration or specific reactive group that was responsible for the production of osteolathyrisms.

Kuloner (1961) proposed that in lathyrisms there was a disturbance
in the general metabolism of nitrogenous components. Levene (1962) projected the idea that "lathyrogenic agents act by blocking carbonyl groups on the collagen molecule, thus preventing cross-linking essential to normal maturation; normal maturation may be restored by the addition of carbonyl groups which act by competing either for the lathyrogen or for functional sites on the collagen molecule." In support of this hypothesis, it was shown that purified lathyritic guinea pig collagen took up less 2:4 dinitrophenylhydrazine than normal collagen, and that while it still possessed the ability to form segment-long spacing collagen, these fibers were much thinner than normal. This was due perhaps to a blockade of groups essential for lateral cross-linking of the tropocollagen units.

Smiley (1962) reported that the increased amount of soluble collagen in lathyrism induced by administration of beta-aminopropionitrile did not arise from the collagen insoluble prior to administration of the drug, but rather that beta-aminopropionitrile acted by blocking the formation of mature collagen fibers, perhaps by preventing the formation of cross-linkages between alpha collagen chains.

Gardner (1960) stated that in experimental lathyrism the homogeneous, translucent, eosinophilic substance deposited particularly in ligaments, was altered collagen (paracollagen). The connective tissues in lathyrism were of a nature between typical collagen and hyaline non-fibrous collagen. With aldehyde fuchsin stain after peracetic acid oxidation, they were shown to contain a significant quantity of a new purple non-collagenous connective tissue fiber. This new connective tissue fiber was resistant to lathyrus toxin, whereas reticulin, collagen and elastic fibers readily
underwent alteration. The possibility still existed that lathyism represented either an alteration in both collagen and ground substance or the formation of a soluble type collagen.

It seemed apparent that there was a change in the state of organization of connective tissues in lathyism. The literature reviewed was confusing as to the nature of these changes, which could very easily be due to the numerous methods employed to study experimental lathyism.
CHAPTER III

MATERIALS AND METHODS

The animals used in this investigation, bullfrog larvae of Rana species, were obtained from the Carolina Biological Supply House, Burlington, North Carolina. They were reared in the laboratories of Atlanta University at room temperature in aerated tap water and fed boiled lettuce. Upon arrival of the shipment, the animals were separated according to length and the emergence of the hind-limb buds. The similarly grouped animals were separately placed in laboratory aquaria (20 per aquarium). Injected animals were placed in large finger bowls in lots of three per bowl.

Semicarbazide hydrochloride, in the concentrations of 0.1 M, 0.01 M, and 0.001 M, was used as the lathyrogenic agent in this investigation. The formula weight of the compound was 111.54 grams and it was obtained from the J. T. Baker Chemical Company, Phillipsburg, New Jersey. All solutions were prepared using 0.65 per cent sodium chloride solution as a solvent.

Two controls were used: one where three animals were each injected with 0.25 cc of a 0.65 per cent sodium chloride solution and another where three animals were not injected. A total of twenty-seven tadpoles were injected with the semicarbazide hydrochloride solution. Each animal was injected into the ventral fin. Animals to be injected were selected as near to the same stage as possible. All injections were made with a luer type Aloe precision hypodermic syringe (#4836) with a capacity of 2 cc, obtained from A. S. Aloe Company, St. Louis, Missouri,
and a Yale one inch, twenty-seven gauge needle.

Nine animals were each injected with 0.25cc of 0.1 M, nine with 0.25cc of 0.01 M, and nine with 0.25cc of 0.001 M semicarbazide hydrochloride. They were observed for periods of twenty-four to seventy-two hours, after which they were sacrificed. The tails were amputated, cut into three pieces (A, upper; B, middle; C lower), and fixed in Bouin's fluid. The pieces were subsequently washed, dehydrated, cleared in xylol, and embedded in paraffin. Sections were cut at ten microns and stained by two methods: Lison's (1954) Alcian blue-chlorantine fast red and hematoxylin and eosin. Photomicrographs were made of selected sections.
CHAPTER IV

EXPERIMENTAL RESULTS

The following results are based on transverse sections through the upper region of the tail (piece A) of bullfrog larvae. This region was selected because it represented both where lateral rounded protuberances appeared and the site of injection of the lathyrogenic agent or salt solution. Sections were stained with hematoxylin and eosin and Alcian blue-chlorantline fast red. According to Lison (1954), by the latter method collagen and osselin stained red and cartilage — bluish-green.

The features of both controls used — those injected with 0.25cc of a 0.65 per cent sodium chloride solution and those that were not injected — were similar. Hence, no further distinction will be made between the two. Upon microscopic examination of the sections of control tissue (Fig. 1), an epidermis (E) several layers in thickness was revealed. The outermost layer of the epidermis consisted of squamous cells and the other layers cuboidal cells. The basement lamella (B) was directly below and in close association with the epidermis. This lamella stained red, suggesting its collagenous nature. Beneath the basement lamella was seen the underlying bluish-green loose connective tissue (C) that contained small blood vessels and melanophores.

The musculature (M) was arranged in bundles that were separated from one another by red staining septa (S) suggesting that the septa and basement lamella were similar in nature. Beyond the muscles (moving inwardly) and continuous with the septa that separated the muscle bundles, a network of red-staining loosely packed fibers (Fig. 2) was observed.
Interspersed among the fibrous network were mesenchymal cells. Continuous with and similar (in stainability) to the reticulum of fibers was a layer which surrounded the outer component of the notochordal sheath.

The notochord consisted of numerous large vacuolated cells (V) with rather faint nuclei and a layer of small peripheral cells (P) containing distinct nuclei. This axial rod was enclosed by a bilaminar sheath: a thick red-staining inner fibrous lamella (IF) and a thin non-staining outer elastic lamella (OE). In the regions above the nerve cord (dorsal fin) and below the caudal blood vessel (ventral fin) was a bluish-green network of loose connective tissue that contained small blood vessels and melanophores (Fig. 3).

The animals injected with the semicarbazide showed no signs of general retardation in movement. They were as active as the control tadpoles. After twenty-four hours there were rounded protuberances on the lateral surface in the upper region of the tail of animals injected with 0.25cc of 0.01 M and 0.001 M semicarbazide. Portions of the tail epidermis underwent decoloration. The animals that were injected with 0.25cc of 0.1 M semicarbazide and observed for seventy-two hours shedded portions of their epidermis. The lower region of the tail (piece C) deteriorated and subsequently the tip dropped off.

Transverse sections (Figs. 4 and 5) through the tail of larvae injected with 0.01 M and 0.001 M semicarbazide showed an epidermis (E) that consisted of several layers of cells that formed a continuous covering over the surface of the tail. The basement lamella (B) stained red and separated the epidermis from the underlying loose connective tissue and musculature.
The loose bluish-green connective tissue (DC) near the site of injection degenerated and appeared as debris when stained with Alcian blue-chloran- 
tine fast red. However, it was eosinophilic when stained with hematoxy- 
lin and eosin.

The protuberances represented a localized pushing out of the epi-
dermis. Upon fixation the protuberances collapsed and when sectioned showed a number of folds (Figs. 6 and 7). However, the side opposite the site of injection remained intact (Fig. 8). There were no obvious changes in the musculature (M). The notochord (NC) was clearly visible and there were no disruptions in its bilaminar sheath (S).

Examination of a transverse section through the tail of a larva injected with 0.1 M semicarbazide revealed a different picture from the control and other experimental animals. The epidermis (Figs. 9 and 10) now appeared in some areas as a single layer and in other areas it was totally absent. In addition there were instances where the basement lamella (B) had become separated from the epidermis. Not only was there evidence of a degeneration of connective tissue (DC), but also of the epidermis, and basement lamella. The musculature (M) consisted of bundles, but it was virtually impossible to distinguish any connective tissue that might be separating these bundles from one another. Again the notochord enclosed by its bilaminar sheath, remained intact. There were no signs of fi-
brous disruptions in the vicinity of the notochord.

PLATE I

(Explanation of Figures)
(Explantation of Figures)

Fig. 1. Photomicrograph of a transverse section through the tail of a normal bullfrog larva of *Rana* species to show: epidermis (e), basement lamella (b), loose connective tissue (c), muscle (m), and septa (s). Alcian blue-chlorantine fast red. X450.

Fig. 2. Photomicrograph of a transverse section through the tail of a normal bullfrog larva of *Rana* species to show: outer elastic lamella (oe), inner fibrous lamella (if), peripheral cells (p), and vacuolated cells (v). Alcian blue-chlorantine fast red. X450.
(Explanation of Figures)

Fig. 3. Photomicrograph of a transverse section through the tail of a normal bullfrog larva of *Rana* species to show the network of loose connective tissue (c) in the area of the ventral fin. Note the epidermis (e) and the basement lamella (b). Alcian blue-chlorantine fast red. X450.

Fig. 4. Photomicrograph of a transverse section through the tail of a bullfrog larva near the site of injection of 0.25cc of 0.01 M semicarbazide hydrochloride and through the lateral protuberance to show: epidermis (e), basement lamella (b), and degenerated loose connective tissue (dc). Alcian blue-chlorantine fast red. X450.
PLATE III

(Explanation of Figures)
(Explanation of Figures)

Fig. 5. Color photomicrograph to more clearly show: epidermis (e), red-staining basement lamella (b), and degenerated loose connective tissue (dc). Alcian blue-chlorantin fast red. X450.

Fig. 6. Photomicrograph of a transverse section through the tail of a bullfrog larva of Rana species injected with 0.25cc of 0.001 M semicarbazide hydrochloride to show the folds resulting from the collapse of the protuberance following fixation. Alcian blue-chlorantin fast red. X450.
PLATE IV

(Explanation of Figures)
(Explanation of Figures)

Fig. 7. Color photomicrograph to more clearly show the folds resulting from the collapse of the protuberance following fixation. Alcian blue—chlorantine fast red. X450.

Fig. 8. Photomicrograph of a transverse section through the tail of a bullfrog larva of *Rana* species to show the side opposite the site of injection. Note the epidermis (e), basement lamella (b), loose connective tissue (c), muscle (m), and notochord (nc) with its intact bilaminar sheath (s). Alcian blue—chlorantine fast red. X450.
PLATE V

(Explanation of Figures)
(Explanation of Figures)

Fig. 9. Photomicrograph of a transverse section through the tail of a bullfrog larva of *Rana* species injected with 0.25cc of 0.1 M semicarbazide to show: basement lamella (b), degenerative connective tissue (dc), muscle (m), and epidermis (e). Alcian blue-chlorantte fast red. X450.

Fig. 10. Color photomicrograph of a transverse section through the tail of a bullfrog larva of *Rana* species injected with 0.25cc of 0.1 M semicarbazide hydrochloride to more clearly show: basement lamella (b), degenerative connective tissue (dc), muscle (m), and epidermis (e). X450.
CHAPTER V

DISCUSSION

Lathyism has been produced experimentally by feeding organisms (particularly mammals) diets containing seeds of *Lathyrus odoratus* or its extracts, placing lathyrogenic compounds in the aqueous environment of amphibians, and introducing such agents onto the chorio-allantois of chick embryos. The present investigation was designed to determine the changes produced by injecting semicarbazide hydrochloride directly into the ventral fin of amphibians. Semicarbazide was used as the lathyrogenic agent because Ramamurti and Taylor (1959) fed weanling rats a diet containing 0.3 per cent semicarbazide and the animals developed the characteristic lesions of osteolathyrism. This condition was the same as that produced by beta-aminopropionitrile, aminoacetonitrile and mercapto-ethylamine.

Since many studies have revealed the gross chemical effects of lathyrogenic compounds on collagen, it became necessary to select a highly reliable indicator of tissue alteration. Lison (1954) suggested that chlorantinine fast red, when added to the Alcian blue mucopolysaccharide staining procedure, selectively stained collagen in a certain state of aggregation. By this method collagen stained red and reticulin (a lower state in the aggregation of collagen in relation to its ground substance) stained bluish-green. The only other red staining was given by ossein. In this investigation the following structures stained red: basement lamella, myosepta, inner fibrous lamella of the notochordal sheath, and the peri-elastic fibers which are continuous with a reticulum of fibers.
between the musculature and the notochord area. Loosely arranged fibers between the basement lamella and musculature (laterally) and in the fin regions (dorsally and ventrally) stained bluish-green. That Lison's procedure is a reliable one is attested to by the following: physical and chemical evidence of the collagenous nature of the basement lamella in larval amphibians (Weiss and Ferris, 1954 - in urodeles; Edds, 1958; Kemp, 1959; Edds and Sweeney, 1961 - in anurans); chemical evidence of the collagenous nature of the inner fibrous lamella of the notochordal sheath and the peri-elastic fibers (Hunter, 1962).

In larvae injected with the semicarbazide, the loose bluish-green connective tissue in the area beneath the basement lamella and near the site of injection degenerated. As a result of the connective tissue degeneration, there was a localized pushing out of the epidermis that appeared as epidermal expansion since the connective tissue was no longer present to hold it intact. There were no signs of mitotic activity that would suggest that the lathyrogenic agent stimulated the epidermis to proliferate. Levy and Godman (1955) observed tumors in the notochord of *Amblystoma punctatum* that were reared in an aqueous solution containing the crystalline factor of *Lathyrus odoratus*. These tumors were superficially similar to the lateral protuberances produced here by injection. The suggestion was made that the tumors arose as a consequence either of a local degeneration of the notochordal sheath leading to secondary ecchordoses or prolapses, or a direct stimulation to proliferation of the notochordal cells. The notochord enclosed by a bilaminar sheath remained undisturbed throughout the present investigation. There is evidence
to indicate that at early stages of development the notochordal sheath did not give the characteristic staining reaction of later stages when stained by Alcian blue-chlorantine fast red. The notochordal sheath at early stages gave the characteristic stain for reticulin and only later did it show characteristic collagen staining (Hunter, 1962). Since the animals used by Levy and Godman were younger than the ones used in this investigation, it might mean that the sheath was exposed to the lathyrogen while it was characterized as reticulin. Thus, it would be attacked by the lathyrogen in a manner similar to the loose bluish-green tissue in the present study. Once the tissue reached the definitive collagen staining state, it no longer was vulnerable to the effects of the lathyrogen (at least not within the time span of this current study). Hence, one may now account for the fact that the notochord enclosed by its thick inner collagenous lamella remained intact throughout the duration of the experiments reported in this thesis. A more adequate test to resolve the differences reported here and by Levy and Godman would be to expose larvae to the lathyrogen before and after the sheath exhibits collagen staining characteristics.

After prolonged exposure to the lathyrogenic agent, the epidermis degenerated in some areas to a single layer while in others it was totally absent; the basement lamella became invaded with cells while the musculature and the connective tissue septa separating the muscle bundles showed degenerative signs. Helff (1930) suggested that the various tissues of the larval anuran's tail inherited their susceptibility to certain histolytic agents which were liberated and became functional at a certain definite
stage of metamorphosis. Could one conclude, therefore, that semicarbazide mimiced these histolytic agents causing the onset of metamorphosis?
1. Some of the characteristics of experimental lathyrisms were studied microscopically following the injection of semicarbazide hydrochloride into the ventral fin of bullfrog larvae of *Rana* species.

2. Rounded protuberances were observed after twenty-four hours on the lateral surface in the upper region of the tail of animals injected with 0.25 cc of 0.01 M and 0.001 M semicarbazide hydrochloride.

3. The loose connective tissue in the area beneath the basement lamella and near the site of injection degenerated.

4. As a result of the connective tissue degeneration, there was a localized pushing out of the epidermis that appeared as epidermal expansion.

5. There was degeneration of the epidermis, basement lamella, and connective tissue in the tail of animals injected with 0.25 cc of 0.1 M semicarbazide hydrochloride following seventy-two hours of exposure to the lathyrogen.

6. The notochord enclosed by its bilaminar sheath, remained intact throughout this investigation.
LITERATURE CITED


